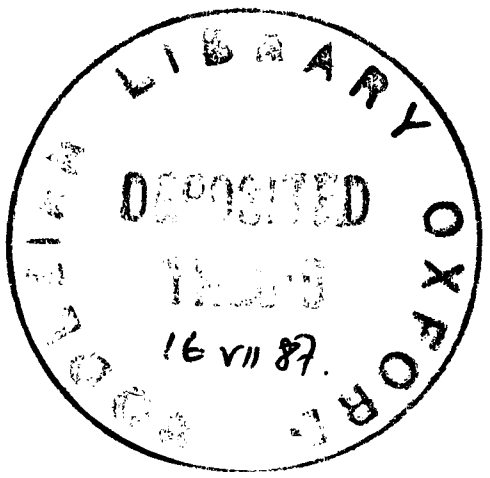
THE PHYTOALEXIN RESPONSE IN *PHASEOLUS VULGARIS* L.



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by
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ABSTRACT

This thesis concerns the initial events leading to the induction of the phytoalexin response in the Phaseolus vulgaris L. - Colletotrichum lindemuthianum L. host - pathogen interaction.

The phytoalexin response is an expression of the resistance of the host to further pathogen invasion. The visible expression of the phytoalexin response is hypersensitive necrosis.

One of the initial events leading to the induction of the phytoalexin response is thought to be the induction of the first enzyme leading to the synthesis of phenylpropanoid phytoalexins, namely L-phenylalanine ammonia-lyase (PAL). Standard methods for determination of PAL activities were found to lead to measurements of both phenylalanine amino-transferase (PAT) and PAL activities together. Further, PAT was found to have a higher affinity for L-phenylalanine than PAL. An isotopic assay was devised for the accurate estimation of PAT and PAL activities separately using a specific inhibitor of PAT, L-aspartic acid. These experiments were carried out in a whole (intact) plant system.

A rapidly isolated cell and protoplast system was specially devised for isolation of the pathogen cell wall constituents responsible for the induction of the phytoalexin response. Special care was taken to minimise mechanical damage in these systems by optimising methods of purification, viability and intactness. The pathogen cell wall component responsible for the induction of the phytoalexin response was found to be an asialoglycoprotein.

The regulation of PAL activities leading to the induction of the phytoalexin response has been variously thought to be due to the de novo synthesis of inactive protein, or of active protein, or to the subsequent activation of an inactive protein, or to end product inhibition, or to substrate supply or substrate availability or to the synthesis of a regulatory protein.

Experiments were designed to test which of these explanations might be correct using a polyclonal antiserum raised to a homogeneous preparation of PAL protein. Results from these experiments indicated the regulation of PAL activity to be due to the de novo synthesis of an inactive protein, and subsequent activation of newly synthesised inactive protein. This process was dependent on both substrate supply (by increased activity and de novo synthesis of PAT protein) and on substrate availability (by a decrease in general protein synthesis), and not on end product inhibition.

The causal sequence of events leading to the decline in protein synthesis associated with the phytoalexin response was found to be associated with the increase in de novo synthesis of phytohemagglutinin (PHA). Phytohemagglutinin was tested for its ability to act as a recognition determinant in the induction of the phytoalexin response. Phytohemagglutinin on the host cell surface was found to bind sugar determinants on an asialoglycoprotein present on the pathogen cell surface. Determinants in the recognition process appeared to be galactose and N-acetyl-galactosamine. The recognition process appeared to be regulated by the rate of turnover of the PHA-asialoglycoprotein complex by de novo synthesis of degradatory enzymes. The host cell wall was found to be an important determinant for the induction of the phytoalexin response.

Attempts were made to relate the sequence of events leading to the induction of the phytoalexin response with increased susceptibility to further invasion, senescence and non-specific induction of the phytoalexin response by abiotic compounds.

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CHAPTER 1

GENERAL INTRODUCTION

Every time moisture and temperature conditions prove favourable to pathogen growth, plant tissues are subjected to attempted infection by potentially parasitic microorganisms. This may happen on numerous occasions during the whole life-cycle of the plant. However, these attempts often fail, and most plants remain healthy. The reasons for failure of successful establishment of a parasite has been extensively studied by pathologists, physiologists, and biochemists.

1.1. PATHOLOGICAL STUDIES

1.1.1. Reasons for failure of parasitism.

Pathological studies describe failure of parasitism to occur either (a) during attempted penetration of the host cuticle and epidermal cell walls by the pathogen (e.g. the Populus tremuloides - Colletotrichum gloeosporoides interaction, Marks et al., 1965) or (b) after penetration of the host cell by the pathogen (e.g. the Solanum tuberosum - Phytophthora infestans interaction, Tomiyama, 1967).

The first case has been found to be due to physical barriers of the host which cannot be broken down by the pathogen. The second case is more complex and has been the subject of extensive study as summarised below.

1.1.2. Failure of parasitism after penetration of the host cell by the pathogen and necrosis.

Ward (1905) found that the failure of parasitism after penetration of the

host cell by rust pathogens was accompanied by the rapid death or necrosis of the host cells in contact with the pathogen. This observation was later confirmed by Stakman in 1915, and afterwards for other fungal pathogens by Müller in 1959 and for bacterial pathogens by Klement & Goodman in 1967. Stakman coined the term 'hypersensitivity' to describe necrosis of resistant host cells to further pathogen invasion.

Not all plants which are resistant to pathogen invasion exhibit necrosis as a component of resistance. For example in the Malus - Venturia inaequalis interaction, resistance to pathogen invasion has been established forty hours before the expression of necrosis (Nicholson et al., 1977).

The method used by most pathologists to study the relation between pathogen invasion and necrosis was simple light microscopy. The light microscope enabled the observation of pathogen zoospore germination after inoculation, penetration of resistant host cell walls, pathogen hyphal growth a few hours later, and the inhibition of further pathogen growth several hours later. A characteristic example of this type of study was described by Tomiyama in 1967 for the Solanum tuberosum - Phytophthora infestans interaction.

The appearance of necrosis of underlying cells accompanied the expression of resistance and the necrotic cells had granular and brown cytoplasm. In some cases, this made it very difficult to observe the time of pathogen penetration and to observe and measure it quantitatively using the light microscope. Skipp & Deverall (1972) experienced considerable difficulty in deciding, by the use of light microscopy, whether the pathogen Colletotrichum lindemuthianum stopped hyphal extension growth before or after penetration of the host (Phaseolus vulgaris) cell wall, because the necrotic cells obscured the view. However, treatment of the tissue (stems, petioles and laminae) with dilute alkali cleared the browning and

granulation from the dead cells and revealed that over 80% of these cells contained short infection hyphae.

A parallel study, using electron microscopy, confirmed the presence of pathogen hyphae inside dead, necrotic or hypersensitive cell protoplasm (Mercer et al., 1974).

In contrast, the hyphae of virulent races of Colletotrichum lindemuthianum are compatible with susceptible bean cells for several days, during which time substantial intracellular growth is made without any adverse effects on the cells (Skipp & Deverall, 1972).

Necrosis of the host cell after pathogen invasion was also observed in other systems, such as the Lactuca sativa - Bremia lactucae interaction (Maclean et al., 1974). These workers observed the disruption of the resistant host cell membrane and cytoplasm four hours after penetration by the pathogen.

1.1.3. Necrosis and inhibition of pathogen invasion.

Using the light microscope to study the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction further, Skipp and Deverall (1972) measured hyphal lengths in inoculated tissues for 18 hours, at which time the host cells became necrotic. They recorded a faster growth rate of pathogen hyphae inside resistant cells before the onset of necrosis as compared to after necrosis. Slow hyphal growth was detected in cells which had become pale brown in colour, and hyphae did not grow out of these necrotic cells. These observations were made in both excised and whole hypocotyl host tissue. This suggested that a progressive inhibition of fungal growth follows necrosis of the host cell. These observations were confirmed by Bailey et al. in 1980 in the same Phaseolus vulgaris - Colletotrichum

lindemuthianum interaction, and the inhibition of pathogen growth several hours after invasion of resistant host cells was found in other systems, such as the Solanum tuberosum - Phytophthora infestans interaction (Shimony & Friend, 1975, 1976).

1.1.4. Conclusion of pathological studies.

The observation of host cell necrosis after pathogen invasion and the continuous growth of the pathogen hyphae, albeit increasingly more slowly in host cells after these cells have undergone necrosis, led pathologists to conclude that necrosis was a cause (and not a consequence) of disease resistance.

1.2. PHYSIOLOGICAL STUDIES

Factors leading both to the failure of further pathogen growth and to expression of hypersensitivity of host cells were further investigated by plant physiologists.

1.2.1. Necrosis and cross-protection.

Between 1971 and 1977, Elliston and coworkers presented further evidence for the induced resistance of host cells to pathogen invasion (Elliston et al., 1971, 1976, 1977). This induced resistance was different from the localised necrosis described above, in that avirulent races of Colletotrichum lindemuthianum caused sites 5mm away from the site of infection on etiolated bean hypocotyls to become resistant to virulent races capable of inducing the anthracnose disease (Elliston et al., 1971). Elliston and coworkers called this phenomenon cross-protection.

Cross-protection was also observed in other host-pathogen interactions,

such as the Cucumis sativus - Colletotrichum lagenarium interaction. Inoculation of the first leaf of the host made the young leaves resistant to the same pathogen when applied one or more weeks later (Kuć et al., 1975).

Similar resistance could also be induced in cells by heat shock (50°C for 30 seconds) around local lesions as has been shown for the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction (Deverall, 1977) and the Nicotiana tabacum - TMV interaction (Ross & Israel, 1970).

1.2.2. Necrosis, cross-protection and phytoalexin formation.

A simple explanation of the types of change in host plants which increase their resistance would be the diffusion of anti-microbial compounds from the host tissues at the sites of inoculation with the protectant organism. Müller envisaged that a hypothetical antifungal priniciple diffused out from necrotic or hypersensitive cells to prevent the development of virulent races of the fungus. The antifungal compound was called a phytoalexin (Müller & Borger, 1940). This concept came to be known as the Phytoalexin Theory. The term 'phytoalexin' meant a warding-off compound produced by the plant [phyton (Greek) = plant; alexos (Greek) = a warding-off substance]. Müller defined phytoalexins as 'antibiotics which are the result of an interaction of two different metabolic systems, the host and parasite and which inhibit the growth of micro-organisms pathogenic to plants' (Müller, 1956).

It was not until 1958 that the chemical nature of phytoalexins was found. This was first shown by Müller in 1958, working with the hypersensitive response of Phaseolus vulgaris tissue to the soft-fruit pathogen Monilinia fructicola. He placed droplets of spore suspension in cavities of opened bean pods deprived of seeds. He observed the germination of spores and

subsequent death of some underlying cells within 24 hours. He then collected the infection droplets at different intervals and tested these for their effects on new spores. He found that the droplets became increasingly antifungal after incubation in seed cavities for 14 hours and completely fungistatic after 24 hours. He was able to extract the substance responsible for the antifungal activity by partition with petroleum spirit, but was unable to characterise it chemically.

It was not until 1962 that phytoalexins were to be chemically identified. In the Pisum sativum - Monilinia fructicola system the phytoalexin was tested for and extracted by the methods described above by Müller (Cruickshank & Perrin, 1960). This compound was isolated, crystallised, characterised as a pterocarpan and named pisatin (Perrin & Bottomley, 1962). Soon after this, the work with Müller's Phaseolus vulgaris system was re-examined and a closely related pterocarpanoid compound was isolated, characterised and named phaseollin (Cruickshank & Perrin, 1963a; Perrin, 1964).

The discovery followed in quick succession of several compounds that were regarded as phytoalexins (Cruickshank, 1963). Amongst these were the sesquiterpenoid compound, ipomeamarone from infected roots of the sweet potato Ipomoea batatas, the compound orchinol from infected tubers of the orchid Orchis militaris and methoxymellein from infected roots of the carrot Daucus carota (Deverall, 1977). Cruickshank (1963) was the first to review the structure, occurrence and biological activity of phytoalexins. Several reviews were to follow: Kuć (1972, 1976), Ingham (1972), Van Etten & Pueppke (1976), Friend (1977; 1981), Stoessl (1980), Dixon et al., (1983a) and Smith & Banks (1986). Thus several different types of chemical compounds (isoflavonoids, terpenoids, stilbenes, and polyacetylenes) were soon recognised as phytoalexins. They were all found to be relatively small molecules.

Further investigation of bean resulted in the characterisation of several chemically related phytoalexins in addition to phaseollin. These additional Phaseolus vulgaris phytoalexins were:-

(1) isoflavones: daidzein, 2′-hydroxydaidzein, genistein, 2′-hydroxygenistein, licoisoflavone A and 2, 3 - dehydrokievitone;

(2) isoflavanones: 2′-hydroxydihydrodaidzein, dalbergioidin, 5-deoxykievitone, cyclokievitone and kievitone;

(3) pterocarpans: demethylmedicarpin, phaseollin and phaseollidin;

(4) isoflavans: demethylvestitol, phaseollinisoflavan and 2′-methylphaseollinisoflavan;

(5) coumestans: coumestrol.

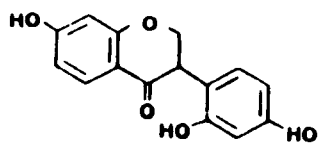
The structures of these compounds are shown overleaf (after Ingham, 1982). Further chemical studies on the phytoalexins of the Leguminosae are described by Ingham (1982).

Many legumes besides Phaseolus vulgaris were found to produce at least one and usually several phytoalexins. Further a number of different legume species were found to produce the same compound (Deverall, 1977).

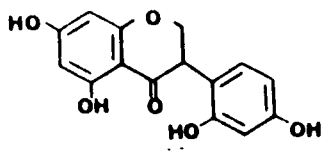
Phytoalexins were found to accumulate in a number of infected plants at about the time that necrosis was first noticed. Classical examples of these studies include those on the Vicia faba - Botrytis cinerea interaction (Mansfield & Deverall, 1974), the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction (Bailey & Deverall, 1971; Bailey, 1974; Rahe,

ISOFLAVANONES

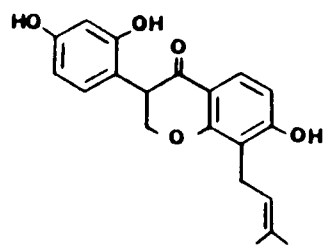
(-)-2'-HYDROXYDIHYDRODAIDZEIN



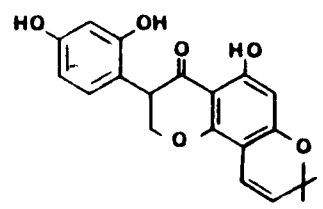
(-)-DALBERGIOIDIN



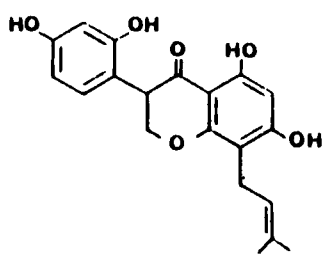
(-)-5-DEOXYKIEVITONE



CYCLOKIEVITONE



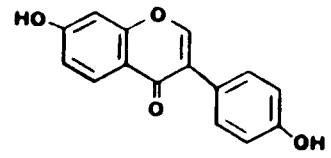
(+) and (-)-KIEVITONE



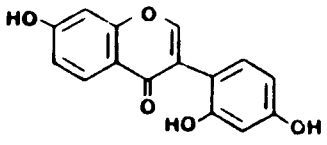
after Ingham (1982)

ISOFLAVONES

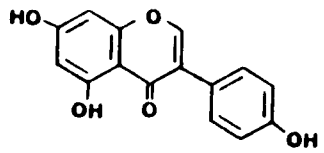
DAIDZEIN



2'-HYDROXYDAIDZEIN



GENISTEIN



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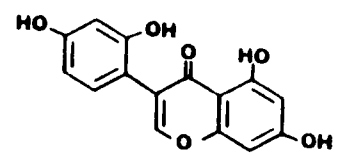
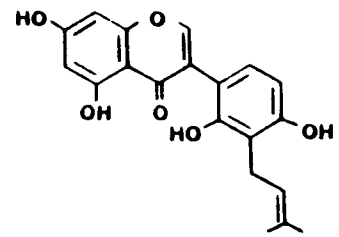
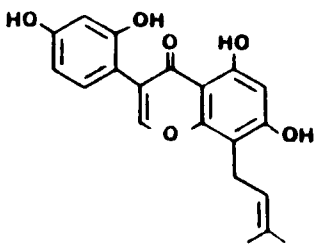


LICOISOFLAVONE A

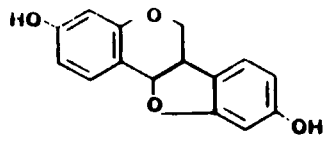


2,3-DEHYDROKIEVITONE



PTEROCARPANS

(-)-DEMETHYLMEDICARPIN



ISOFLAVANS

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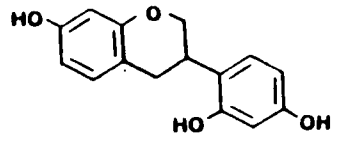
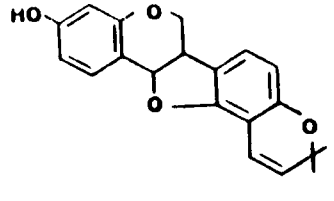
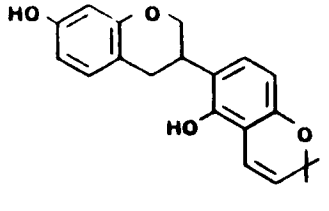


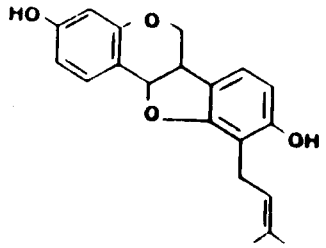
(-)-PHASEOLLIN



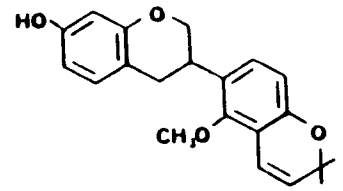
(-)-PHASEOLLINISOFLAVAN



(-)-PHASEOLLIDIN

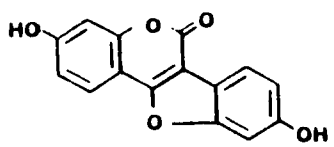


(+)-2'-O-METHYLPHASEOLLINISOFLAVAN



COUMESTANS

COUMESTROL



after Ingham (1982)

1973b) and the Phaseolus vulgaris - Uromyces appendiculatus interaction (Bailey & Ingham, 1971).

Further studies led to the finding that phytoalexins are localised at the site of induction, an important feature of the Phytoalexin Theory (Müller & Borger, 1940). Phytoalexins were found to be produced by cells invaded by or juxtaposed to challenging organisms but were absent or greatly reduced in quantity in nearby tissues (see Bailey, 1982). For example, phaseollin was restricted to the small areas of necrotic tissue at infected sites, but its location inside necrotic cells and/or their immediate neighbours has not been firmly established. These results were obtained only with resistant but not susceptible interactions between Phaseolus vulgaris and Colletotrichum lindemuthianum. The higher concentrations in resistant compared with susceptible types result from earlier accumulation in resistant bean host tissues as compared to susceptible tissues. These results implied that susceptibility is due to a prolonged biotrophic phase of growth by the fungal pathogen, avoidance of host cell injury and hence delayed phytoalexin production (Bailey & Deverall, 1971; Rahe, 1973b; Bailey, 1981). These results were confirmed by Hahn et al. in 1985 in tissues of Glycine max infected with Phytophthora megasperma f. sp. sojae. Radioimmunoassay and immunofluorescence studies showed a much higher accumulation of glyceollin I in resistant as opposed to susceptible tissues. Where large amounts of glyceollin I are produced, further growth of the pathogen is prevented and resistance is expressed.

It was possible that phytoalexins are produced by either dead or dying cells in the necrotic region or live host cells surrounding the necrotic region. Dead or dying cells cannot be expected to synthesise phytoalexins and are thought rather to be involved in hydrolysis and oxidation of phytoalexins such as phaseollin (Rathmell & Bendall, 1972). In the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction, Rahe

(1973b) noted that the amounts of phaseollin accumulating were more closely related to the surface areas of the lesions. He suggested that the stimulus from the dead cells causes phaseollin formation in live cells around the edge of the lesion.The live cells around the edge of the lesion have been found to be resistant to normally compatible hyphae (Skipp & Deverall, 1973), and have a dense cytoplasm (Mercer et al., 1974). In the Vicia faba - Botrytis cinerea interaction, the cells around the edge of the lesion have been found to emit fluorescence spectra of compounds similar to wyerone and wyerone acid (phytoalexins). These elegant microspectrofluorometric studies were carried out by Mansfield et al. (1974).

Hargreaves & Bailey demonstrated in 1978 that phaseollin and other isoflavonoid phytoalexins are probably synthesised in tissues around necrotic cells and are adsorbed and accumulate in the dead tissue containing intracellular hyphae, but unequivocal proof of cellular localisation of phytoalexin production is very difficult to obtain experimentally.

From the above studies, it seems possible that phytoalexin formation is induced as a consequence of necrosis or cell death in host-pathogen interaction. Indirect evidence in support of this finding is the induction of phytoalexin formation by simple physical injury used to simulate the damage caused by infection. Examples include (1) the induction of necrosis and phytoalexin formation within 24 hours after moderate bruising of leaves of Vicia faba (Deverall & Vessey, 1969) and (2) the induction of phytoalexin (phaseollin) formation by freezing injury to French bean tissues (Rahe & Arnold, 1975).

Mansfield et al. (1974) speculated that phytoalexin accumulation itself may enhance the death of host cells. Phaseollin induces cell death and reduces

the growth of Phaseolus aureus and Phaseolus vulgaris in cell suspension cultures (Skipp et al., 1977; Glazener & Van Etten, 1978). This was because phaseollin inhibited respiration of bean cells (Skipp et al., 1977). Phaseollin and many flavonoids inhibited the formation of ATP in mitochondria from cucumber hypocotyls (see Van Etten & Pueppke, 1976). Phaseollin also lysed red blood cells in vitro (Van Etten & Bateman, 1971; Van Etten, 1972). However, phytoalexins may also be metabolised by host tissues. For example Phaseolus vulgaris suspension cultures transformed phaseollin to phaseollinisoflavan (Hargreaves & Selby, 1978). Whether this occurs in the intact host plant is not known.

1.2.4. Phytoalexin formation and inhibition of pathogen invasion and host cell death.

In the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction, phaseollin forms at inoculated sites several hours after host cell death. Phaseollin begins to accumulate 2 to 3 days after the first symptoms of necrosis. The accumulation of phaseollin was found to coincide with the period when germ-tubes of the fungus were seen to slow their growth rate and to become restricted inside the necrotic cells (Skipp & Deverall, 1972). The concentration of phaseollin six days after inoculation was more than 3000 $\mu g.ml^{-1}$, a concentration far higher than that required to prevent germ-tube growth in vitro (10 $\mu g.ml^{-1}$)(Bailey & Deverall, 1971). These studies were confirmed by Bailey and coworkers in 1980 using a Phaseolus vulgaris - Colletotrichum lindemuthianum interaction. This system had the unique advantage of exhibiting inducible resistance at 25°C and induced susceptibility at 16°C, allowing an accurate estimation of the time factor in the phytoalexin response (Bailey et al., 1980). The disadvantage of this system was the possibility of accelerated host cell death at 25°C which may cause phytoalexin formation. Bailey and coworkers therefore concluded that the accumulation of phytoalexins probably caused the restriction of fungal

growth during the resistance of beans to Colletotrichum lindemuthianum (Bailey et al., 1980).

The antifungal activity of phytoalexins has been the subject of much intensive study. The commonly used biosassays assess the radial growth of mycelium on solid medium, spore germination, germ tube growth, or accumulation of fungal mycelium in liquid culture. Isoflavonoid phytoalexins are active generally at concentrations from 1 to 100 $\mu g.ml^{-1}$ (Bailey & Burden, 1973; Cruickshank, 1962; Cruickshank & Perrin, 1971; Perrin & Cruickshank, 1969; Van Etten, 1976). Of 27 fungi tested, phaseollin was found to be less inhibitory to pathogens of Phaseolus vulgaris than non-pathogens (Cruickshank & Perrin, 1971). The fungus that was most inhibited by phaseollin was Colletotrichum lindemuthianum (Bailey & Burden, 1973). However, of 16 bacteria tested, phaseollin was found not to be inhibitory to as many as 13 (Cruickshank & Perrin, 1971; Stholasuta et al., 1971). Of the phytoalexins produced by Phaseolus vulgaris, coumestrol lacked antifungal activity (Bickoff et al., 1969; Perrin & Cruickshank, 1969) but had weak antibacterial activity (Keen & Kennedy, 1974).

The effects of phytoalexins on pathogens have been extensively studied. Both bean pod diffusates and phaseollin induce the swelling and bursting of zoospores of Phytophthora infestans and Aphanomyces euteiches in as short a time as one minute (Müller, 1956, 1958; Van Etten, 1976). Phaseollin induces the swelling and distortion of germ tubes of Colletotrichum lindemuthianum and hyphae of Neurospora crassa and Rhizoctonia solani (Slayman & Van Etten, 1974; Van Etten & Bateman, 1971). The inhibitory effects involve a leakage of mycelial constituents and inhibition of respiration.

It should be noted that there may be errors in estimating damaging

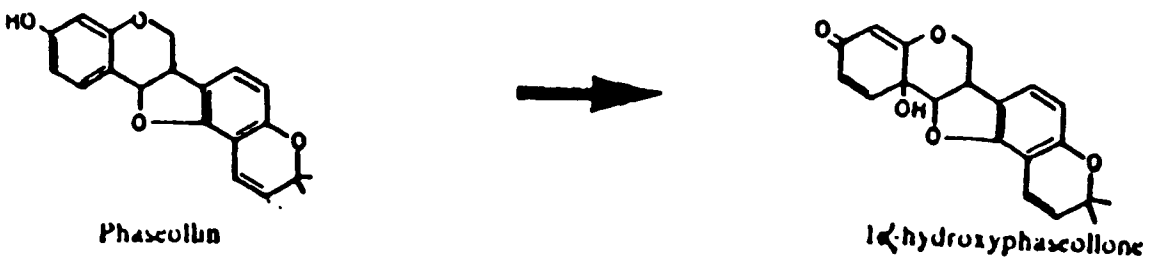
concentrations of phytoalexins *in vitro* as compared to *in vivo* because of unknown factors in the host tissue, which may reduce or enhance the effectiveness of the phytoalexin (Van Etten & Pueppke, 1976). One of the reasons suggested in the literature is the nature of the assay used to study inhibition. For example radial growth bioassays are more relevant to *in situ* events, because phytoalexin production is a post-infectional phenomenon and therefore occurs after spore germination of the fungi tested (e.g. *Colletotrichum lindemuthianum* or *Monilinia fructicola* (Cruickshank & Perrin, 1971).

The term phytoalexin has therefore now come to include a range of low molecular weight broad spectrum antimicrobial compounds synthesised by the host from primary metabolites in response to pathogen invasion (Paxton, 1981).

1.2.5. *Phytoalexin formation and susceptibility to pathogen invasion.*

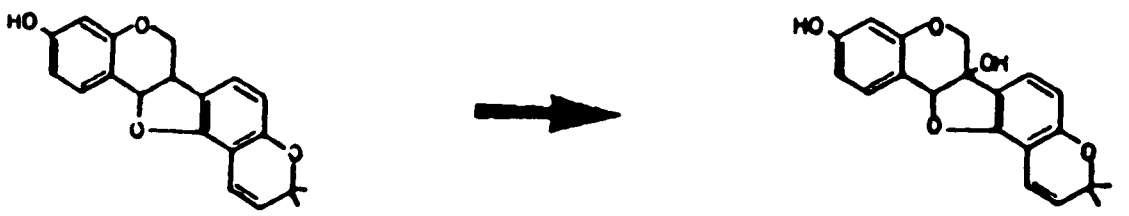
If phytoalexins were inhibitory to pathogen growth, it would also be possible that pathogens had evolved a system by which they could overcome an inhibition due to phytoalexin production and become virulent on their hosts. This aspect became a subject of extensive research. While studying the *Vicia faba* - *Botrytis fabae* (a virulent pathogen) interaction, Mansfield and his colleagues found a rapid decline in concentrations of the phytoalexin wyerone acid at points of further pathogen invasion (Mansfield & Deverall, 1974). Further investigation revealed a conversion of wyerone acid to a reduced form of wyerone acid less antifungal than the phytoalexin, first in the lesion and then in the whole leaf (Mansfield & Widdowson, 1973). It was further found that the pathogen would convert exogenous wyerone acid to the same reduced wyerone acid form *in vitro*. Several virulent pathogens have been found to metabolise phytoalexins of their potential hosts *in vitro*. For example *Colletotrichum lindemuthianum*

and Botrytis cinerea can metabolise phaseollin to 6α-hydroxyphaseollin (reaction 1; Burden et al. 1974; Van den Heuvel & Glazener, 1975), while Fusarium solani f.sp. phaseoli and Cladosporium herbarum hydroxylate phaseollin to 1α-hydroxyphaseollone (reaction 2; Van den Heuvel et al., 1974; Van den Heuvel & Glazener, 1975). Do these pathogens metabolise phytoalexins in situ? The formation of 1α-hydroxyphaseollone in infected



Phaseollin → 1α-hydroxyphaseollone

reaction 1



Phaseollin → 6α-hydroxyphaseollin

reaction 2

Examples of fungal detoxification of phaseollin (after Van Etten et al. 1982).

bean host tissue suggests that Fusarium solani f.sp. phaseoli probably does metabolise phytoalexins in situ (Van Etten & Smith, 1975) but unequivocal evidence is in most cases lacking since a combination of host and pathogen products in situ leads to results which are difficult to interpret.

1.2.6. Conclusions of physiological studies.

Bearing in mind the association of phytoalexin production with necrosis or cell death and slow pathogen growth (section 1.2.4), it seems reasonable to conclude that phytoalexin accumulation restricts the growth of the pathogen during resistant interactions. It must be noted, however, that phytoalexins are not thought to be the only method of induced biochemical defense. Evidence is accumulating further which indicates the importance of induced physical cell surface barriers (lignification) in defense in certain plant-pathogen interactions (Friend, 1973, 1976, 1980). Examples include the Solanum tuberosum - Phytophthora infestans interaction (Friend, 1976; Henderson & Friend, 1979) and the Cucumis sativa - Colletotrichum lagenarium / C. cucumerinum - interaction (Hammerschmidt & Kuć, 1980). The major unresolved question concerns the stimulus for phytoalexin formation, and the factors leading to phytoalexin formation have been much studied biochemically.

1.3. BIOCHEMICAL STUDIES

1.3.1. Phytoalexin induction by pathogen components.

The factors leading to phytoalexin formation has been the subject of much speculation. The first factor in the chain leading to phytoalexin formation was found to be in the pathogen. The first compound to which this role was attributed was a simple polypeptide, monilicolin A.

This polypeptide, isolated from the pathogen Monilinia fructicola, stimulated the production of the phytoalexins, phaseollin and phaseollidin in its host Phaseolus vulgaris (Cruickshank & Perrin, 1968; Cruickshank et al., 1974). The molecular weight of monilicolin A was approximately 8 x 10^3. It was effective at concentrations of 1.6µg.ml^{-1} in producing as much as 15µg phaseollin.ml^{-1} pod diffusate. It was specific to its host and did not affect either Pisum sativum or Vicia faba. Several investigators have isolated from a number of pathogens other molecules that induce phytoalexin formation in various ways. These are as follows:

(i) Host-cell wall degrading enzymes. Preparations containing polygalacturonases from the pathogen Rhizopus stolonifer stimulated the formation of casbene synthase which is responsible for casbene (a phytoalexin) formation in Ricinus communis. The component resulting in casbene synthase induction was a glycoprotein (molecular weight 3.2 x 10^4), and both the carbohydrate and peptide moieties were needed for activity. Since the preparation was not pure polygalacturonase, it was not known whether phytoalexin induction was by enzyme activity or some other interaction with the host cell (Lee & West, 1981). The enzymes themselves were found to be active at concentrations of 0.64µg.ml^{-1}. They were glycoproteins and both carbohydrate and protein components were required for activity (Stekoll & West, 1978). An endopeptidase (a protease) isolated from Nectria galligena induced benzoic acid formation in the host, apple (Malus spp.) (Swinburne, 1975). Cell wall degrading enzymes from Erwinia carotovora (Albersheim et al., 1981) and Monilinia fructigena (see Bailey, 1982) caused production of phytoalexins in their hosts, Glycine max and Phaseolus vulgaris respectively. These enzymes have been thought to act by inducing the rapid release of cellular constituents and hence cell death on contact with the host tissue.

(ii) Polysaccharides: Ayers et al. (1974, 1976a,b,c) and

Anderson-Prouty & Albersheim (1975) partially characterised the phytoalexin-inducing components from Phytophthora megasperma and Colletotrichum lindemuthianum. Similar components have been obtained from a range of fungi (Albersheim & Valent, 1978) and even from commercial extracts of Saccharomyces cerevisiae (Hahn & Albersheim, 1978). The active components were carbohydrates. Further analysis revealed them to be heterogeneous neutral polysaccharides coated with glycoproteins. They were heat-stable and had high molecular weights ranging from 5×10^3 to 2×10^5. They had a β-1,3-glucan backbone with branches at the C-4 and C-6 atoms and also contained a few mannosyl residues. The branched links were important for the efficient induction of phytoalexin formation. The carbohydrates were active as low as 0.2 to 0.5μg.ml^{-1} in Phaseolus vulgaris (Albersheim & Valent, 1978). Cotyledons of soybean receiving only 10ng elicitor produced sufficient glyceollin to inhibit growth of Phytophthora megasperma. The glucans were non-specific inducers of phytoalexin formation; for example, the glucan from Phytophthora megasperma f.sp. glycinea caused production of several chemically diverse phytoalexins in a variety of host plants including glyceollin in Glycine max, phaseollin in Phaseolus vulgaris and rishitin in Solanum tuberosum (Albersheim & Valent, 1978; Cline et al., 1978). Unlike these β-glucans, other water soluble glucans of Phytophthora infestans inhibit phytoalexin accumulation (Garas et al., 1979; Doke & Tomiyama, 1980). However, since the effective concentrations were 1 to 10 mg. ml^{-1}, these glucan suppressors are probably not physiologically important (see Darvill & Albersheim, 1984). The host plant may have β-glucanases which can destroy β-glucans and thus prevent them from inducing phytoalexins, rendering the plant susceptible irrespective of the properties of the pathogen. This point is further discussed in section 1.3.2 (i). Yoshikawa et al. (1983) reported the presence of membrane binding sites in soybean for the β 1,3-glucan of Phytophthora megasperma var sojae. These receptors were not characterised but were thought to be either glycoproteins or proteins. This finding may

also be relevant to the argument in section 1.3.2 (i).

(iii) Amino-sugars and sugar alcohols. An amino sugar chitosan, isolated from cell walls of Fusarium solani, induced pisatin in its host Pisum sativum. The effective concentration of chitosan was 3µg.ml^{-1} (Hadwiger & Beckman, 1980). Chitosan is a β 1,4-linked glucosamine and is therefore a polycation. Polycations are thought to induce phytoalexin formation by binding to polyanionic constituents of the host cell wall such as polygalacturonic acid (Darvill & Albersheim, 1984) or perhaps directly injuring cells (Young et al., 1982). On the other hand ^{3}H-labelled chitosan appeared to move into the plant cell and accumulate in the nucleus (Hadwiger et al., 1981).

A sugar alcohol, hexa-(β-d-glucopyranosyl-D-glucitol) was isolated from cell walls of the pathogen Phytophthora megasperma var sojae and found to induce accumulation of compounds absorbing in the region of 286nm (phytoalexins?) in its host, soybean (Ossowski et al., 1984; Sharp et al., 1984a,b,c). The mode of phytoalexin induction by this compound remains uncertain (Sharp et al., 1984a,b,c).

(iv) Glycoproteins: Besides the host cell wall degrading enzymes of glycoprotein nature described above, glycoproteins of non-enzymatic nature have also been isolated from several fungi. The glycoproteins from Cladosporium fulvum produced the phytoalexin rishitin in its host, tomato. The glycoproteins from Monilinia fructicola produced the phytoalexin pisatin in its host, pea and the glycoproteins from Phytophthora megasperma produced the phytoalexin glyceollin in soybean. Preliminary analysis of glycoprotein fractions of Cladosporium fulvum showed them to be a mixture of molecules varying in molecular weight from 3×10^4 to 2.5×10^5. They were heat stable and contained glucose, mannose and galactose as carbohydrate moieties (Dow & Callow, 1979a; Lazarovits et al., 1979; De Wit

& Kodde, 1981). The glycoproteins were present in culture filtrates of the pathogen and their activity was not affected by changes in the galactofuranosyl residues that occured during pathogen growth. The protein, but not the carbohydrate, was found to be important for their activity. On the contrary the glycoprotein from Phytophthora megasperma var. sojae bound concanavalin A, was inactivated by periodate and showed no loss of activity with pronase and it was therefore concluded that carbohydrates were the basis of its activity (Keen & Legrand, 1980). Detailed characterisation of the glycoprotein elicitors isolated from cell walls of Phythophthora megasperma (Wade & Albersheim, 1979; Keen & Legrand, 1980) have not been reported. Thus glycoproteins isolated from different pathogens may differ in the requirement for carbohydrate or protein moieties for induction of the phytoalexin response. The concentration of glycoproteins required for elicitation are commonly between 1 and 5 $mg.ml^{-1}$. Yields of phytoalexins from tissues treated with isolated glycoprotein are often low (less than 50 $\mu g.g\ tissue^{-1}$); they may be a reflection of the potential of the tissue treated or of altered activity of the glycoprotein, especially as it is isolated under harsh conditions (cold alkali extraction) (Keen & Legrand, 1980). Albersheim and coworkers while studying the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction also found glycoprotein constituents on the surface of cell wall glucans (polysaccharides) isolated from the pathogen. The nature of these glycoproteins remains to be characterised. (Ayers et al., 1974, 1976a,b,c; Anderson-Prouty & Albersheim, 1975).

The glycoproteins studied were usually not host-specific. The glycoprotein isolated from Cladosporium induced phytoalexin formation in Lycopersicum, Pisum sativum and Glycine max (De Wit & Roseboom, 1980). Glycoproteins are thought to induce phytoalexin formation by altering host cell membrane permeability and inducing cell death. For example, a glycoprotein from Cladosporium fulvum induced rishitin formation in Lycopersicon esculentum

and also caused a rapid leakage of ions and death of treated cells (Dow & Callow, 1979b). The glycoprotein inducer of phytoalexin formation from Phytophthora infestans was found to be toxic to the cells of many plants (Doke et al., 1979).

and (v) Lipids: Arachidonic acid and eicosapentaenoic acid isolated from Phytophthora infestans induced the formation of rishitin and lubimin in the host potato at concentrations of 3µg.ml^{-1} (Preisig & Kuć, 1985). The mode of action of lipids in bringing about phytoalexin formation is unknown.

Several pathogen constituents which interact with the host cell wall and are associated with phytoalexin formation remain to be characterised. An interesting example is the purified cell wall component of Fusarium solani f. sp. pisi (Hadwiger et al., 1981). This component was shown to enter the host (Pisum sativum) cell within 15 minutes and accumulate inside the cell wall at the same time as fungal growth was inhibited.

These studies imply some form of recognition between molecules on the pathogen surface with the host. The nature of the recognition may or may not be specific to the interaction.

1.3.2. Phytoalexin induction and mediation by host components.

Following their discovery of pisatin and phaseollin in Phaseolus vulgaris, Cruickshank & Perrin (1963a) found the induction of pisatin by ions of some heavy metals. It subsequently transpired that several organic molecules also induce phytoalexins in different potential host plants (e.g. Perrin & Cruickshank, 1965; Bailey, 1969 ; Hadwiger & Schwochau, 1970). The inducers came to be described as 'Pandora's box' by Van Etten & Pueppke (1976), largely because they included a list of diverse molecules that included

salts, plant hormones, peptides, toxins and heavy metals as well as ultraviolet light. Specific examples included the induction of phaseollin in Phaseolus vulgaris tissues by antibiotics, metabolic inhibitors (Cruickshank & Perrin, 1971; Hess et al., 1971), triton surfactants (Hargreaves, 1981) and mercuric chloride (Hargreaves, 1979). The method by which the pathogen molecules transmitted their phytoalexin inducing signal became a subject of much speculation in the early 1980s. Some workers believed in the interaction of the pathogen with a constituent (constitutive component) of the host directly involved in the induction of the phytoalexin. Other workers believed in the interaction of the pathogen with a bound constituent of the host to release a free and active constituent (endogenous component) of the host directly involved in the induction of the phytoalexin.

Examples of components of the host thought to be directly involved in the induction of the phytoalexin follow.

(i) Enzymes which degrade pathogen cell walls. Enzymes of Pisum sativum host tissue degrade pathogen (Fusarium solani) cell walls and release chitosan, an inducer of phytoalexin accumulation (Nichols et al., 1980). Similarly enzymes of Glycine max host tissue degrade pathogen (Phytopththora megasperma f.sp. glycinea) cell walls and in less than two minutes these release a glucan, an inducer of glyceollin accumulation in soybean (Cline & Albersheim 1981; Yoshikawa et al., 1981). It has been suggested that host metabolism of fungal constituents may be important for (a) cleavage of the phytoalexin inducing component to allow its passage through the host cell wall, (b) subsequent destruction of activity of the phytoalexin inducing component to ensure a localised host response (Cline & Albersheim, 1981) and (c) race specificity in the linkage between the phytoalexin inducing component and the fungal cell wall (Bailey, 1982). Lyon & Albersheim (1982) obtained a heat-labile component of enzymic nature

from freeze-thawed host (soybean) hypocotyls which induced glyceollin in host (soybean) tissues. The mechanism is thought to involve the release of an unknown endogenous heat stable component from the cell walls of host tissue.

and (ii) Glycoproteins. Glycoproteins in host cell walls of host plants are thought to recognise specific determinants on the pathogen (Keen, 1982). The evidence for the presence of glycoproteins has been indirect. Dow & Callow (1979b) found radiolabelled culture filtrate molecules from the pathogen Cladosporium fulvum to bind to cell membranes of its host Lycopersicon esculentum. The nature of this binding component was thought to be a glycoprotein. Further indirect evidence for the involvement of glycoproteins comes from work on the suppression of glucan induction of phytoalexins by race-specific oligosaccharide moieties in the Phytophthora infestans - Solanum tuberosum interaction (Doke et al., 1979). Lamb and coworkers found one of the polypeptides synthesised during phytoalexin induction in the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction to recognise an antiserum raised to phytohemagglutinin (PHA), the lectin of the host Phaseolus vulgaris (Lamb, personal communication).

Besides lectins, hydroxyproline-rich glycoproteins (extensin) have been thought to be involved in the induction of phytoalexin formation. These constituents are present in the host cell wall and stimulate the formation of pathogen cell wall degrading enzymes. For example, chitinase (β-1,3-glucanase) was stimulated by extensin in the Solanum tuberosum - Heterodera rostochiensi and Cucumis melo - Colletotrichum lagenarium interactions (Esquerre-Tugaye et al., 1979).

Early examples of constituents of the host thought to be released as free and active constituents involved in the induction of the phytoalexin response include polyamines and ethylene. Both these constituents induce

the phytoalexin response in pea and soybean respectively (Hadwiger et al., 1974; Paradies et al., 1979). However, the absence of changes in endogenous levels of polyamines (Teasdale & Hadwiger, 1977) and the failure to prevent glyceollin production despite inhibition of ethylene production (Paradies et al., 1980), led to a search for other possible host constituents.

These were cell wall pectic fragments and proteins specific to phytoalexin production.

(i) Cell wall pectic fragments. Studies by other workers lend support to the finding that infection and physiological stress cause the release of extracts containing cell wall fragments containing galacturonic acid residues and that these induce the accumulation of phytoalexins in leguminous tissue. An example is the water-soluble extract present in infected bean tissues containing galacturonic acid residues. Its presence in frozen tissue, its heat-stability, its molecular weight ($< 10^4$), its presence in aqueous extracts of either dead or living host (Phaseolus vulgaris) tissues and its ability to stimulate the production of phaseollin and phaseollidin in Phaseolus vulgaris hypocotyls (Hargreaves & Bailey, 1978) or cultured cells (Hargreaves & Selby, 1978) lend further support to its role as a mediator of the phytoalexin response. Similar results (namely the induction of glyceollin formation in cut soybean cotyledons) were obtained with walls or wall-containing extracts of other host plants such as soybean, tobacco or sycamore by Hahn et al., (1981). The inducing constituent in the above experiments was thought to be of pectic nature, but was not characterised fully. In soybean cell walls Nothnagel et al. (1983) identified a dodeca-α-1,4-D-galacturonide elicitor (also found in citrus pectin). It is this component which has been found to be released from pectin by pathogen polygalacturonic acid lyase. This is an established case where an elicitor is actually released from the host. Support for pectic compounds being mediators of the phytoalexin response comes from the

work of Ryan and coworkers who found chitin (a pathogen compound) to induce pectic compounds (e.g. rhamnogalacturan I) which acts as a protease-inhibitor inducing factor closely associated with phytoalexin formation and host cell death during host-pathogen interaction (Walker-Simmons & Ryan, 1984). These compounds would be released following wound-injury by a pathogen, could cause phytoalexin production in surrounding healthy cells and accumulate in progressively dying host cells (Bailey et al., 1980).

(ii) Protein synthesis. Hadwiger and coworkers found an increase in protein synthesis and certain RNA fractions to accompany phytoalexin (e.g. pisatin) production in host tissues (e.g. Pisum sativum). They suggested there was a requirement for protein synthesis to precede phytoalexin synthesis. This conclusion was supported by indirect evidence, namely the induction of phytoalexin synthesis by compounds which act at the level of DNA e.g. DNA intercalating compounds (Hadwiger & Schwochau, 1971); bromodeoxyuridine (Sander & Hadwiger, 1979); low concentrations of inhibitors of RNA and protein synthesis (Schwochau & Hadwiger, 1968, 1969) and ultraviolet light (Hadwiger & Schwochau, 1971). Compounds thought to function at the level of DNA were also implicated; examples were heavy metal salts, polypeptides and polyamines (Hadwiger & Schwochau, 1970; Hadwiger et al., 1973) and antiviral, antimalarial and antihistamine drugs (Hadwiger, 1972).

Hadwiger and his associates proposed that all these components de-repress certain genes coding for enzymes responsible for the synthesis of phytoalexins (Schwochau & Hadwiger, 1968). The mechanism remains unknown. It still remains possible that these compounds induce phytoalexin accumulation via cell injury by exerting toxic side effects.

1.3.3. Phytoalexin induction and biosynthesis.

The formation of phytoalexins could have been due to either de novo synthesis or release from precursors. Isoflavonoid phytoalexin (e.g. phaseollin and pisatin) synthesis would result from substrates such as phosphoenol-pyruvic acid and erythrose-4-phosphate, while release would result from precursors such as flavanoid glycosides (Deverall, 1977). Rathmell (1973) found no change in flavonoid glycoside concentrations during synthesis of phaseollin in Phaseolus vulgaris. Further, no alternative precursor form of phaseollin was found (Deverall, 1977). This evidence together with the slow release of phaseollin, suggested isoflavonoid phytoalexin formation by de novo synthesis.

Existing understanding of isoflavonoid (the major class of bean phytoalexins) biosynthesis in plants has been well summarised by Dixon et al., (1983a) and Smith & Banks (1986). Grisebach (1965) demonstrated that the basic flavonoid skeleton was derived from the products of two metabolic pathways, the acetate-malonate and the shikimic acid routes. Phenylalanine is produced via shikimic acid and is then deaminated by the enzyme phenylalanine ammonia-lyase to cinnamic acid. Cinnamic acid is then prepared for condensation with acetate units to form either a chalcone or its isomeric flavanone in parsley (Grisebach & Hahlbrock, 1974). Subsequent steps in the formation of isoflavans and pterocarpans have been demonstrated by logical deduction and chemical analogies, and not by experimental verification.

Evidence was found for the operation of this metabolic pathway in plants producing phytoalexins. Isotopic ^{14}C from phenylalanine appears in the isoflavonoids pisatin and phaseollin (Hadwiger, 1967; Hess et al., 1971). In immature pea pods [U-^{14}C]-phenylalanine and [1-^{14}C]-t-cinnamic acid were readily incorporated into pisatin following treatment of the tissues with salt solutions ($CuCl_2$) or spore suspensions of the pathogen Monilinia

fructicola (Hadwiger, 1966, 1967). Phenylalanine and cinnamic acid were similarly good precursors of phaseollin in excised pods (Hess & Schwochau, 1969; Hess et al., 1971) and tissue cultures (Dixon & Fuller, 1977) of Phaseolus vulgaris. [^{14}C]-phenylalanine was incorporated into glyceollin, the isoflavone daidzein, and the coumestans, coumestrol and sojagol in soybean hypocotyls infected with an incompatible race of the fungal pathogen Phytophthora megasperma var sojae (Keen et al., 1972) and into medicarpin in $CuCl_2$-treated seedlings of Trifolium pratense (Dewick, 1975).

That this isoflavonoid pathway and not general phenylpropanoid synthesis occurs during isoflavonoid phytoalexin synthesis was found by Rathmell and coworkers. Their conclusion was based on changes in isoflavonoids and absence of changes in flavonoid and general endogenous phenols during isoflavonoid synthesis (Rathmell & Bendall, 1971; Rathmell, 1973; Dixon & Bendall, 1978a).

1.3.4. Phytoalexin induction and induction of enzymes involved in biosynthesis of phytoalexins.

Although much information is available concerning the metabolic pathways by which isoflavonoid and terpenoid compounds are produced in some organisms, biochemists have had to look closely at activity of these pathways in diseased plants. An example of such a pathway is shown in Fig. 1 overleaf.

Several enzymes involved in the synthesis of isoflavonoids have been found to be induced with phenylpropanoid-derived phytoalexin formation. These are as follows:

(i) L-phenylalanine ammonia-lyase (PAL). Increased PAL activity is often observed prior to or concurrent with increased phenolic synthesis (Camm & Towers, 1973). Rathmell (1973) observed a marked increase in PAL activity 24 hours before the attainment of maximum phytoalexin (phaseollin

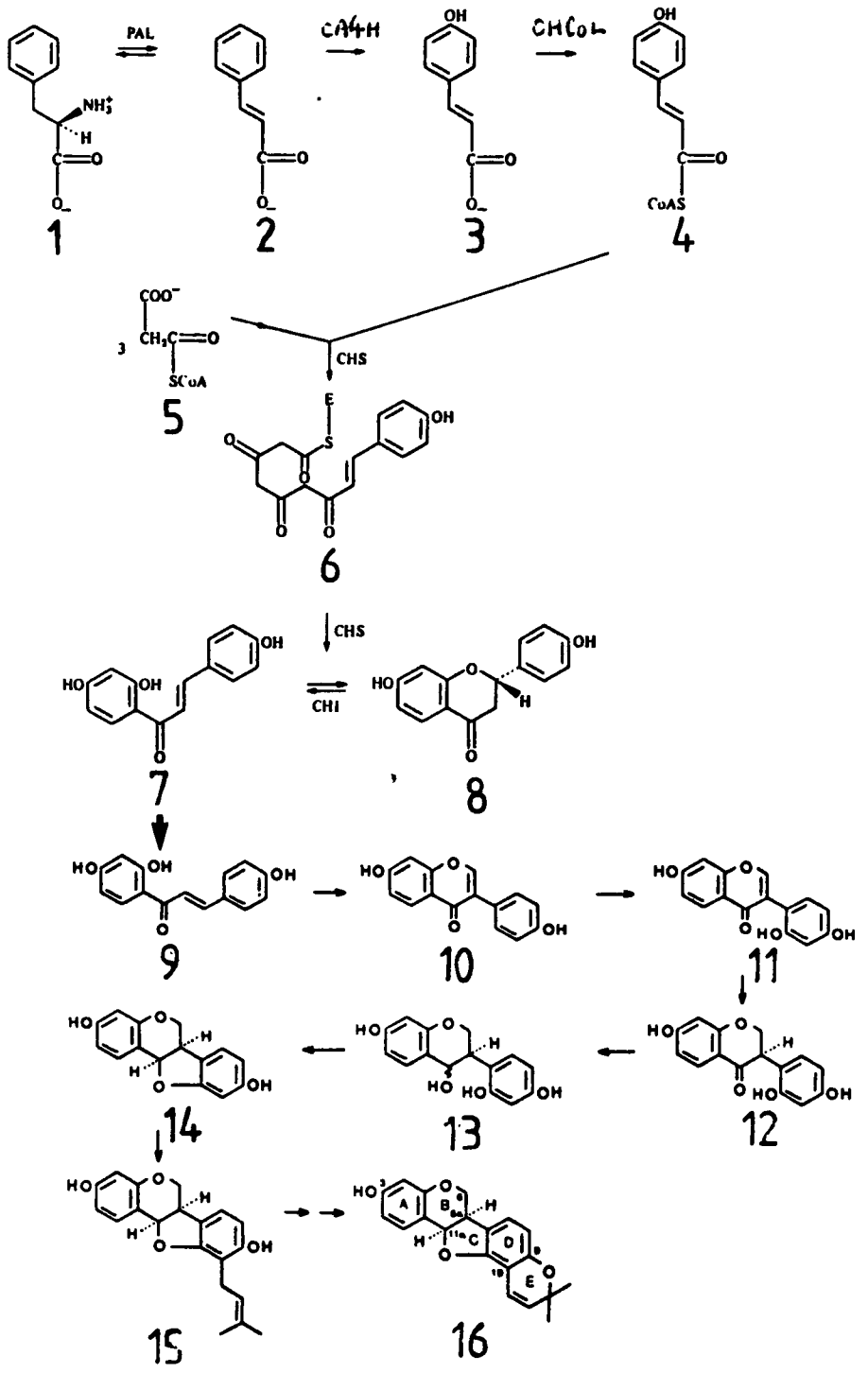


Fig. 1 Biosynthesis of phaseollin (Dewick & Steele 1982; Smith & Banks 1986):

1. L-phenylalanine; PAL = phenylalanine ammonia-lyase;
2. t-cinnamic acid; CA4H = cinnamic acid 4-hydroxylase;
3. 4-hydroxycinnamic acid; OH CoL = hydroxy-cinnamoyl CoA ligase;
4. 4-coumaroyl CoA;
5. malonyl CoA; CHS,E = chalcone synthase;
6. $C_6C_3C_6$ intermediate;
7. isoliquiritigenin; CHI = chalcone/flavanone isomerase;
8. liquiritigenin
9. 2',4'4-trihydroxy chalcone (isoliquiritigenin);
10. daidzein;
11. 7,2',4'-trihydroxyisoflavone;
12. 7,2',4'-trihydroxyisoflavanone
13. 7,2',4'-trihydroxyisoflavonol intermediate;
14. 3,9-dihydroxypterocarpan;
15. phaseollidin;
16. phaseollin.

and coumestrol) concentrations in the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction. PAL was temporally and spatially associated with phytoalexins and necrotic lesions. Lamb and coworkers used the same interaction, but in a different form, to study phytoalexin synthesis: namely cell suspension cultures of the host Phaseolus vulgaris treated with autoclaved ribonuclease and biotic components present in culture filtrates and heat-released from Colletotrichum lindemuthianum (Dixon & Bendall, 1978b; Dixon & Lamb, 1979; Lawton et al., 1980; Lawton et al., 1983a). They observed a marked but transient increase in the activity of PAL concomitant with the onset of phaseollin accumulation. The induction of PAL was also found to precede isoflavonoid phytoalexin formation in cell suspension cultures of Glycine max in response to heat-released components from the cell walls of the pathogen Phytophthora megasperma var sojae (Ebel et al., 1976; Dixon & Bendall, 1978b). Henderson & Friend (1979) found increase in PAL activity with induced physical cell surface barrier (lignin) formation in the Solanum tuberosum - Phytophthora infestans interaction. Hadwiger and coworkers found the induction of PAL to precede isoflavonoid phytoalexin formation in Pisum sativum in response to a wide range of abiotic compounds and ultraviolet light (e.g. Hadwiger & Schwochau, 1970, 1971).

(ii) Cinnamic acid 4-hydroxylase (CA4H). Fewer studies have been made on CA4H and its induction during phytoalexin synthesis than on PAL. Major studies on modulation of CA4H activity have been in illuminated storage potato tissues by Lamb & Rubery (1976a) and Lamb (1977). Induction of CA4H activity has been reported after treatment of Glycine max with heat-released cell wall components of Phytophthora megasperma f.sp. glycinea, producing the phytoalexin glyceollin (Ebel, 1979). In the cultured cell suspension system of Phaseolus vulgaris producing phaseollin in response to denatured ribonuclease A, CA4H activity was found to increase linearly for up to 48 hours after application (Dixon & Bendall, 1978b). CA4H responded at a slower rate than the transient increase observed for PAL. In illuminated potato storage tissues however, Lamb

(1977) found PAL and CA4H to increase in parallel.

In cultured cell suspensions of Petroselinum hortense treated with heat released cell wall components of the pathogen Phytophthora megasperma f.sp. glycinea, Hahlbrock and coworkers observed an increase in both PAL and CA4H activity. The two enzymes exhibited a similar dose-dependency. However, this induction does not lead to phytoalexin formation (Hahlbrock et al., 1981). Recent studies suggest the presence of furanocoumarin phytoalexins in the parsley system (see Dixon et al., 1983a).

(iii) Hydroxy-cinnamoyl CoA ligases. The hydroxy-cinnamoyl CoA ligases are of two types depending on the substrate acted upon. One is active on ferulic and sinapic acids and leads to lignin synthesis; the other is active on 4-coumaric acid and caffeic acid and leads to flavonoid and isoflavonoid synthesis (Knobloch & Hahlbrock, 1975). We are therefore only concerned with studies on the second form of hydroxy-cinnamoyl CoA ligases in phytoalexin formation. Hydroxy-cinnamoyl CoA ligase (active on 4-coumaric acid) has been found to be induced in two host-pathogen interactions accompanying the induction of phytoalexin synthesis. These are the hypocotyls and cell suspensions of Glycine max treated with a heat-released component of the pathogen Phytophthora megasperma f.sp. glycinea leading to glyceollin formation (Ebel, 1979) and cell suspensions of Phaseolus vulgaris treated with denatured ribonuclease and a heat-released component of the pathogen Colletotrichum lindemuthianum leading to phaseollin formation (Dixon & Bendall, 1978b).

(iv) Chalcone synthase (CHS). The enzyme CHS catalyses the first step leading to isoflavonoid and flavonoid biosynthesis. Few studies have been made on its activity in plant cells producing phytoalexins. In the cotyledons of Glycine max treated with cell wall components of Phytophthora megasperma f. sp. glycinea leading to glyceollin formation, the CHS reached a maximum one day after treatment (Zahringer et al., 1978). CHS followed

the induction of PAL activity and preceded the accumulation of the phytoalexin glyceollin by a few hours. In the cell suspensions of Phaseolus vulgaris treated with denatured ribonuclease or a heat-released pathogen component of Colletotrichum lindemuthianum interaction, CHS increased more rapidly and transiently, preceding the formation of the phytoalexin phaseollin. The kinetics of CHS induction were similar to those of PAL. Significant differences were observed in activities of PAL and CHS with dose of pathogen component. 17.5 and 50µg carbohydrate (measured as glucose equivalents = carbohydrate equivalents) were required for optimal PAL induction, while ⌣ 100µg carbohydrate was required for optimal CHS induction. Changes in CHS activity exactly paralleled changes in phaseollin concentration with dose of pathogen component (Lawton et al., 1980; Dixon et al., 1981; Lawton et al., 1983a,b). Transient increases in both CHS and PAL were also observed by Robbins et al. (1985) for kievetone formation in the same host-pathogen interaction. Changes in CHS activity were also found to parallel changes in 5-hydoxyflavonoids in cotyledons of Phaseolus vulgaris. The CHS was induced by wounding as was also observed for PAL (Whitehead et al., 1982).

(v) Chalcone isomerase (CHI). Chalcone isomerase catalyses the formation of isoflavones from chalcones (Grisebach, 1965). In hypocotyls of Glycine max treated with cell wall components of Phytophthora megasperma f.sp. glycinea, leading to glyceollin formation (Partridge & Keen, 1977), and in suspension cultures of Phaseolus vulgaris treated with denatured ribonuclease (Dixon & Bendall, 1978b) and heat-released pathogen components of Colletotrichum lindemuthianum interaction leading to phaseollin formation (Dixon & Lamb, 1979), CHI increases slowly, but does increase above a relatively higher basal level when compared to CHS or PAL. CHI was inhibited more by phytoalexins (kievetone and coumestrol) than by phytoalexin-precursors in vitro (Dixon et al., 1982).

It is generally the case that induction of phytoalexin accumulation is

correlated with increases in the activity levels of the appropriate biosynthetic enzymes. For example, in the Ricinus communis - Rhizopus stolonifer interaction leading to the accumulation of the diterpenoid phytoalexin casbene, the enzyme casbene synthase was found to be induced (Lee & West, 1981).

1.3.5. Phytoalexin induction and control of biosynthesis by enzyme activity.

The marked increase noted in some enzymes over low basal levels described above led Lamb's school to the conclusion that these enzymes regulate the flux through a metabolic pathway leading to phytoalexin synthesis (Dixon et al., 1983a). They found increases in activities of PAL, CA4H, 4-coumarate:CoA ligase, and CHS over low basal levels in Phaseolus vulgaris cell suspension cultures concomitant with phaseollin accumulation following treatment with heat-released Colletotrichum lindemuthianum components (Dixon & Bendall, 1978b; Lawton et al., 1983a; Dixon & Lamb, 1979). In contrast CHI is present at relatively high levels in control cultures and responds only sluggishly to elicitor treatment, and would therefore not be considered important in control of phaseollin accumulation in this system (Dixon & Bendall, 1978b; Dixon & Lamb, 1979).

Major control sites within this group of enzymes (PAL, CA4H, 4-coumarate:CoA ligase and CHS) were selected according to whether or not they undergo broadly concomitant changes in activity levels with phytoalexin formation. Lamb & Rubery (1976a) illustrated this finding in illuminated tissues of Solanum tuberosum. On this basis, Lamb's school selected PAL and CHS, rather than CA4H and 4-coumarate:CoA ligase as major control sites in phaseollin production in Phaseolus vulgaris cell cultures (Dixon & Bendall, 1978a). The location of PAL and CHS in the metabolic pathway suggested PAL to be a primary control point for phenylpropanoid-derived phytoalexins and CHS a secondary control point for

biosynthesis of a specific branch of the specific phenylpropanoid-derived product, flavonoids and isoflavonoids (Schroder et al., 1979), as suggested by preliminary research work by Lamb (1977) in illuminated potato discs. This view was supported by the induction of wall bound phenolics in addition to phaseollin in suspension cultures of Phaseolus vulgaris during induction with pathogen compounds (Dixon & Bendall, 1978a), inhibition of light-induced CHS activity by pathogen molecules in Petroselinum hortense cultures (Hahlbrock et al., 1981) and additional weak induction of PAL activity concomitant with glyceollin accumulation in wounded hypocotyls of Glycine max (Partridge & Keen, 1977).

1.3.6. Phytoalexin induction and control of biosynthesis by substrate supply.

Although increase in extractable PAL activity preceded phenylpropanoid-derived phytoalexin accumulation in most instances (e.g. Hadwiger & Schwochau, 1970; Rathmell, 1973), the association of PAL with phenylpropanoid-derived phytoalexins was inconsistent. In both cotyledons and hypocotyls of Phaseolus vulgaris phytoalexin elicitation sometimes occurred even when PAL was suppressed below the wounded control levels (Whitehead et al., 1982).

These discrepancies in temporal association were followed by discrepancies in spatial association between PAL and the phenylpropanoid-derived phytoalexins that accumulate. For example, in tissues of Vigna sinensis, application of salt solutions of cupric chloride, actinomycin D and cycloheximide resulted in an increase in PAL activity in both upper and lower halves of hypocotyls while kievetone accumulated only in the upper half (Munn & Drysdale, 1975).

Although these results could be explained by our lack of understanding of the influence of cellular and subcellular sites of accumulation of

phytoalexin synthesis, especially under altered environmental conditions, Margna in 1977 found no explanation in the literature for the consistent observation that PAL activities in Phaseolus vulgaris, and many other plant species were much too high to account for levels of phenylpropanoid-derived phytoalexins that accumulated (Margna, 1977).

In 1983, Dixon et al (see Dixon et al. 1983a) suggested these high levels could be due to errors in estimating enzyme activities of PAL in vitro. Amrhein et al. in 1976, devised an indirect in vivo assay for PAL activity. This assay consisted of feeding ^{3}H labelled phenylalanine and measuring the accumulation of ^{3}H in water as ammonia is released by PAL activity. Errors due to inefficient extraction, proteolytic enzyme degradation, or phenolic oxidation could explain decreased but not increased activities of PAL measured. It was suggested that different isoenzymic forms of PAL (Bolwell et al., 1986) may account for some of these discrepancies (Smith & Banks, 1986).

The suggested role of PAL as the primary controller of phenylpropanoid-derived phytoalexin formation was the subject of much criticism by Margna in 1977. The high increase in PAL activities, 10 to 100 times more than that accountable for the phenylpropanoid phytoalexins accumulated, led Margna to argue in favor of substrate (L-phenylalanine) supply as being a primary controller of the flux through the phenylpropanoid phytoalexin pathway. The evidence in support of this argument was the importance of L-phenylalanine as a structural component of phenylpropanoids and phenylpropanoid-derived phytoalexins. L-phenylalanine contributes to the second, third and fourth carbon atoms of the flavonoids (Camm & Towers, 1973) and to ring B. L-phenylalanine also forms an important structural component of isoflavonoid phytoalexins; for example, [^{14}C]-phenylalanine is incorporated into phaseollin in vitro and in vivo as mentioned above. However, Margna found that endogenous concentrations of unbound L-phenylalanine in the leaf tissue remained as low as 0.1 to $0.2\mu M.gFW^{-1}$ and, further, that the L-phenylalanine pool size remained

constant during rapid fluctuations in phenylpropanoid synthesis. If tRNAs for L-phenylalanine are not sensitive to feedback inhibition, it seemed likely to Margna that the available L-phenylalanine was under direct competition between the increased PAL activity and by the enzymes of protein synthesis. Margna further investigated the association between PAL activity and protein synthesis both in various systems. In agreement with a literature survey he had made he found a decrease in protein synthesis associated with an increase in phenylpropanoid production. Margna therefore concluded that although the pool sizes did not change, the high levels of PAL activity were determined by L-phenylalanine made available by inhibition of protein synthesis.

Margna's view was the subject of much criticism by Lamb's school (Lamb & Rubery, 1976 a, b, c ; Dixon et al., 1983a). For them, Margna's view did not explain (i) the physiological role of the negative cooperativity exhibited by PAL; (ii) the often constant phenylalanine pool sizes measured during rapid fluctuations in the rates of phenylpropanoid synthesis (Amrhein & Zenk, 1971) and (iii) the limited evidence against changes in the levels or extractable activities of enzymes of the shikimic acid pathway during phenylpropanoid biosynthesis (Minamikawa & Uritani, 1967). All these points could be explained by the theory proposed by the school of Lamb and coworkers namely, the regulation of phenylpropanoid-derived phytoalexin formation by enzyme activity, primarily PAL (Dixon et al., 1983a).

1.3.7. Phytoalexin formation and control of biosynthesis by synthesis of enzyme protein.

In section 1.3.2. indirect evidence was provided for protein synthesis as a pre-requisite for phytoalexin synthesis, especially by Hadwiger and associates.

Further evidence for this observation comes from the finding that inhibitors of protein synthesis prevent necrosis and phytoalexin accumulation in response to infection (Doke & Tomiyama, 1975; Kojima & Uritani,1976; Vance & Sherwood, 1976; Tani & Yamamoto, 1978, 1979; Yoshikawa et al., 1978), although there were a few exceptions (Doke & Tomiyama, 1975). In addition, an increase in overall rate of RNA synthesis occurs in a number of systems following infection (e.g. Yoshikawa et al., 1978) and inhibitors of RNA synthesis have been observed to inhibit necrosis (Yoshikawa et al., 1978), phytoalexin accumulation and induction of appropriate biosynthetic enzymes (Yoshikawa et al., 1978; Sander & Hadwiger, 1979).

Inhibitors of RNA and protein synthesis did not always give the results described above. Low as opposed to high concentrations of actinomycin D or cycloheximide induced pisatin formation in Pisum sativum and this induction was often accompanied by a decrease in total incorporation of precursors into RNA (Schwochau & Hadwiger, 1968), or protein (Hadwiger 1972). Further, Biggs in 1972 presented evidence for discrepancies that may arise when the labelled precursors used to follow RNA and protein synthesis or the RNA and protein synthesis inhibitor were added at different times. These results could be explained by possible side effects that may arise during inhibition of RNA and protein synthesis (Ellis & McDonald, 1970).

Following the above studies, Lamb and coworkers decided to adopt an isotope labelling technique to explore the possible control of biosynthesis of phytoalexin formation by synthesis of enzyme protein.

In vivo density labelling experiments with ^{2}H followed by analysis of PAL activity after CsCl density gradient centrifugation were conducted on suspension cultures of Phaseolus vulgaris treated with denatured ribonuclease or heat-released pathogen constituents of Colletotrichum lindemuthianum to induce phaseollin formation. The results obtained

demonstrated a marked increase in the rate of labelling of PAL (Lamb & Dixon, 1978; Dixon & Lamb, 1979). These results were confirmed using a higher-resolution density gradient solute, KBr, which unlike CsCl allowed quantitative measurement of the amounts of ^{2}H-labelled and ^{2}H-unlabelled enzyme independent of assumptions about, or measurement of, the specific activity of label in the amino acid pool from which the enzyme was synthesised (Lawton et al., 1980). In addition, different responses were obtained at different doses of pathogen. At low concentrations of pathogen component, an increase in PAL activity occurred associated with an increase in rate of de novo synthesis but accompanied by a constant rate of removal of active enzyme. At higher concentrations of pathogen component, the increase in PAL activity was accompanied by a marked apparent stabilisation of the enzyme in vivo, and the rapid but transient increase in enzyme activity was achieved by reciprocal changes in the rate constant for de novo enzyme production and the rate constant for removal of enzyme activity (Lawton et al., 1980).

Lamb and coworkers were aware of the limitations of the density labelling technique. The first limitation was the need for adequate separation of labelled and unlabelled enzyme, and hence the need for a stable enzyme. The second limitation was the non-applicability of the technique to short pulses as ratios of labelled to unlabelled enzyme had to be significant, and the third was the absence of information on possible changes in the synthesis of active or inactive PAL protein (Lamb & Rubery, 1976c).

A technique which proved ideal in meeting these short-comings was the simple immunoprecipitation of radiolabelled protein followed by SDS-polyacrylamide gel electrophoresis of the immunoprecipitates and estimation of the incorporation of label into the enzyme subunits (Dixon et al., 1983).

Re-examination of the same system by Lamb and coworkers using this technique

with ^{35}S-labelled methionine confirmed the results obtained with density labelling and further showed marked but transient increases in the rates of synthesis of both PAL and CHS concomitant with the phase of rapid increase in enzyme activity at the onset of phaseollin accumulation. Increased rates of synthesis of both PAL and CHS enzymes were observed 20 minutes after treatment. The patterns of induction of synthesis of PAL and CHS were broadly similar with respect to concentration of pathogen and time of application. The results obtained with respect to concentration of pathogen confirmed the results described in section 1.3.4 (iv). The maximum rates of synthesis of PAL and CHS were between 2.5 and 3.0 hours after elicitor treatment, CHS occurring 20 to 30 minutes earlier than PAL. The synthesis of PAL and CHS each accounted for between 0.5 and 1.0% of the total protein synthesis in tissue of the host, Phaseolus vulgaris (Lawton et al.,1983a,b).

Using a similar technique, Loschke et al. (1981) found a similar rapid, transient increase in the rate of synthesis of PAL correlated with rapid increase in enzyme activity in the Pisum sativum - Fusarium solani f. sp. pisi / F. solani f. sp. phaseoli interactions (Loschke et al., 1981). Hahlbrock et al. (1981) obtained similar results using the interaction between suspension cultures of Petroselinum hortense and high molecular weight constituents of the pathogen Phytophthora megasperma f.sp. glycinea which lead to phytoalexin formation (Hahlbrock et al., 1981).

Lamb and coworkers went on to study in vitro protein synthesis using a rabbit reticulocyte lysate translation system coupled with immunoprecipitation of PAL subunits. In the systems described above, namely Petroselinum hortense (Hahlbrock et al., 1981) and Phaseolus vulgaris, (Lawton et al., 1983a,b) there was an increase in the activity of mRNA encoding for PAL prior to phytoalexin production. Pathogen-induced changes in the activity levels of these polysomal mRNAs closely followed changes in the rate of enzyme synthesis as measured by the in vivo labelling

experiments described above. It was also shown that the increase in enzyme synthesis observed did not reflect the selective recruitment of these mRNAs into polysomes (Lawton et al.,1983b). In 1984, Lamb and coworkers reported an early localised increase in CHS mRNA activity in Phaseolus vulgaris hypocotyls resisting Colletotrichum lindemuthianum, prior to phaseollin accumulation (Bell et al., 1984). In susceptible interactions there was a delayed increase in CHS mRNA activity, and a widespread increase as lesions developed. Smith & Banks (1986) suggested that these results rather overemphasised the importance of CHS in the resistant reaction. Increase in CHS mRNA was also observed for the Petroselinum interaction described above by Ryder et al. (1984). In 1983, Loschke et al. obtained a similar increase in the mRNA which translated PAL in vitro. The system used was the pea endocarp and the inducers of pisatin synthesis were chitosan, actinomycin D, ultraviolet light, 4,5',8-trimethylpsoralen or infection with the pathogen Fusarium solani f.sp. phaseoli (Loschke et al., 1983). Ebel et al., in 1984 also obtained an increase in PAL and CHS mRNA synthesis in the Glycine max - Phytophthora megasperma var. sojae interaction. The increase in PAL and CHS mRNA correlated with increased activities of PAL and CHS (Ebel et al., 1984).

These results suggested the importance of PAL and CHS protein synthesis in the regulation of phytoalexin formation.

1.3.8. Phytoalexin induction and the transient control of PAL activity.

Results described above, especially those obtained by density labelling studies in section 1.3.7 showed a transience in PAL activity with time and dose of pathogen constituent. This transience was also found by Faye in radish seedlings activated by light instead of pathogen (Faye, 1975). He observed PAL activities to increase over an 18 hour period, remain at a maximum for 48 hours and rapidly decline over another 18 hour period (Faye, 1975). Neither density labelling nor immunoprecipitation explained this

transience in PAL activity and to date, the molecular mechanisms which determine the inactivation of active PAL protein have not yet been satisfactorily resolved. Catalytically inactive and active forms of PAL have been reported in other systems by Engelsma in 1967, Zucker in 1968, Attridge & Smith in 1973, Creasy et al in 1974, Faye in 1975 and Creasy in 1976. There are several views that have been put forward to explain the activation of the newly synthesised inactive PAL protein implied in above studies:

(i) Post-translational control. Lamb and coworkers suggested the transience to be regulated at the post-translational level (Lawton et al., 1980). Possible post-translational controls could be the addition of the active site (thought to be a dehydroalanyl residue, Hanson & Havir, 1981); or an S=S ---> -SH interconversion of a sulphydryl enzyme as suggested by the dependence of de novo synthesised PAL protein on cysteine residues, which are though to activate it (Hanson & Havir, 1981).

(ii) End-product regulation. Lamb and coworkers also found evidence for end-product inhibition of PAL activity by t-cinnamic acid in vitro. They suggested that PAL activity was controlled in situ in this way. Using density labelling experiments, they found exogenous t-cinnamic acid to inhibit both the de novo synthesis of PAL and also to stimulate the rate of removal of active enzyme. There were therefore 2 mechanisms by which PAL was regulated by t-cinnamic acid (Shields et al., 1982). Although the molecular mechanisms underlying these effects remain to be elucidated, it has been established that this dual-feedback control mechanism operates in vivo following endogenous production of cinnamic acid. Changes in the levels and compartmentalisation of cinnamic acid and biosynthetic derivatives during phytoalexin accumulation might then account for modulation of the apparent stability of the enzyme in vivo following elicitor treatment.

(iii) Inhibitor/activator regulation. It was possible that activation and inactivation of PAL could also be due to the presence of inhibitors specific or non-specific for PAL, e.g. the de novo synthesis of a regulatory protein or another effector

(Engelsma, 1967; Blondel et al., 1973; French & Smith, 1975; Tanaka et al., 1977; Billett et al., 1978). Such high molecular weight inhibitors of PAL have been reported in other systems but the mechanism of their role in control of PAL activity remains unknown (Creasy, 1976; Tanaka et al., 1977; Billett et al.,1978). The regulation is thought to depend on the differences in the rate of turnover of the regulatory molecule and PAL protein (Zucker, 1968; Smith et al., 1977).

(iv) Substrate (L-phenylalanine) availability. Evidence for this proposal is indirect. De novo PAL protein synthesis occurs under conditions of limited nitrogen or depletion of nitrate (Havir, 1981). Addition of exogenous sucrose in the absence of light also triggers de novo synthesis of PAL protein (Creasy, 1976). These workers have suggested the dependence of PAL protein and activity on L-phenylalanine availability, which is in turn dependent on light and the flux into protein synthesis. Further, the inactivation of de novo synthesised active PAL protein is triggered by the onset of darkness and the consequent loss of photosynthetic products (Zucker, 1968; 1972).

The availability of L-phenylalanine depends on light and photosynthesis as follows: During the light phase the 'source' (leaf chloroplast) traps electromagnetic energy of sunlight to generate ATP and NADPH which are used to convert CO_2 to carbohydrates (starch and sucrose) and to fatty acids

(acetyl CoA and malonyl CoA) for temporary storage in primary sinks (cytoplasm). Light activates enzymes of the reductive pentose phosphate pathway in proportion to the light intensity and photosynthetic rates (Fridland & Kaler, 1984). High CO_2 fixation rates, high triose phosphate and minimal Pi concentrations outside the chloroplast permit synthesis of starch and diversion of carbohydrates into nitrogen compounds, amino acids and into protein synthesis (Schulze-Siebert et al., 1984). When high nitrogen levels are present these conditions lead to a decrease in sucrose phosphate synthase (Huber et al., 1984a,b). Translocation to secondary sinks (fruits, storage organs, roots) is limited in the light. Key enzymes of the glycolytic pathway (e.g.phosphofructokinase) and the oxidative pentose phosphate cycle (e.g.glucose-6-phosphate dehydrogenase) are deactivated (Buchanan et al., 1981; Hampp et al., 1985). At the same time substrates of the shikimate pathway (E4P and PEP) are made available within the chloroplast (Schulze-Siebert et al., 1984). The additional availability of PEP leads to preferential synthesis of aromatic amino acids within the chloroplast (Schulze-Siebert et al., 1984). CO_2 and shikimic acid are incorporated into aromatic acids by chloroplast rich preparations of Spinacia oleracea leaves (Bickel et al., 1978; Bickel & Schultz, 1979; Buchholz & Schultz, 1980). Enzymes responsible for the synthesis of shikimic and chorismic acids are present in the chloroplast (Mousdale & Coggins, 1985). Light and photosynthesis are indeed necessary for synthesis of phenylpropanoid-derived phytoalexins.

But during the dark, the carbohydrates stored in the primary sinks (cytoplasm) are converted into sucrose and transported to secondary sinks. The activities of key enzymes of the glycolytic pathway (e.g.phosphofructokinase) and oxidative pentose phosphate cycle (e.g.glucose-6-phosphate dehydrogenase) are enhanced. These pathways lead to energy generation (ATP and NADPH) during the dark period. Enzymes involved in the reductive pentose phosphate cycle no longer fix CO_2. Low triose phosphate and maximal Pi concentrations outside the chloroplast do

not permit the diversion of carbon products into nitrogen metabolites (amino acids and proteins). Low nitrogen levels lead to an increase in sucrose phosphate synthase (Huber et al., 1984a,b). Carbohydrates are translocated to secondary sinks (fruits, storage organs etc.) while nitrogenous products like glutamine, aspartate and alanine are diverted into the glycolytic pathway and Krebs cycle. Substrates of the shikimate pathway leading to L-phenylalanine, PEP and E4P, are not readily available from the chloroplast reductive pentose phosphate pathway (by the triose-Pi translocator) and the cytosolic oxidative pentose phosphate pathway. High Pi levels in the cytoplasm are not available to promote the exchange. PEP may also be fed into the respiratory pathways providing a source of ATP and NADPH necessary for the shikimate pathway.

1.3.10. Conclusions of biochemical studies.

Biochemical exploration of the factors leading to phytoalexin formation led to the finding that some form of recognition occurs between molecules on the pathogen surface with the host cell surface. It is not clear whether the interaction is specific or non-specific. The transmission of factors within the host cell leading to phytoalexin formation elicited by pathogen constituents remains to be characterised. Protein synthesis was in some way involved in the interaction of pathogen constituents with the host for induction of phytoalexin synthesis. Some of the proteins synthesised and of importance to the induction of phytoalexin synthesis were enzymes responsible for phytoalexin synthesis. The primary control point of phenylpropanoid-derived phytoalexin synthesis was L-phenylalanine ammonia-lyase (PAL). The activity of PAL was regulated by the formation of newly synthesised PAL protein and by the transient formation of active and inactive PAL protein. The mechanisms of activation and inactivation remain unknown, although end product inhibition remains a possibility. The role of substrate (L-phenylalanine) availability in the regulation of PAL activity remains to be demonstrated. A factor essential for the synthesis of

L-phenylalanine is known to be the presence of an active 'source'.

OUTLINE OF RESEARCH WORK.

The experiments reported in this thesis were designed to understand further the induction of the phytoalexin response. The emphasis would be on the initial events leading to the induction of the phytoalexin response, and on the regulation of enzyme activity leading to the induction of the phytoalexin response.

The host-pathogen system chosen for this study was the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction. The reason for choosing this system was mainly because this interaction is one of the oldest known interactions and has been well characterised, as has been described above. Further,the phytoalexins formed in this interaction (as described above) are phenylpropanoid-derived isoflavonoid phytoalexins. The pathway leading to the synthesis of isoflavonoids is well characterised, sharing a common pathway with another enzymologicaly well defined pathway leading to general phenylpropanoid synthesis.

The areas studied in more detail were

(1) The association between necrosis or lesion formation, phytoalexin formation and PAL activity (Chapter 2, section 2.3.1).

(2) The mechanism of regulation of phenylpropanoid-derived phytoalexin formation (Chapter 2, section 2.3.2; chapter 5).

(3) The nature of the phytoalexin-inducing factor from the pathogen (Chapter 4) and elucidation of the mechanism of induction of the phytoalexin response (Chapter 2).

Most of the work was devoted to looking at the mechanism of regulation of phytoalexin formation. The sequence of studies presented is

(i) a study of the de novo synthesis of PAL protein (Chapter 2, section 2.3.2.2),

(ii) a search for the active and inactive forms of PAL protein (Chapter 6 and Chapter 7),

(iii) determination of the kinetics of PAL (Chapter 5),

(iv) investigation of the negative cooperativity of PAL with regard to substrate (Chapter 5),

(v) regulation of PAL by substrate availability and substrate supply (Chapter 2, section 2.3.2.3).

Each section of the work was investigated under conditions expected to be favorable (e.g. optimal 'source' metabolism) or unfavorable (e.g. high temperature) for the expression of necrosis and phytoalexin formation.

CHAPTER 2

INDUCTION OF HYPERSENSITIVE NECROSIS AND PHYTOALEXIN SYNTHESIS IN THE *PHASEOLUS VULGARIS* L. - *COLLETOTRICHUM LINDEMUTHIANUM* L. INTERACTION : INITIAL EVENTS

2.1 INTRODUCTION:

This chapter describes experiments to test the various responses associated with hypersensitive necrosis and the phytoalexin response in the *Phaseolus vulgaris* - *Colletotrichum lindemuthianum* interaction as described in Chapter 1. These are

(i) the ability of the host to respond to the pathogen by induction of hypersensitive necrosis, phytoalexin formation and PAL activity. To establish whether these responses were (a) correlated (b) age dependent and (c) related to photoperiod,

(ii) to study the mechanism of regulation of PAL activity, (in particular, its regulation by end-product inhibition, substrate supply, substrate availability and *de novo* synthesis of PAL protein) and relate these mechanisms to hypersensitive necrosis and the phytoalexin response,

and (iii) to establish the ability of the host to respond to the pathogen by recognition of specific determinants on the pathogen cell surface and to establish whether any links exist between the

recognition phenomena and hypersensitive necrosis, induced PAL activity and the phytoalexin response.

The Phaseolus vulgaris - Colletotrichum lindemuthianum interaction has been studied in two experimental systems:

(i) the whole (intact) host system and living and non-living pathogen mycelium,

(ii) the rapidly isolated leaf cell or leaf protoplast system and an abiotic pathogen mycelial component.

Whole (intact) host system - living and non-living pathogen mycelium:

This system was used because of the following experimental advantages:

(a) the response could be studied under natural developmental and environmental conditions,

(b) the response could be studied under conditions of normal interaction between cells and tissues of different types, existing in a normal `source´-`sink´ relationship,

(c) the response could be studied under conditions of minimal mechanical damage to the host tissue. Minimal damage to host tissue results in a minimal non-specific induction of phenylpropanoid phytoalexin synthesis,

(d) the response could be studied using the natural pathogen inoculum, viz. pathogen spores and mycelium,

(e) the response could be studied with minimal disturbance to phenylpropanoid phytoalexin biosynthesis,

and (f) the response could be studied under conditions requiring photoperiod.

On the other hand, the whole (intact) plant system had the following disadvantages:

(a) the response could not be quantified i.e. pathogen interaction per host cell,

and (b) the response could be a reflection of contamination by other pathogenic micro-organisms in the environment, such as bacteria.

Rapidly isolated leaf cell and protoplast system - abiotic pathogen mycelial component:

This system was used because it had some of the advantages (and not disadvantages) of the whole (intact) system described above:

The disadvantages (a) and (b) described above were overcome by using this system, coupled with the following additional advantages:

(a) the response could be studied with minimal disturbance to phenylpropanoid-derived phytoalexin biosynthesis, leading to the production of similar phenylpropanoid-derived phytoalexins as those produced by the whole (intact) host plant,

(b) the response could be studied under conditions requiring photoperiod,

(c) the response could be studied under conditions of minimal damage to the host tissue. Minimal damage to host tissue results in a minimal induction of phenylpropanoid-derived phytoalexin synthesis,

and (d) the response could be studied under conditions of a normal `source´-`primary sink´ relationship.

Besides having the above advantages, rapidly isolated single cells and protoplasts have been found to synthesise proteins similar to those of the whole (intact) host system (Fleck et al. 1979, 1980, 1982).

These advantages suggested that similar experiments could be conducted in the isolated cell and protoplast system as in the whole (intact) system, but with some disadvantages:

(a) the response could not be studied under natural developmental and environmental conditions but under controlled conditions,

(b) the response could not be studied under conditions of normal

interaction between cells and tissues of different types,

and (c) the response could not be studied using the natural pathogen inoculum, viz. pathogen spores and mycelium.

The results described in this chapter are in 3 sections,

(i) the interaction between the whole (intact) host system and living and non-living pathogen mycelium,

(ii) the interaction between the rapidly isolated leaf cell system and an abiotic pathogen mycelial component,

and (iii) the interaction between the isolated leaf protoplast system and an abiotic pathogen mycelial component.

2.2. MATERIALS AND METHODS:

2.2.1. METHODS:

2.2.1.1. Growth of plant material:

Seeds of Phaseolus vulgaris L. var. Prince were sown in autoclaved graded horticultural perlite, and maintained under greenhouse conditions for 1 week. Seedlings were transferred to pots containing Levington soilless potting compost. Seedlings for whole plant experiments were maintained under greenhouse conditions for 2 weeks.

Abbreviations: CCD, counter-current distribution; CoA, coenzyme A; DEAE, diethyl aminoethyl; DTT, dithiothreitol; MES, methyl-ethyl-sulphonate; MSH, 2-mercaptoethanol; NAD, nicotine adenine dinucleotide; PAL, phenylalanine ammonia-lyase; PAT, phenylalanine amino-transferase; PHA, phytohemagglutinin; PVP, polyvinylpyrrolidone; SDS-PAGE, lauryl sulphate (sodium salt)- polyacrylamide gel electrophoresis; T, percentage total monomer i.e. grammes acrylamide plus bisacrylamide; TCA, trichloroacetic acid; TPP, thiamine pyrophosphate.

PMSF, p-methylsulphonyl fluoride; POPOP, 2,2 -p-phenylene-bis[5-phenyloxazole]; PPO, 2,5,diphenyloxazole

Seedlings for isolation of single cells and protoplasts were grown in a `propatray´ at 23°C, 64 to 75% relative humidity, 20 $w.m^{-2}$ with warm white fluorescent tubes (Iris fluor, 9L, MCFE, Griffin & George Ltd., Wembley, Middlesex) under a daylength of 16 hours, for up to 5 weeks. The 2 basal leaves of the plant were used in all experiments. Plants were kept at 90% relative humidity for 6 hours prior to isolation of protoplasts.

2.2.1.2. Rapid isolation of cells by removal of middle lamella from intact leaf tissue:

Cells were isolated by the method of Servaites & Ogren (1977) using the apparatus shown in Fig. 24. Leaves were rinsed in sterile distilled water, the midrib removed, cut into 1 x 0.1cm strips and vacuum infiltrated for 15 seconds in enzyme solution [20mM MES, pH 5.8, 12.5mM K_2SO_4, 2% (w/v) PVP-40 and 3% (w/v) Macerase]. The enzyme solution was discarded and leaf material placed in the maceration chamber (Fig. 24). 50ml enzyme solution [0.3M sorbitol, 20mM MES, pH 5.8, 12.5mM K_2SO_4, 2% (w/v) PVP-40 and 3% (w/v) Macerase] was circulated through the maceration chamber at 10ml.min^{-1} using a peristaltic pump (Fig. 24). Cells (devoid of middle lamella) were collected on a 25µm nylon mesh and washed in medium [50mM Tris-HCl, pH 7.8, 0.2M sorbitol, 5mM KNO_3, 2mM $CaNO_3$ and 1mM $MgCl_2$]. Washes were repeated twice in Mc´Cartney tubes by centrifugation at 780 x g for 5 minutes at 18 to 25°C. Cells were finally washed and suspended in medium [50mM Tris-HCl, pH 7.8, 0.2M sorbitol, 5mM KNO_3, 2mM $CaNO_3$, 1mM $MgCl_2$ and 5mM DTT].

2.2.1.3A. Isolation of protoplasts:

Protoplasts were isolated by modification of the procedure of Pelcher et al. (1974) as follows: Leaves were washed in 10% (v/v) Domestos [10.5% (v/v) commercial sodium hypochlorite, 0.3% (w/v) Na_2CO_3, 10.0% (w/v) NaCl, 0.5% (w/v) NaOH, patented thickener, "softened" water to 100%] containing 0.05% (w/v) Tween-80 for 5 minutes followed by immersion in 70% (v/v) EtOH for 2 minutes, then washed in two changes of osmotic solution [0.3M mannitol and $2.4ng.ml^{-1}$ bromocresol purple, made to pH 5.8 with 0.2N KOH using bromocresol purple as pH indicator]. Leaves were cut into 1 x 1 cm sections and lower epidermis peeled with forceps. Sections were placed in petri dishes containing 10ml enzyme solution [0.3M mannitol, $2.4ng.ml^{-1}$ bromocresol purple, 0.25% (w/v) Driselase and 0.25% (v/v) Pectinase adjusted to pH 7.0 with 0.2N KOH using bromocresol purple as visual indicator of pH] and incubated in the dark for 18 hours at 23 to 25°C. Protoplasts were separated from debris by passage through an 85µm nylon mesh and suspended in McCartney tubes containing isotonic Pelcher's B_5 medium adjusted to pH 5.8 using bromocresol purple as visual indicator of pH (Table 1).

Table 1. Isotonic and hypotonic Pelcher's B_5 medium used for the isolation of protoplasts:

Medium component	Concentration
Major nutrients:	
KNO_3	24.73mM
$CaCl_2 2H_2O$	7.82mM
$NaH_2PO_4 H_2O$	1.09mM
$(NH_4)_2SO_4$	1.02mM
$MgSO_4 7H_2O$	1.02mM
Minor nutrients:	
Na_2EDTA	0.10mM
$FeSO_4 7H_2O$	10^{-4} M
$MnSO_4 H_2O$	10^{-5} M
H_3BO_3	5.92×10^{-6} M
$ZnSO_4 7H_2O$	4.85×10^{-6} M
KI	6.96×10^{-6} M
$Na_2MoO_4 2H_2O$	4.52×10^{-6} M
$CoCl_2 6H_2O$	1.03×10^{-7} M
$CuSO_4 5H_2O$	1.05×10^{-7} M
	1.00×10^{-7} M
Vitamins:	
thiamine-HCl	2.96×10^{-5} M
nicotinic acid	8.12×10^{-6} M
pyridoxine-HCl	4.86×10^{-6} M
Nitrogen source:	
casein hydrolysate	$1g.l^{-1}$
hydroxy-L-proline	0.76mM
Growth hormones:	
kinetin	9.29×10^{-6} M
2,4-dichlorophenoxyacetic acid	4.52×10^{-6} M
Carbon source:	
mannitol	300.01mM
sucrose*	58.43mM
D-glucose*	100.00mM
D-ribose*	3.33mM
D-xylose*	3.33mM
myo-inositol	0.56mM
pH indicator:	
bromocresol purple	$8.0mgs.l^{-1}$

*sugars excluded to form a hypotonic medium.

2.2.1.3B. Purification of protoplasts:

Protoplasts were washed 3 times in modified Pelcher's B_5 medium containing bromocresol purple by centrifugation at 45 x g at 18 to 25°C. The protoplast pellet was suspended in 0.6 ml counter-current distribution (CCD) buffer [0.3M sorbitol, 0.05M Tris-KOH, pH 8.0, 5mM $MgCl_2$ and 0.1% (w/v) BSA]. CCD solution [1.1ml 30% (w/v) polyethylene glycol MW 6,000, 3.0ml 20% (w/w) dextran T_{40}, 0.3ml 0.5M Na_2HPO_4-NaH_2PO_4, pH 7.5 and 1.0ml 1.62M sorbitol] was placed in 13 x 100mm test-tubes at 4°C and thoroughly mixed by inversion (20 times). 0.6ml protoplast suspension was layered on top of the solution and the tubes centrifuged at 300 x g for 6 minutes at 4°C. The interface and the top layer was then layered on a similar gradient and the process repeated. The interface containing the intact protoplasts was suspended in CCD buffer [0.3M sorbitol, 0.05M Tris-KOH, pH 8.0, 5mM $MgCl_2$ and 0.1% (w/v) BSA], and centrifuged at 400 x g for 90 seconds at 18 to 25°C. The pellet containing purified protoplasts was suspended in modified Pelcher's B5 medium.

2.2.1.4. Growth of pathogen material:

Growth of pathogen material for live mycelia:

The pathogen Colletotrichum lindemuthianum L. isolate CMI 112166 was grown as agar slants in McCartney tubes, in nutrient medium [3.26mM glucose, 5.0mM $MgSO_4$, 20mM KH_2PO_4 and 0.2% (w/v) Neopeptone at pH 4.9, Mathur *et al.*, 1950].

Cultures were grown in the dark, at 22°C. The inoculum consisted of 10-day-old mycelium suspended in distilled water at a concentration of 10^{-3} gDW.ml^{-1}.

Growth of pathogen material for isolation of pathogen asialoglycoprotein:

The pathogen Colletotrichum lindemuthianum L. was grown in a nutrient medium containing 3.26mM glucose, 5.0mM $MgSO_4$, 20.0mM KH_2PO_4 and 2g.litre^{-1} Neopeptone at pH 4.9 (Mathur *et al.*, 1950). Cultures were maintained in a 50 litre fermentor vessel under 14 hours light at 5 to 7 w.m^{-2} with warm white fluorescent tubes (Sylvania, Germany) for 8 days at 20°C.

2.2.1.5 Isolation of pathogen cell wall asialoglycoprotein:

2.2.1.5.1. Isolation of pathogen cell walls:

Mycelia from 8-day-old cultures were harvested on a glass-sintered funnel and homogenised for 1 minute with 5 ml distilled water g.FW^{-1} mycelia. The mycelial extract was filtered through a glass-sintered funnel and the residue subjected three times to the same treatment. This was followed by washing the cell walls in chloroform:methanol (1:1 v/v) by homogenisation for 1 minute and filtration through a glass-sintered funnel. The residue was finally homogenised with acetone for 1 minute and filtered through a glass-sintered funnel. An osteriser was used for homogenisation. The final residue containing

fungal cell wall material was air-dried and stored at -20°C.

2.2.1.5.2. Isolation of pathogen cell wall constituents:

A 1% (w/v) cell wall suspension in distilled water was autoclaved for 20 minutes at 121°C. The extract was filtered through a glass-sintered funnel and further clarified by passage through a 5.0μm, 0.45μm and 0.22μm millipore filter. The extract was freeze-dried and stored at -20°C.

2.2.1.5.3. Purification of pathogen cell wall constituents:

The extract was made to 0.02M Tris-HCl with 0.02%(w/v) sodium azide and subjected to protein precipitation with cold acetone to 80% at 4°C for 1 to 12 hours. Samples were centrifuged at 20,000 x g for 10 minutes at 4°C and protein pellet suspended in buffer [2.8M NaCl in 24mM Na_2HPO_4-NaH_2PO_4 buffer, pH 7.2] at a concentration of 0.5mg.ml^{-1} carbohydrate. The protein was precipitated with 5mg carbohydrate^{-1}.mg concanavalin A and the precipitate quantified by techniques used by Clarke and Denborough (1971). Concanavalin A was separated from the glycoprotein by passage through an anion exchange DEAE-Sephacel column (80 x 2.2cm) in presence of 0.2M to 0.5M methyl-α-D-mannoside according to the method of Leon (1967).

2.2.1.6. Induction of hypersensitive necrosis:

Plants were treated at different ages by infiltrating a suspension of mycelia using a Hagborg device (Hagborg, 1970). This device (Fig. 2) consisted of a pair of blunt forceps with rubber bungs mounted in the jaws. A circular cavity (about 2 cms deep) was made in one of the rubber bungs and a blunt hypodermic needle passed through from the opposite end so as to just enter the cavity. The lower side of the leaf was held firmly between the forceps and the inoculum forced through a hypodermic syringe, through the needle, into the cavity and then through the open stomata into the mesophyll. Areas around the major vein were the sites of inoculation. The plants were incubated at 100% relative humidity to stimulate hyphal growth. Control inoculations were carried out with distilled water. Symptom expression was recorded for 2 weeks. The number of lesions.gFW^{-1} leaf material were used to quantitate the degree of hypersensitive response. Hypersensitive necrosis was defined as dark green areas at inoculated sites, 1mm or more in diameter. Dark brown areas accompanied by rapid senescence were representative of leaves susceptible to the pathogen.

2.2.1.7. Estimation of phaseollin:

Leaf material was frozen in liquid N_2 ($-198.5^{o}C$) and ground in a mortar and pestle in 80% (v/v) EtOH at a concentration of $1.5ml.gFW^{-1}$ leaf material. The extract was filtered through Whatman No. 1 filter paper and the filtrate dried <u>in vacuo</u> in a rotary evaporator at $40^{o}C$. The residue was dissolved in 5ml distilled water, and partitioned 4

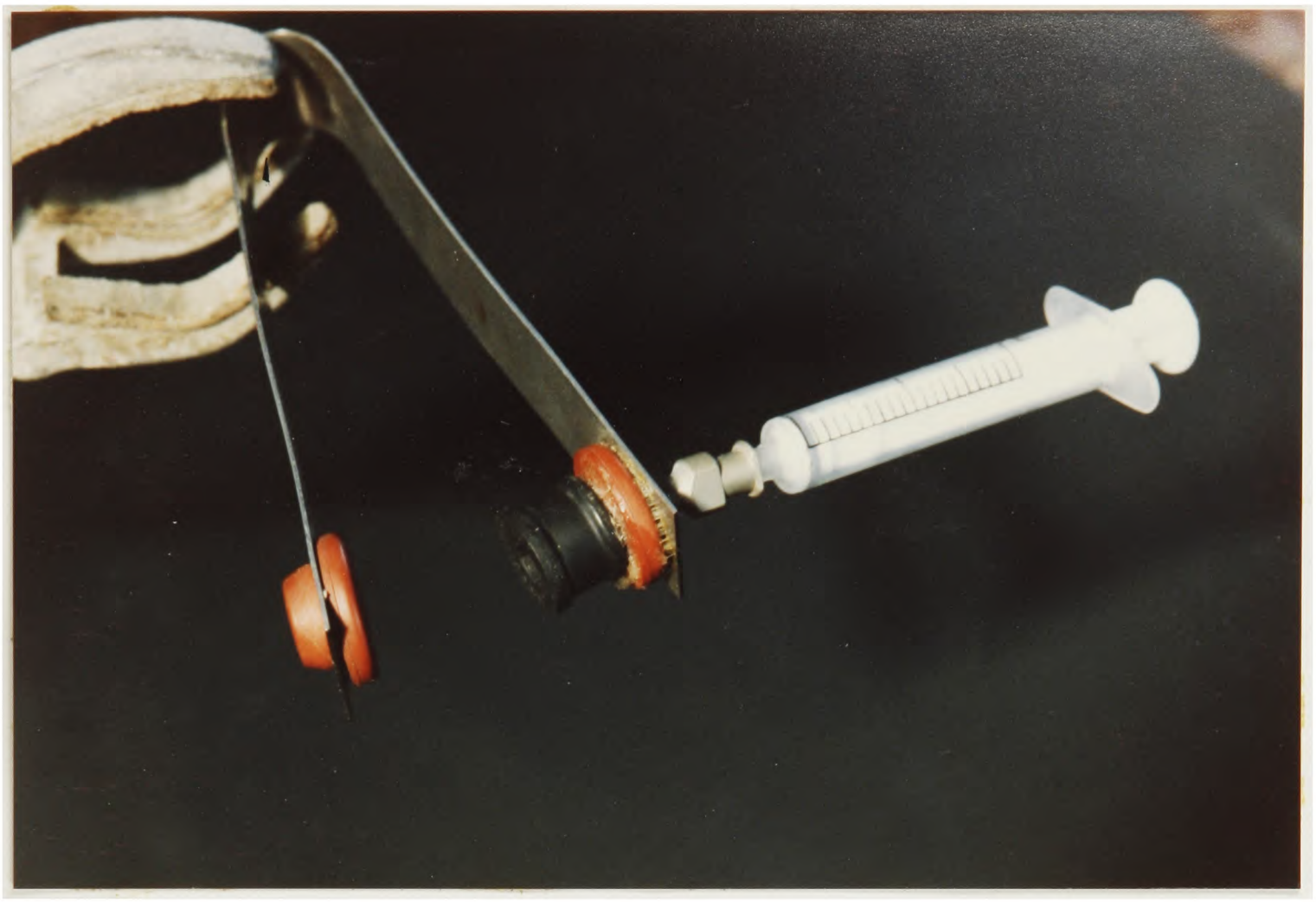


Fig. 2 Hagborg device used for induction of hypersensitive necrosis and the phytoalexin response in the leaves of the host plant Phaseolus vulgaris L. to the fungal pathogen Colletotrichum lindemuthianum L.

For further details see `methods´.

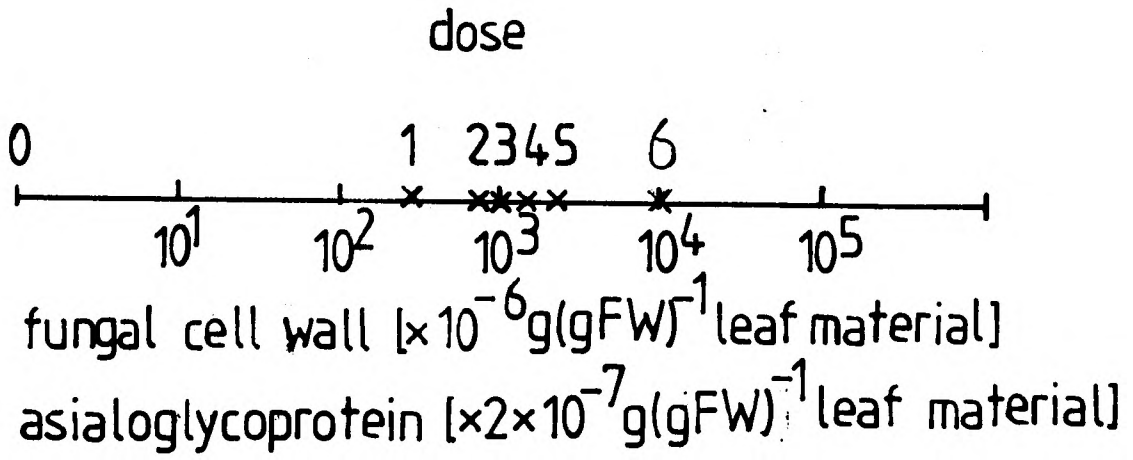


Fig. 3 Dosage scale of fungal pathogen cell wall asialoglycoprotein used to study initial events in the induction of the hypersensitive necrosis and the phytoalexin response in the host plant. (continued on facing page)

times against an equal volume of petroleum ether (40 to 60°C). The petroleum ether fractions were pooled together, and dried *in vacuo* in a rotary evaporator at 25°C. The residue was dissolved in 90% (v/v) EtOH and samples spotted on silica gel/UV254 TLC plates using disposable 20µl pre-calibrated pipettes. TLC plates were run in $CHCl_3$:MeOH [50:2 (v/v)]. Under these conditions, phaseollin had an R_f of 0.71 to 0.75. Samples were eluted in 90% (v/v) EtOH, the spectrum was scanned and the absorption read in a Perkin-Elmer 551S UV/VIS spectrophotometer at 280nm. Phaseollin was quantified using the molar extinction coefficient 9.3×10^3 $mol^{-1}.cm^{-1}$ of phaseollin at 280nm (Cruickshank & Perrin, 1971).

2.2.1.8. Determination of PAL activity:

Preparation of homogenates:

Leaf material was ground in a mortar and pestle (previously chilled to -70°C), in liquid N_2 (-198.5°C). 1/10 (w/v) insoluble PVP was added in extraction buffer [0.1M $Na_2B_4O_7$-HCl buffer, pH 8.8 and 5mM L-ascorbic acid] at a concentration of $5ml.gFW^{-1}$ leaf material. Potato discs were extracted in extraction buffer [0.1M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.5] at a concentration of $5ml.gFW^{-1}$ plant material. The extract was thawed to 4°C and filtered through 3 layers of cheesecloth followed by centrifugation at 20,000 x *g* for 15 minutes in a Sorvall RC5C centrifuge. The supernatant was desalted by centrifugation for 1 minute at 625 x *g* and 0.5 minutes at 1875 x *g* through a Sephadex G-25 (5 x 1cm) column in a swinging-bucket centrifuge. Homogenates were assayed for PAL activity.

Estimation of PAL activity:

Aliquots of homogenate were incubated with 10 mM to 20mM L-aspartic acid, 20mM ∝-ketoglutarate and 20µM pyridoxal-5´-phosphate in a final volume of 0.76 ml in extraction buffer [0.1M $Na_2B_4O_7$-HCl buffer, pH 8.8 and 5mM L-ascorbic acid]. The reaction mixture was incubated at 30 to 40°C for 0.5 hours, followed by the addition of 5 to 10mM L-phenylalanine containing 0.09nM L-phenylalanine-1-^{14}C at 57 $mCi.mmole^{-1}$ [2.11GBq.$mmol^{-1}$] and the final volume made up to 1.0ml. Control samples contained the above reaction mixture either devoid of homogenate or with heat-killed enzyme. The reaction mixture was incubated for 2 hours at 30 to 35°C in a capped tube. The reaction was stopped with 0.1ml, 50% (w/v) TCA preceded by 0.1ml, 0.1% (w/v) t-cinnamic acid in 0.05M NaOH. Samples were left at 18 to 25°C for 10 minutes, and centrifuged at 6000 x g for 5 minutes in a microfuge. The supernatant (reaction mixture) was transferred to an insert and the insert incorporated in a glass vial containing 0.12ml 20% (w/v) KOH. The glass vials were sealed with a rubber stopper. 0.1ml 30% H_2O_2 was injected into the reaction mixture using a Hamilton syringe. Samples were incubated for 30 minutes at 18 to 25°C. The insert and reaction mixture were removed and the KOH solution, now +$KHCO_3$, made to 5 ml with scintillation cocktail [0.7% (w/v) butyl-PBD, 8% (w/v) naphthalene, 60% (v/v) toluene and 40% (v/v) 2-methoxy-ethanol]. Samples were counted at -4°C in an LKB 1210 liquid scintillation counter. These values were an indication of the level of PAT activity present in homogenates. The reaction mixture (supernatant) was extracted in 2.0ml toluene, and centrifuged at 1670 x g for 10 minutes

in a swinging-bucket centrifuge at 18 to 25°C. A 1.0 ml aliquot of the toluene phase was transferred to a vial containing 1.0ml scintillation cocktail [66.6% (v/v) toluene, 33.3% (v/v) Triton X-100 (scintillation grade), 0.003% POPOP and 0.33% (w/v) 2,5-diphenyloxazole]. For concentrations above 17nM t-cinnamic acid a second extraction with toluene was necessary. Samples were counted at 4°C in an LKB-1210 liquid scintillation counter. Specific activity was recorded as nmoles t-cinnamic acid produced $min^{-1}.10^{-3}$ g protein.

2.2.1.9. Determination of PAT activity:

Preparation of homogenates:

Plant material was ground in a mortar and pestle (previously chilled to -70°C) in liquid N_2 (-198.5°C). 1/10 (w/v) insoluble PVP was added in extraction buffer [0.05M Na_2HPO_4-NaH_2PO_4 buffer, pH 8.0, 10µM Na_2EDTA and 10µM MSH.ml^{-1}]. The standard quantities of material used were 0.2gFW.ml^{-1} extraction buffer. The extract was thawed to 4°C and filtered through 3 layers of cheesecloth followed by centrifugation at 20,000 x g for 15 minutes at 4°C in an Sorvall RC5C centrifuge. The supernatant was desalted by centrifugation at 625 to 1875 x g through a Sephadex G-25 (50 to 150 µm) (5 x 1cm) column in a swinging-bucket centrifuge and the homogenates assayed for PAT activity. Specific activity was recorded as nmoles phenylpyruvic produced min^{-1} 10^{-3} g protein or nmoles NADH produced $min^{-1}.10^{-3}$ g protein (in the assay given below).

Estimation of PAT activity:

PAT was assayed as part of the method described above for PAL. An alternative assay was devised for confirming measurements on PAT activity. The assay used to determine PAT activity was a modification of the method of Brown and Perham (1980) for determination of ∝-ketoglutarate dehydrogenase. 20mM L-glutamic acid, 20µM pyridoxal phosphate and the homogenate were mixed together and incubated at 35°C for 7 minutes. 2.5mM NAD, 0.2mM TPP, 1.0mM $MgCl_2$, 2.6mM CoA and 2.6mM cysteine, 20 to 80mM L-phenylpyruvic acid and 0.1 unit ∝-ketoglutarate-dehydrogenase. The assay was run in a Perkin-Elmer 551S UV/VIS spectrophotometer maintained at 30°C, and the reaction followed at 340nm or 366nm (the latter wavelength being less affected by contaminants in the crude extract). Controls contained all components except the homogenate and the dehydrogenase, or heat killed enzyme.

2.2.1.10.De novo protein synthesis:

Incorporation of label into protein:

Glass vials containing 1 ml protoplasts / cells suspended in their respective media at concentrations of 1 x 10^6 protoplasts / cells. ml^{-1} were incubated at 25°C in an orbital incubator at 120 $rev.min^{-1}$ under a constant illumination of 30.wm^{-2} with warm white fluorescent tubes (F30 T12/WW/RS, Sylvania, W. Germany). Protoplasts / cells were pre-incubated for 1 hour prior to addition of L-methionine-^{35}S (860 to 1460 $Ci.mmol^{-1}$ (31.8 to 54.0 $TBq.mmol^{-1}$).

Fungal asialoglycoprotein was added at a concentration described on the dose scale shown in Fig. 3 during the time course, when necessary. The time and duration of incubation with label ranged from 0.5 to 17 hours. At the end of the labelling period, protoplasts / cells were recovered by gentle vacuum filtration on 20µm neutral millipore filters. The filters were frozen in liquid N_2 (-198.5°C) and stored frozen at -70°C. The culture constituted the extraprotoplast or extracellular fraction. Protoplasts / cells were homogenised in grinding buffer containing 50mM Tris-HCl,pH 7.4, 10mM KCl, 1mM Na_2EDTA, 5mM $MnCl_2$, 60mM MSH, 1mM PMSF, 0.4mM DL-methionine and 10% (w/v) sucrose (Rottier et al. 1980). Cellular debris was removed by centrifugation at 625 x g for 10 minutes. The pellet constituted the heavy membrane fraction. The supernatant was further centrifuged by ultra-centrifugation at 100,000 x g for 90 minutes in a Beckman Type 65 fixed angle rotor. The pellet constituted the light membrane fraction. The supernatant constituted the soluble cytoplasmic fraction. The extracellular / protoplast proteins were precipitated with 10% (w/v) TCA at 4°C. Chlorophyll was removed from protein pellets with cold acetone. Precipitation and washes were carried out by centrifugation at 7250 to 9650 x g for 2.5 minutes in a microfuge. The heavy and light membrane fractions were used directly for estimation of further experiments. The soluble cytoplasmic fraction was dialysed versus 1mM DL-methionine and freeze-dried prior to use in further experiments. Proteins were solubilised in sample buffer containing 0.1875M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15% (w/v) MSH and 0.1% (v/v) 1% (w/v) bromophenol blue, (Laemmli, 1970). Measurement of incorporation and uptake of label were made by

addition of an aliquot of sample to a scintillation cocktail [66.6% (v/v) toluene, 33.3% (v/v) Triton X-100 (scintillation grade), 0.003% POPOP and 0.33% (w/v) 2,5-diphenyloxazole]. Samples were counted at 4°C in a LKB-1210 liquid scintillation counter.

Analysis of de novo synthesised proteins:

Proteins were analysed by one-dimensional separation on the discontinuous SDS-PAGE system described by Laemmli (1970). The resolving gel was 10%T, the stacking gel was 4% T. The above protein samples and molecular weight marker ^{14}-C-methylated protein mixture [^{14}C-methylated lysozyme (MW 14,300), ^{14}C-methylated carbonic anhydrase (MW 30,000), ^{14}C-methylated ovalbumin (MW 46,000), ^{14}C-methylated BSA (MW 69,000), ^{14}C-methylated phosphorylase b (MW 92,500) and ^{14}C-methylated myosin (MW 200,000), were dissolved at 2mg .ml^{-1}] in sample buffer containing 0.1875M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15% (w/v) MSH and 0.1% (v/v) 1% (w/v) bromophenol blue (Laemmli, 1970). Samples were heated in a boiling water bath for 2 minutes and cooled to room temperature prior to loading on the gel. Electrophoresis was performed in buffer [0.03M Tris-base, 193mM glycine and 0.1% (v/v) SDS, pH 8.3] at a constant current of 35mA for 4.5 hours in the anodic direction at 18°C. The gels were subsequently treated for fluorography with En^{3}hance as follows: Gels were fixed in fixative [30% (v/v) MeOH, 10% (v/v) glacial acetic acid and 10% (w/v) TCA for a minimal period of 1 hour, followed by impregnation with En^{3}hance for 1 hour. Fluorescent material inside the gel was precipitated with distilled water for 1

hour. A horizontal shaker was used for the treatment. The gels were dried under vacuum at 80°C using a gel dryer. Fluorographs were obtained by exposure of gels to pre-flashed Fuji film RX-P at -70°C. The film was developed in a 1:3 dilution of developer for 3 minutes, and fixed in a 1:3 dilution of fixative for 6 minutes. The film was rinsed thoroughly in distilled water and air-dried. Fluorographs were scanned at 632.8nm using an LKB-2202 ultrascan densitometer connected to a Hewlett-Packard 3390A integrator and in this way quantified.

2.2.1.11. Pre-existing proteins and glycoproteins:

Treatment of cells / protoplasts:

The treatment of cells / protoplasts was as described in section 2.2.1.10, except that no label was added.

Analysis of proteins and glycoproteins:

Proteins were analysed by one-dimensional separation on the discontinuous SDS-PAGE system described by Laemmli (1970). The resolving gel was 10% T. The stacking gel was 4% T. The protein samples and the molecular weight marker protein mixture [α-lactalbumin (MW 14,200), soybean trypsin inhibitor (MW 20,100), trypsinogen (MW 24,000), bovine erythrocyte carbonic anhydrase (MW 29,000), rabbit muscle glyceraldehyde-3-phosphate (MW 36,000), egg albumin (MW 45,000) and bovine albumin (MW 66,000), were dissolved at 2mg $.ml^{-1}$] in sample buffer containing

0.1875M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15% (w/v) MSH and 0.1% (v/v) 1% (w/v) bromophenol blue (Laemmli, 1970), heated in a boiling water bath for 2 minutes and cooled to room temperature prior to loading on the gel. Electrophoresis was performed in buffer [0.03M Tris-base, 193M glycine and 0.1% (w/v) SDS, pH 8.3] at a constant current of 35mA for 4.5 hours in the anodic direction at 18^{o}C. The gels were subsequently stained with silver stain using a horizontal shaker, according to the technique of Morrissey (1981) modified as follows: Gels were fixed in fixative [50% (v/v) MeOH and 10% (v/v) glacial acetic acid] for 30 minutes followed by a wash in solution [5% (v/v) MeOH and 7% (v/v) glacial acetic acid] for 30 minutes. The gels were then fixed for 45 minutes in 10% (v/v) glutaraldehyde and washed extensively for 2 to 12 hours in distilled water. Gels were immersed in 0.5mg.ml^{-1} DTT for 30 minutes, 0.1% $AgNO_3$ for 45 minutes and rapidly rinsed with distilled water. Gels were developed in developing solution [3% sodium carbonate and 0.5 μl.ml^{-1} 37% formaldehyde] until the desired level of staining intensity was attained. Staining was stopped by the addition of 23mM citric acid. After 10 minutes, gels were washed several times in distilled water over a 30 minute period and dried under vacuum at 80^{o}C using a gel dryer. Gels were scanned in an ultrascan densitometer. Glycoproteins were analysed by staining gels with periodic acid and Schiffs reagent, as described in section 2.2.1.13.

2.2.1.12. Immunoprecipitation of <u>de novo</u> and non <u>de novo</u> synthesised proteins and glycoproteins:

Cells which had been treated as above were ground in liquid N_2 (-198.5°C) with extraction buffer [0.1M sodium borate, pH 8.3, 5mM L-ascorbic acid and 1mM PMSF] and centrifuged at 20,000 x g in a Sorvall RC5C centrifuge for 10 minutes at 4°C. 0.125ml. ml^{-1} PAL-antiserum (prepared as described in Chapter 5) was added to the supernatant. The pellets (containing cell walls) were ground in grinding buffer [50mM Tris-HCl, pH 7.4, 10mM KCl, 1mM Na_2EDTA, 5mM $MnCl_2$, and 10% (w/v) sucrose]. Samples were centrifuged at 20,000 x g for 10 minutes at 4°C. $0.125ml.ml^{-1}$ PHA-antiserum was added to the supernatant. Samples containing antiserum were incubated for 2 hours at 26°C for 10 to 18 hours at 4°C. $0.125ml.ml^{-1}$ goat-anti-rabbit IgG immunobead second antibody (prepared by rehydration of immunobead second antibody at $4mg.ml^{-1}$ in 0.1M $Na_2B_4O_7$-HCl, pH 8.0) was added to each sample and vials incubated for 2 hours at 18 to 25°C for 30 minutes. Samples were made to 3 ml (to facilitate washing) and centrifuged at 1,000 x g for 6 minutes at 4°C. Pellets (immunoprecipitates) were dissolved in sample buffer containing 0.1875M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, 15% (w/v) MSH and 0.1% (v/v) 1% bromophenol blue (Laemmli, 1970). Measurement of incorporation and uptake of label were made by addition of an aliquot of sample to a scintillation cocktail [66.6% (v/v) toluene, 33.3% (v/v) Triton X-100 (scintillation grade), 0.003 % POPOP and 0.33% (w/v) 2,5-diphenyloxazole]. Samples were counted at 4°C in a LKB-1210 liquid scintillation counter.

2.2.1.13. Binding of asialoglycoprotein to phytohemagglutinin (PHA):

The amount of asialoglycoprotein bound to de novo and pre-existing phytohemagglutinin was determined by disruption of the asialoglycoprotein-PHA complex in the cell wall and rough ER fraction of the cell by SDS-PAGE described in section 2.2.1.12 and 2.2.1.13 respectively. A schematic diagram of the procedure used is shown in Fig. 4 and densitometric scan in chapter 4. Quantification of PHA was by immunoprecipitation of PHA using PHA-antiserum and the methods described in section 2.2.1.12. Quantification of PHA was by staining gels with periodic acid and Schiffs reagent. Periodic acid Schiff's (PAS) staining was carried out as follows (Fairbanks et al. 1971): Gels were fixed in 25% (v/v) isopropyl alcohol and 10% (v/v) glacial acetic acid overnight. They were rinsed in 10% (v/v) isopropyl alcohol and 10% (v/v) glacial acetic acid for 6 to 9 hours, and incubated in 10% (v/v) glacial acetic acid overnight or till a clear background was obtained. Gels were stained with 0.5% (v/v) periodic acid for 2 hours, followed by washes in 0.5% (w/v) sodium arsenite and 5% (v/v) glacial acetic acid for 30 to 60 minutes and 0.1% (w/v) sodium arsenite and 5% (v/v) glacial acetic acid for 20 minutes, 0.1% (w/v) sodium arsenite and 5% (v/v) glacial acetic acid for 20 minutes and glacial acetic acid for 10 to 20 minutes. Gels were stained with Schiff's reagent overnight and destained in 0.1% (w/v) sodium metabisulfite in 0.01N HCl for several hours till the solution failed to turn pink with addition of formaldehyde. Gels were scanned at 490nm and stained asialoglycoprotein bands cut out and quantified by weighing in a balance. Incorporation of label into de novo synthesised PHA in the gel was by fixing the gel in 50% (v/v) MeOH and 10% (v/v) glacial acetic acid, cutting the gel into 1mm segments, and incubating these segments in

Fig 4 CONTD.

Cells were treated with ^{35}S-methionine in presence of different concentrations of pathogen cell wall asialogycoprotein. The PHA-asialoglycoprotein fraction was extracted and disrupted by SDS-PAGE. The asialoglycoprotein associated with PHA was quantified by PAS staining and gel scanning of the PAS-stained band. The PHA associated with the asialoglycoprotein was quantified by immunoprecipitation with PHA antiserum followed by scintillation counting of the labelled immunoprecipitate.

Fig. 5. CONTD.

Leaves of the host plant were infiltrated with a suspension of mycelia using a Hamborg device. The plants were incubated at 100% relative humidity to stimulate hyphal growth. The number of lesions (indicated by arrows) were used to quantify the degree of hypersensitive response. For further details see 'methods'.

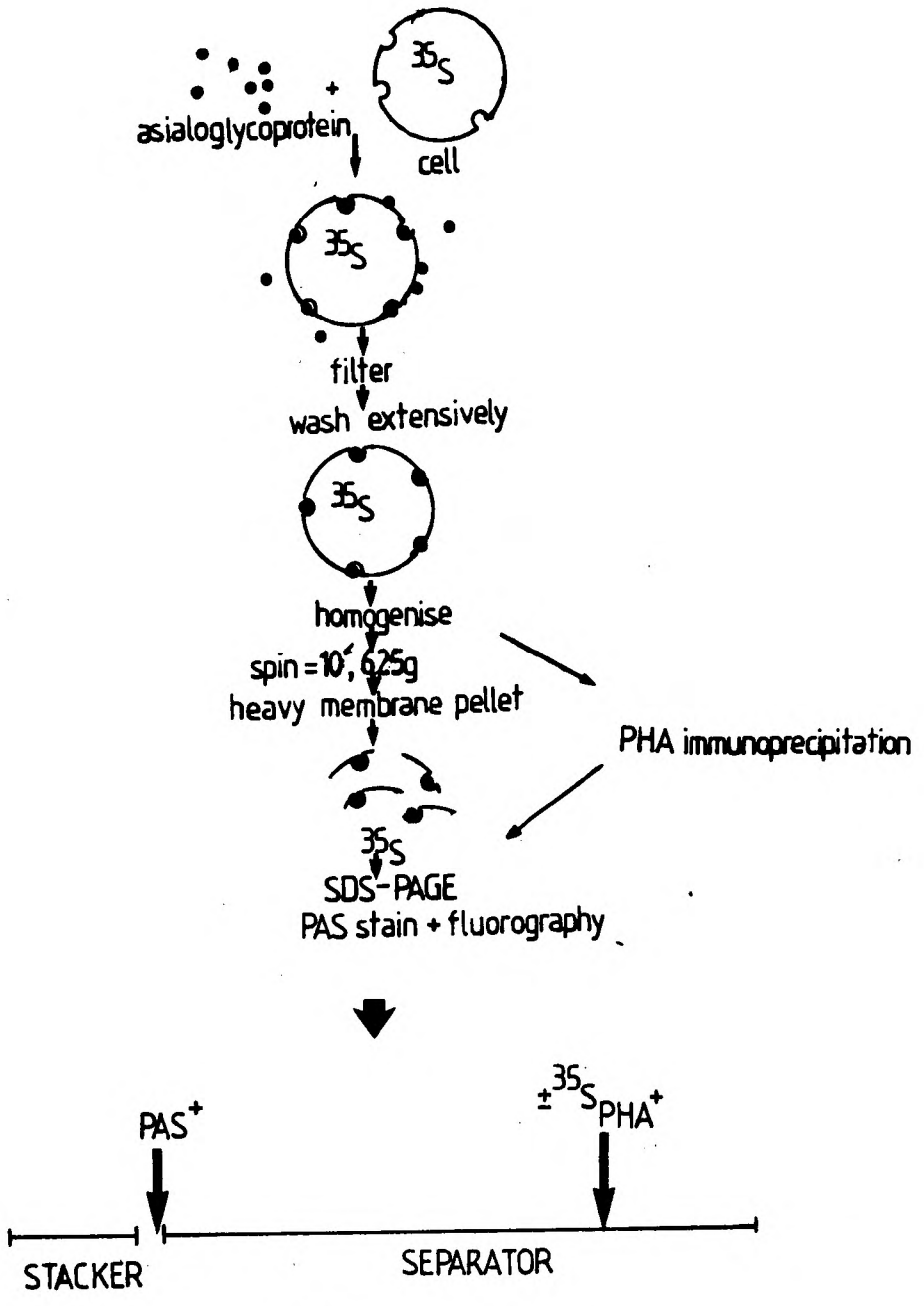


Fig. 4 Schematic representation of method used to quantify the interaction between pathogen cell wall asialoglycoprotein and de novo and non de novo synthesised phytohemagglutinin in the cell walls and rough ER of the host plant. (continued on facing page)

Fig. 5 The response of the leaves of the host plant Phaseolus vulgaris L. to the fungal pathogen Colletotrichum lindemuthianum L. - induction of hypersensitive necrosis. (continued on facing page)

scintillation vials containing 0.250ml 30% (v/v) H_2O_2. Vials were tightly capped and placed at 50 to 55°C for 18 hours. Samples were cooled to room temperature, and 4.5ml scintillation cocktail [Triton X-100 : toluene scintillation fluid (5.0g PPO + 0.5g POPOP.l^{-1} toluene) and counted at 4°C in a LKB liquid scintillation counter.

2.2.2. MATERIALS:

Seeds of the host plant, Phaseolus vulgaris L. var. Prince were from Clause (UK) Ltd., Charvil, Reading and the fungal pathogen Colletotrichum lindemuthianum L. isolate CMI 112166 from the Commonwealth Mycological Institute, Kew, Surrey.

The `propatray' was from Humex, Byfleet, Surrey; peristaltic pump from Watson-Marlow Ltd., Falmouth, Cornwall; nylon mesh from Henry Simon Ltd., Cheshire; graded horticultural perlite was from Silvaperl Products Ltd., Harrogate, North Yorkshire; Levington soilless potting compost from Fisons Horticultural Division, Ipswich, Suffolk; silica gel/UV254 TLC plates from Macher-Nagel & Co., Doren, W. Germany and pre-calibrated pipettes from Modulohm 1/S, Vasekaer, Denmark. The spectrophotometer was from Perkin-Elmer & Co., GMBH/Uberlingen, Bundesrepublik Deutschland and the rotary evaporator from Buchi Laboratoriums-Technik AG, Flawil, Switzerland. The orbital incubator was from A. Gallemkamp & Co., London; swinging-bucket centrifuge from MSE Scientific Instruments, Crawley, West Sussex; centrifuge from DuPont Co., Wilmington, Delaware; ultra-centrifuge from Beckman Instruments, Palo Alto, California; freeze-dryer from Edwards High

Vacuum, Crawley, Sussex; ultrascan densitometer and liquid scintillation counter from LKB-Produkter AB, Bromma, Sweden; gel electrophoresis equipment (apparatus GE-2/4) and gel dryer from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden; horizontal shaker from Northern Media Supply Ltd., Humberside and the integrator from Hewlett-Packard, Avondale, Pennyslyvania. Millipore filters were from Millipore Corporation, Bedford, Massachusetts; 'Gelbond' from FMC Corporation, Rockland, Maine and X'ray film from Fujimex Ltd., Swindon, Wiltshire.

All glassware used for induction of hypersensitive necrosis, studies on phaseollin production and isolation of pathogen cell wall asialoglycoprotein was rinsed in EtOH and dried prior to usage. Inoculations were carried out with sterile, disposable equipment and equipment sterilised by autoclaving at 121°C for 20 minutes prior to use. All glassware used for analysis and immunoprecipitation of de novo and pre-existing protein was washed in sterile 1M NaOH, sterile 2% (v/v) Decon-90, sterile distilled water, siliconised with 'Repelcote' and autoclaved at 121°C for 20 minutes. This procedure was used to remove any ribonuclease present.

Chemicals used were of the analar grade.

'Repelcote' was obtained from Hopkins and Williams, Chadwell Heath, Essex; Neopeptone was obtained from Difco Laboratries, Detroit, Michigan; Domestos from Lever Bros. Ltd., London; Macerase (cellulase)

from Calbiochem-Behring Corp., C.P. Labs. Ltd., Hertfordshire; Driselase from Unwin & Co. Ltd., Hertfordshire; dextran-T_{40} from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden, butyl-PBD from Fisons Scientific Apparatus, Loughborough, Leicestershire; goat-anti-rabbit IgG immunobead second antibody from Biorad, Richmond, California; radioactive chemicals from Amersham International, Amersham, Buckinghamshire; developing and fixing solutions were from Kodak Ltd., Hemel Hempstead, Hertfordshire; α-ketoglutaric acid, α-ketoglutaric acid dehydrogenase, L-phenylalanine, L-aspartic acid, pyridoxyl phosphate, PMSF, MSH, soluble PVP 40, insoluble PVP, concanavalin A, PHA antiserum, DEAE-Sephacel, methyl-α-D-mannoside, pectinase, DTT, $AgNO_3$, non-radioactive molecular weight markers, Tris-base and Tris-HCl from Sigma Chemical Co. Ltd., Poole, Dorset and En^{3}hance from New England Nuclear, Dreiech, W. Germany. Other chemicals were from BDH Chemicals Ltd., Poole, Dorset.

All solutions and equipment were autoclaved at 121^{o}C for 20 minutes prior to use. When heat-sterilisation was not posible, solutions were filter-sterilised using an acrodisc filter assembly (0.2μm) (Gelman Sciences Ltd., Northampton).

2.3. RESULTS:

When Phaseolus vulgaris plants were grown and infected with live mycelium of the fungal pathogen Colletotrichum lindemuthianum, as described in methods, section 2.2.1.6., the following results were obtained:

2.3.1. Whole (intact) host system - living and non-living pathogen mycelium:

2.3.1.1. Hypersensitive necrosis was found in the leaves of the intact host plant after infiltration with live mycelium of the fungal pathogen. An example of such an infected leaf is shown in Fig. 5. As can be seen in this photograph, necrotic lesions, the darkened areas on the surface of the leaf, are characteristic symptoms produced after pathogen invasion. These darkened areas did not appear in control leaves and appeared 10 days after application of inoculum or assumed pathogen invasion. The darkened areas (as opposed to surrounding pale green areas) contained pathogen mycelium, and were restricted to areas less than 1mm in diameter when stained with cotton blue. The darkened areas were considered to be an expression of resistance by the host plant to further pathogen invasion, constitutive of the major visible symptom of hypersensitive necrosis.

Leaves of the older plants infiltrated with live mycelium of the fungal pathogen, showed a smaller degree of hypersensitive necrosis (Fig. 7). Subsequently, the host plant in presence of the pathogen did

not change the number or size of lesion formation.

This confirms that the host plant responds to the pathogen by induction of hypersensitive necrosis after pathogen invasion [Chapter 1, section 1.1].

2.3.1.2. Isoflavonoid phytoalexin (phaseollin) formation was found in leaves of the host plant after infection with live mycelium of the fungal pathogen, including areas expressing hypersensitive necrosis. Fig. 6 shows the concentrations of phaseollin obtained 10 days after infection by the pathogen. This response may be seen to correlate with the onset of hypersensitive necrosis in host plants of different ages (Fig. 7). It can be seen that younger host plants synthesise phytoalexins while older host plants do not.

This confirms that the host plant responds to the pathogen by producing phytoalexins (phenylpropanoid-derived phytoalexins in this system) [Chapter 1, section 1.2].

2.3.1.3. PAL activity was found in leaves after infiltration with live pathogen mycelium, not earlier than 3 days after pathogen invasion, in areas showing induced phaseollin formation and hypersensitive necrosis. Fig. 6 shows the activities of PAL obtained 10 days after infiltration by the fungal pathogen. It may be seen that younger host plants show induced levels of PAL activity while older plants fail to do so within 10 days.

Fig 6. CONTD.

The host plant was infected with the pathogen as described in Fig. 5. Control inoculations were carried out with 'distilled water. The phytoalexin phaseollin was extracted from leaves exhibiting hypersensitive necrosis, by procedures described in 'methods'. Each spot represents the mean of results obtained from 10 such plants. The bars indicate the maximum and minimum values obtained. The absence of bars indicates that the variation is contained within each spot.

Fig. 7. CONTD.

The host plant was infected with fungal pathogen as described in Fig. 5. The number of necrotic lesions. $g\ FW^{-1}$ leaf material was used to quantify the degree of hypersensitive response. Control inoculations were carried out with distilled water. Each spot represents the mean of results obtained from 10 such plants. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot. For further details see 'methods'.

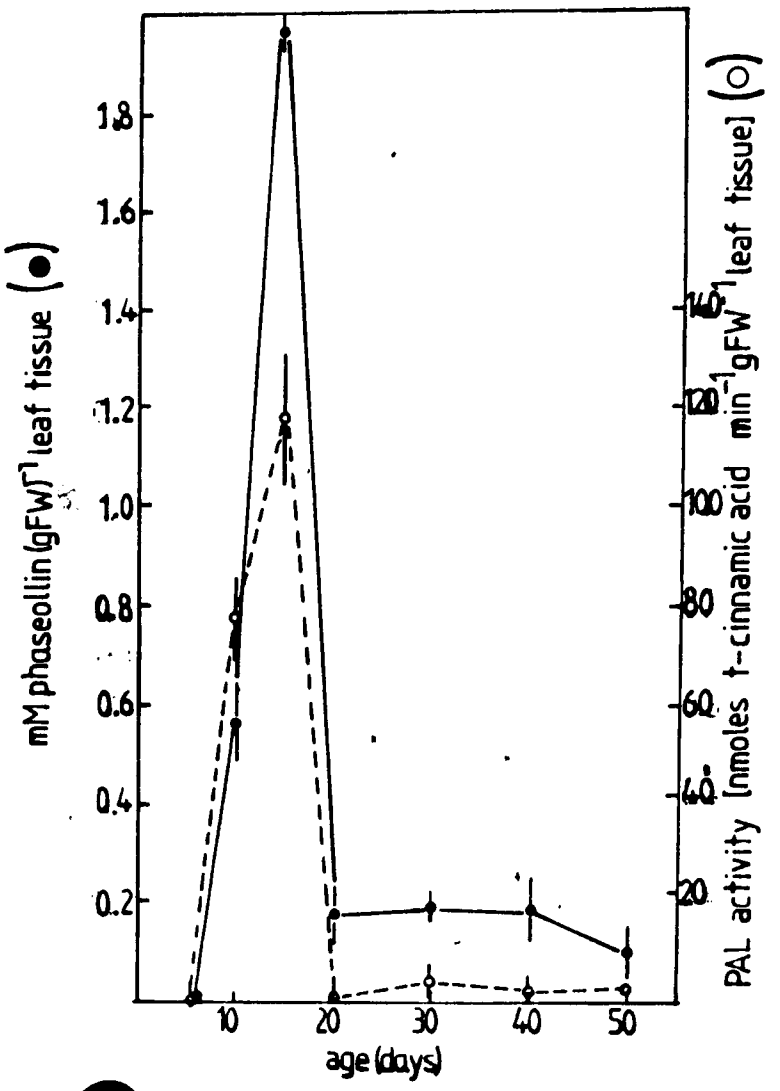


Fig. 1 The response of the leaves of the host plant Phaseolus vulgaris L. to the fungal pathogen Colletotrichum lindemuthianum L. - induction of the phytoalexin response (phaseollin formation) and PAL activity in leaves of various ages. (continued on facing page)

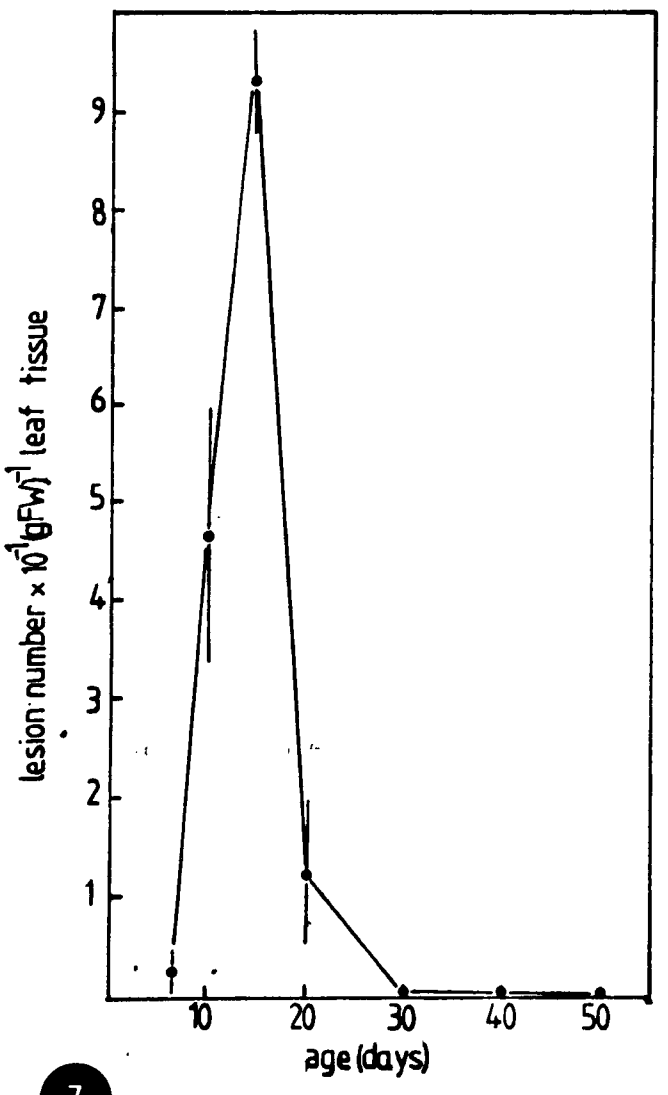


Fig. 7 The response of leaves of the host plant Phaseolus vulgaris L. to the fungal pathogen Colletotrichum lindemuthianum L. - induction of hypersensitive necrosis in leaves of various ages. (continued on facing page)

This shows that the host plant responds to the pathogen by inducing enzyme activities in the metabolic pathway leading to the production of phytoalexins [chapter 1, section 1.3].

Hypersensitive necrosis, phytoalexin production and PAL activity showed a correlative increase with time and age of host plant. These changes were not a result of wounding as minimal damage to the host tissue was produced by using the Hoagborg device for infiltration of pathogen mycelium (Fig. 2). Further control plants treated in the same way but infiltrated with distilled water or pathogen cell wall asialoglycoprotein showed no necrotic lesion formation, no phenylpropanoid-derived phytoalexin (phaseollin) formation and no induction of PAL activity.

2.3.1.4. The growth of young plants (below 2 weeks old) under continuous low light intensity resulted in expression of necrotic lesions more than 1mm in diameter, (Fig. 9) within 10 days after pathogen invasion. No phenylpropanoid-derived phytoalexin formation or increased PAL activity was observed within 10 days. During this period, the host plant underwent rapid senescence and died. Host plants incubated in absence of pathogen and in continuous high light intensity showed lesions greater than 1mm in diameter, concomitant with transient increases in PAL activity not correlated in time with hypersensitive necrosis and not accompanied by phenylpropanoid-derived phytoalexin formation (described more fully in chapter 6).

This experiment suggests that the host plant responds to the pathogen

Fig. 8 CONTD

Cells were treated with different concentrations of pathogen cell wall asialoglycoprotein as shown in Fig. 3, for 1, 5 and 10 hours. PAL and PAT activities were estimated by procedures described in 'methods'. Each spot represents the mean of 7 different experiments. The bars indicate the maximum and minimum values in an experiment. The absence of bar indicates that the variation is contained within each spot. Values plotted for concentrations other than those in the dose scale in Fig. 3 were obtained by drawing a regression line of values of the ordinate on the abscissa, fitted by the methods described by Campbell (1974).

Fig. 9 CONTD.

Leaves of the host plant were infiltrated with a suspension of mycelia using a Hagborg device. The plants were incubated at 100% relative humidity to stimulate hyphal growth and kept in the shade. The dark brown areas accompanied by rapid senescence were representative of leaves susceptible to the pathogen. For further details see 'methods'.

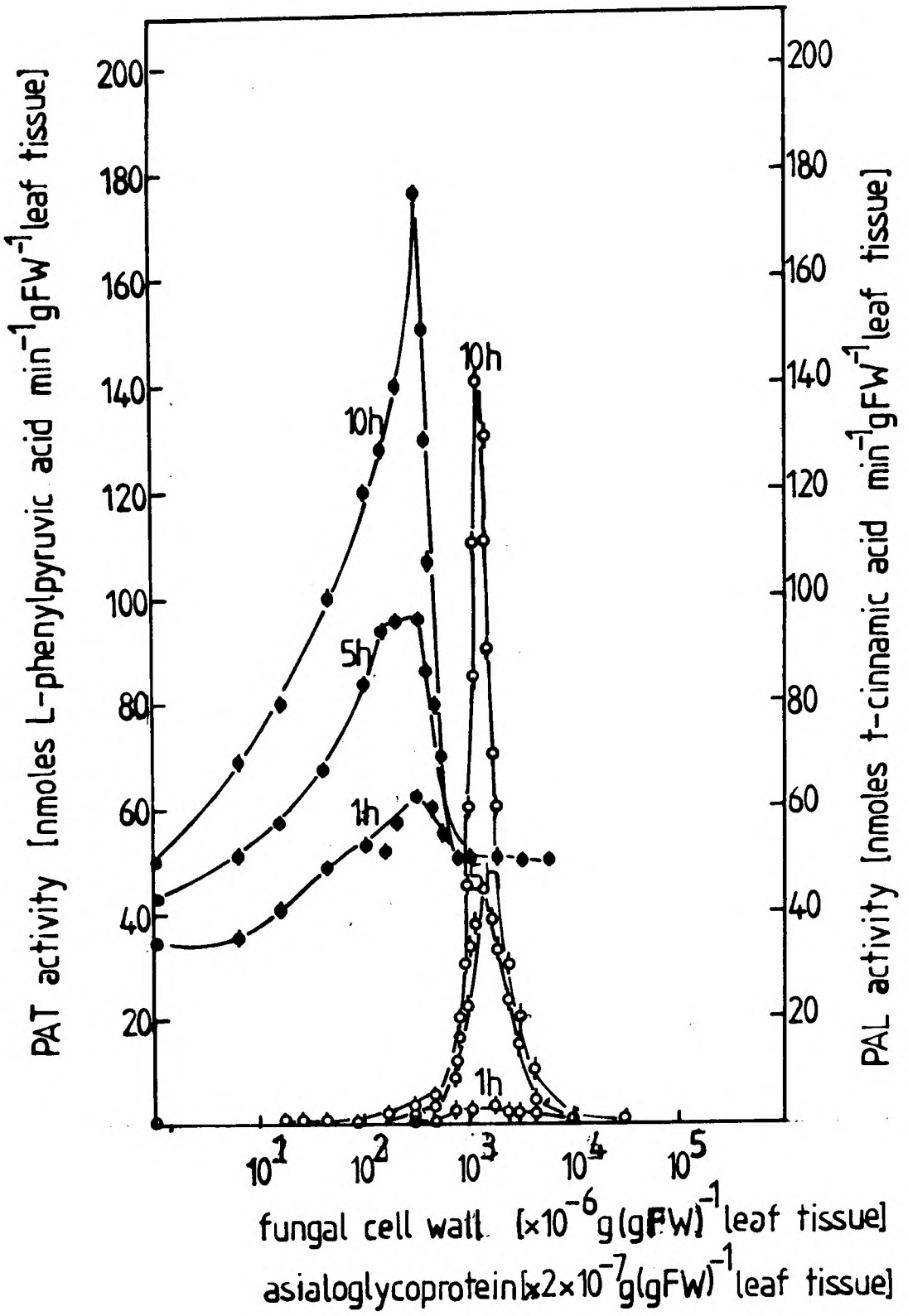


Fig. 8 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - PAL (O——O) and PAT (●——●) activities. Age of host plant: 10 days. (continued on facing page)

Fig. 9 The response of the host plant Phaseolus vulgaris L. to the fungal pathogen Colletotrichum lindemuthianum L. in the dark - induction of susceptibility. Age of host plant at time of inoculation : 10 days. (continued on facing page)

under conditions of optimal `source´ metabolism [chapter 1, section 1.3 (iv)].

2.3.1.5. No induction of hypersensitive necrosis, phenylpropanoid-derived phytoalexin (phaseollin) formation or induction of PAL activity was found in the young whole host plant after inoculation with non-living fungal pathogen mycelium, i.e. air-dried mycelium, which failed to grow in suspension culture. This indicates that enzymatic activity(ies) of the pathogen on the host is required for the host responses. No induction of hypersensitive necrosis, phenylpropanoid-derived phytoalexin (phaseollin) formation or induction of PAL activity was obtained in the young, whole host plant when leaves were inoculated with the major cell wall non-enzymatic asialoglycoprotein of the fungal pathogen. This was indicative of possible enzymatic activity of the pathogen on the host, and showed that living mycelia may be needed to produce the responses.

These observations are consistent with the view that the host plant responds to the fungal pathogen by recognition of specific determinants and intercellular signals [Chapter 1, section 1.3].

2.3.2. Rapidly isolated leaf cell system - abiotic pathogen mycelial component:

In order to clarify further the events taking place in the early stages of infection, cells isolated from bean plants were used (see

methods, 2.2.1.2).

2.3.2.1. The removal of cellulose and pectin from the middle lamella of the young host plant cell wall (to form single cells) permits the host to respond to the major cell wall non-enzymatic cell wall asialoglycoprotein of the mycelial pathogen by an induction of PAL activity (Fig. 8). This response was not due to wounding, as no significant leakage (>10%) of de novo synthesised proteins occurred during the incubation period (described more fully in chapter 3). These observations additionally confirmed the finding above (chapter 2, section 1.3.2).

2.3.2.2. Cells isolated from the host plant responded to the pathogen cell wall asialoglycoprotein by inducing simultaneously both the de novo synthesis and subsequent activation of PAL protein (Fig. 8; Fig. 10; Fig. 16, tracks 4,7,8 and 9; Fig.18). Controls showed no induction of de novo synthesised active PAL protein (Fig. 8; Fig. 10; Fig. 16, tracks 3 and 19; Fig. 18). Further, the de novo synthesis of active PAL protein occured only in cells isolated from host plants younger, but not older than 2 weeks (Fig. 20). No de novo synthesis and activation of PAL protein (and no induction of PAL activity) occurred when young host cells were incubated with pathogen cell wall asialoglycoprotein in the dark (Fig. 16, tracks 20 and 29). Light was necessary for induction of PAL. These results coincided with results obtained on interaction of live pathogen mycelia with the intact host plant mentioned above (Fig. 6; Fig. 7). The occurrence of these responses between 5 and 10 hours after incubation in presence of abiotic pathogen constituents and cell wall removal strongly support

Fig. 10 CONTD

Cells were treated with ^{35}S-methionine for 1, 5 and 10 hours in presence of diffferent concentrations of pathogen cell wall asialoglycoprotein as shown in Fig. 3. PAL was extracted and immunoprecipitated with PAL-antiserum. the immunoprecipitate was quantified by scintillation counting.

PAT, active and inactive PAL protein were quantified following SDS-PAGE and fluorography by scanning the relevant peptide bands at 22, 27, 30 and 32K. Each spot represents the mean of 7 different experiments. The bars indicate the maximum and minimum values in an experiment. The absence of a bar indicates that the variation is contained within each spot. Values plotted for concentrations other than those in the dose scale in Fig. 3 were obtained by drawing a regression line of values of the ordinate on the abscissa, fitted by the method described by Campbell (1974). For further details see 'methods'.

Fig. 11 CONTD

Cells were treated with ^{35}S-methionine for 10 hours in presence of different concentrations of pathogen cell wall asialoglycoprotein as shown in Fig. 3. Membrane, cytoplasmic and extracellular fractions were separated by techniques described in 'methods'. Proteins from these fractions were solubilised in sample buffer as described in 'methods'. The incorporation of label into protein in various fractions was estimated by scintillation counting. Each spot represents the mean of 7 different experiments. The bars indicate the maximum and minimum values in an experiment. The absence of a bar indicates that the variation is contained within each spot. Values plotted for concentrations other than those in the dose scale shown in Fig. 3 were obtained by drawing a regression line of ordinate values on the abcissa, fitted by the methods described by Campbell (1974). Viability of cells at 0 and 10 hours were determined by the fluorescence diacetate method of Widholm (1972). Each spot represents the mean of 36 determinations.

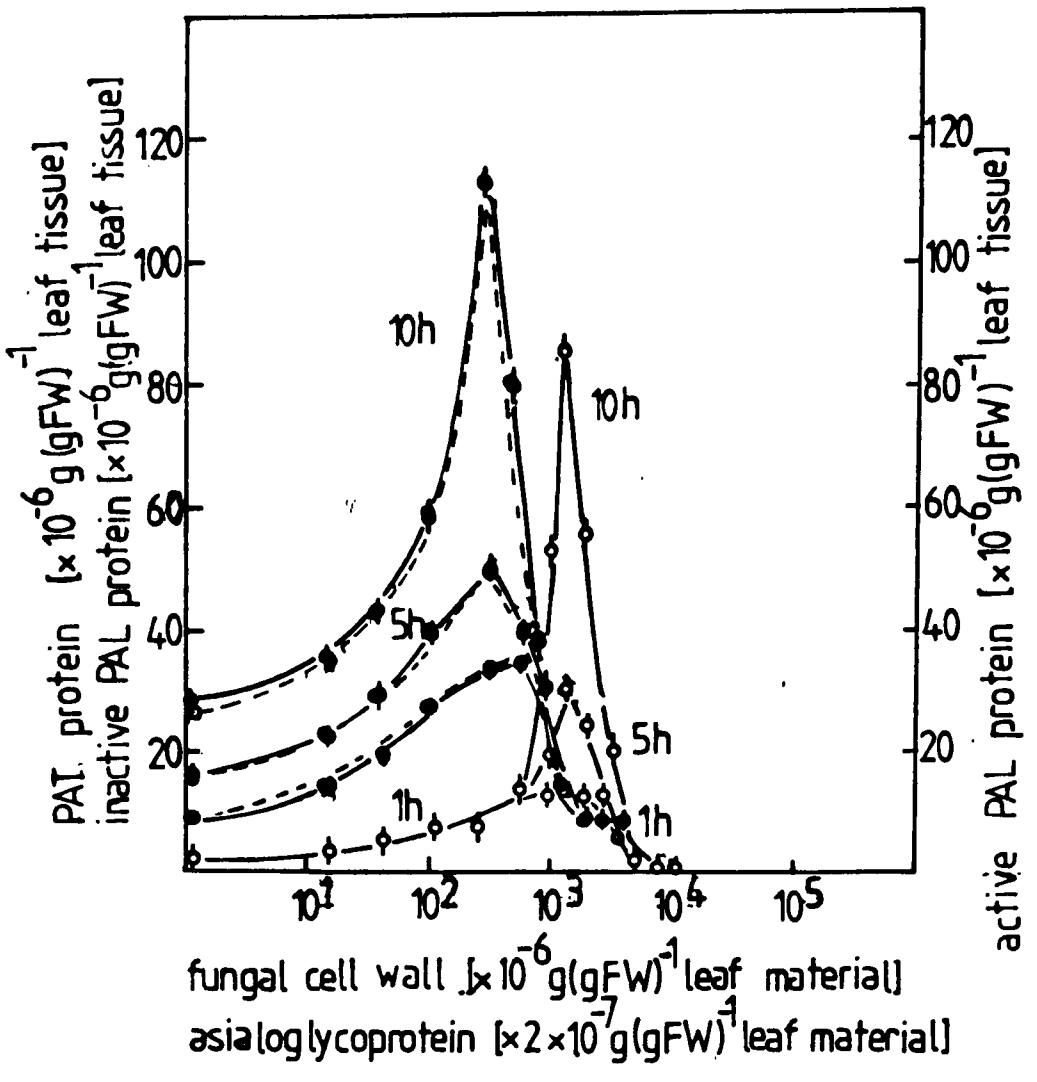


Fig. 10 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - quantities of inactive PAL (○---○) and active PAL (○——○) and PAT (●——●) protein. Age of host plant : 10 days. Quantification was by scanning fluorographs. (continued on facing page)

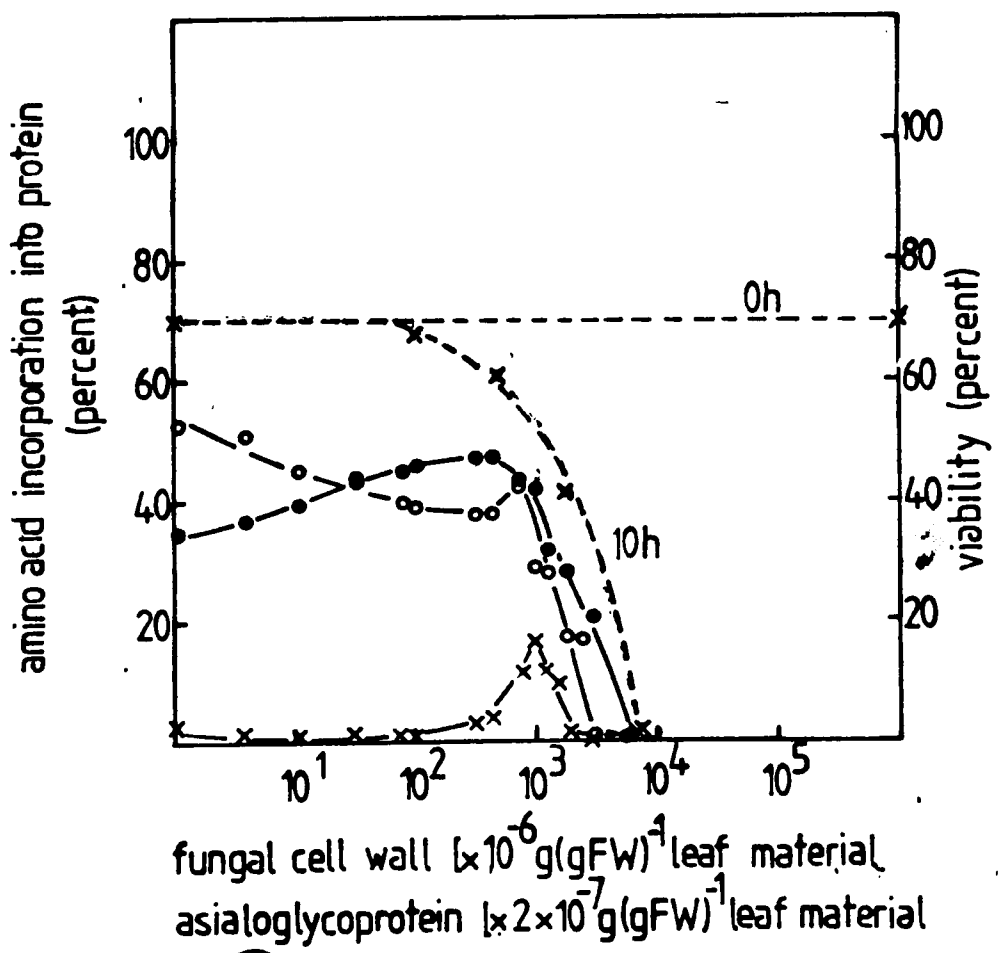


Fig. 11 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - protein synthesis (i.e. labelled amino acid incorporation in membranes (●—●—●) and cytoplasm (○—○—○), leakiness (labelled amino acid incorporation in to proteins in the extracellular medium (×—×—×) and viability (×---×---×) of host cells. Age of host plant : 10 days.

(continued on facing page)

Fig. 12 CONTD

Cells were treated with ^{35}S-methlonine for 1, 5 and 10 hours in presence of different concentrations of pathogen cell wall asialoglycoprotein as shown in Fig. 3. PHA was extracted and immunoprecipitated with PHA-antiserum. The immunoprecipitate was quantified by scintillation counting. Each spot represents the mean of 3 different experiments. The bars indicate the maximum and minimum values in a gel. The absence of a bar indicates that the variation is contained within each spot. Values plotted for concentrations other than those in the dose scale shown in Fig. 3. were obtained by drawing a regression line of ordinate values on the abscissa, fitted by the methods described by Campbell (1974).

Fig 13. CONTD

Cells were treated with different concentrations of pathogen cell wall asialoglycoprotein as shown in Fig. 3. for 1, 5 and 10 hours. The PHA-asialoglycoprotein-containing fraction was extracted and disrupted by SDS-PAGE. The asialoglycoprotein associated with PHA was quantifed by PAS staining and gel scanning of PAS stained bands. Each spot represents the mean of 3 different experiments. The bars indicate the maximum and minimum values determined. The absence of a bar indicates that the variation is contained within each spot. Values plotted for concentrations other than those in the dose scale shown in Fig. 3 were obtained by drawing a regression line of ordinate values on the abcissa, fitted by the methods described by Campbell (1974). For further details see 'methods'.

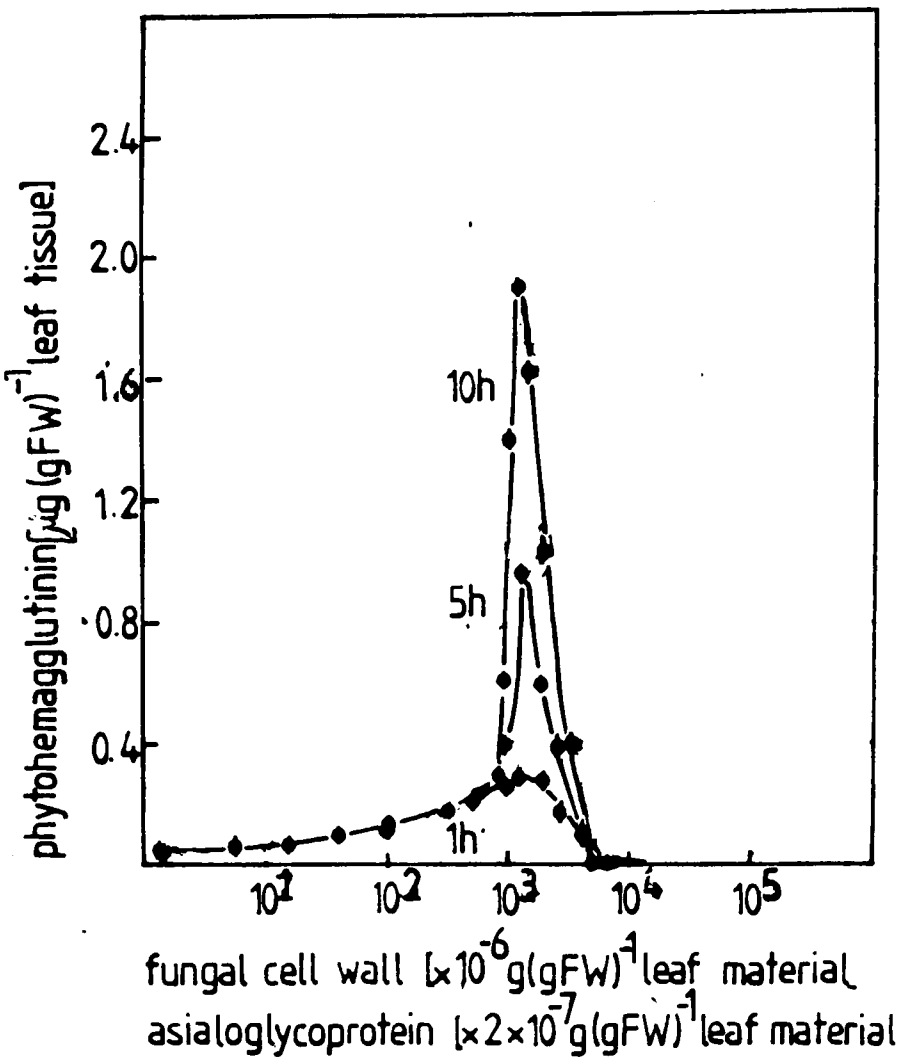


Fig. 10 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - concentrations of immunoprecipitated, radiolabelled phytohemagglutinin. Age of host plant : 10 days. Quantification was by scanning fluorographs and counting labelled immunoprecipitate. (continued on facing page)

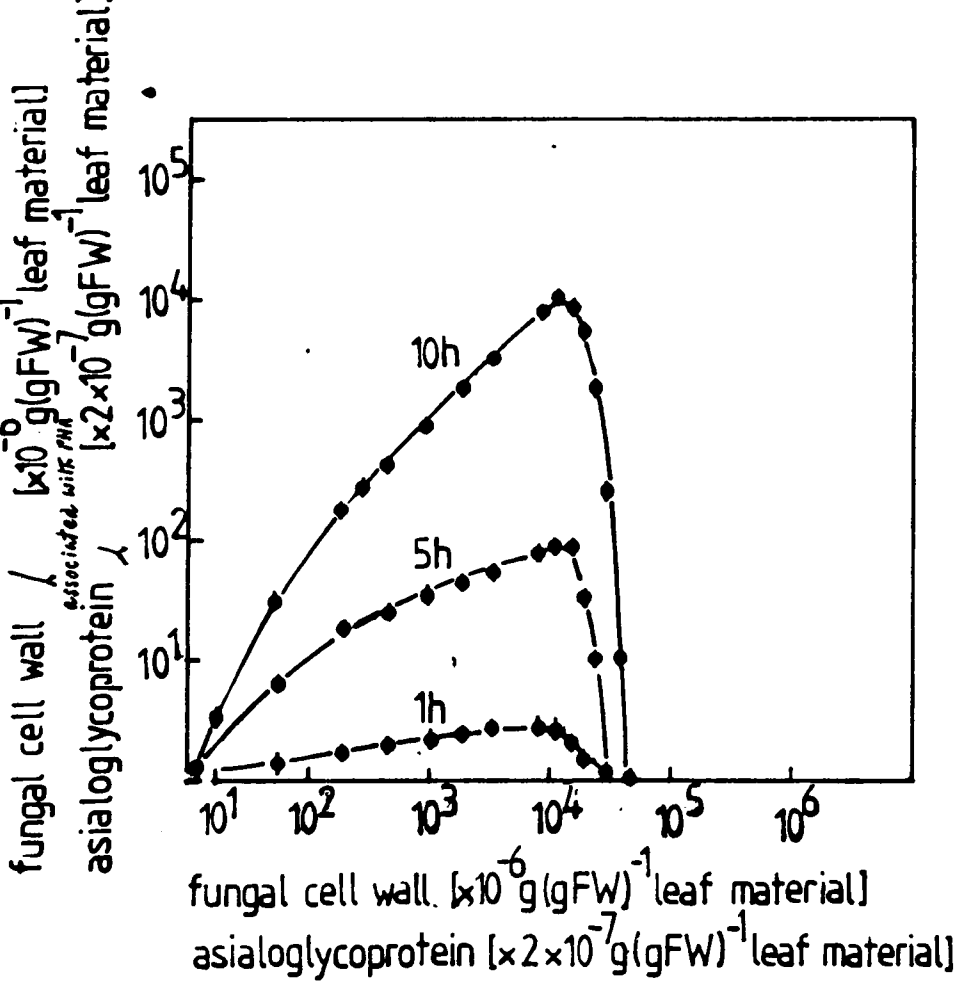


Fig. 11 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - concentrations of asialoglycoprotein associated with phytohemagglutinin (PHA) in the host cell wall. Age of host plant: 10 days. Quantification was by weighing scanned PAS-stained band. (continued on facing page)

Fig 14 CONTD.

This figure was derived from values presented in Fig. 13, using the equation of Aducci et al (1984) to achieve best fit as described in 'results'.

Fig 15 CONTD.

This figure was derived from values presented in Fig. 13.

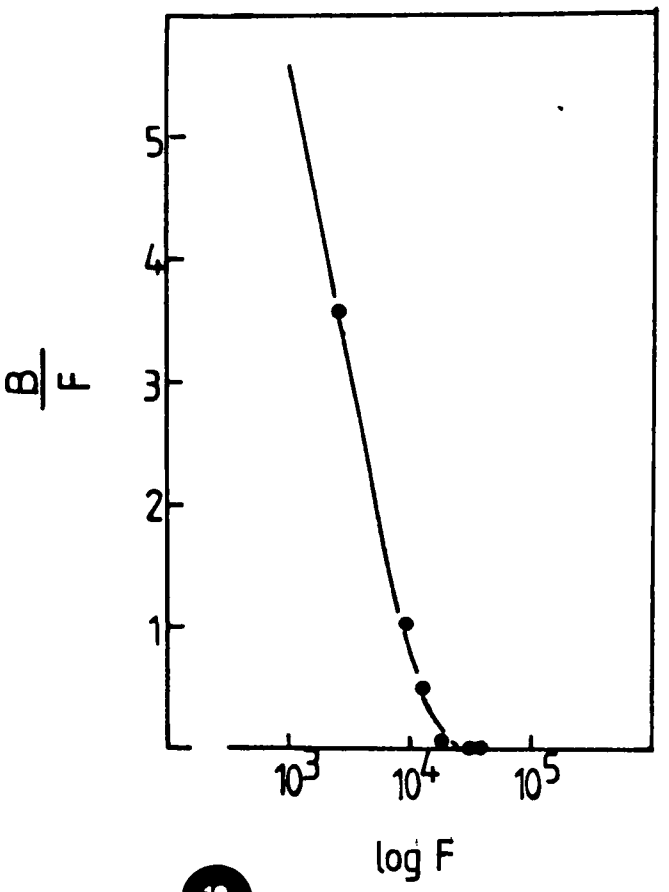


Fig. 10 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - the Scatchard plot of the binding of asialoglycoprotein to phytohemagglutinin in the host cell wall. Age of host plant : 10 days. (continued on facing page)

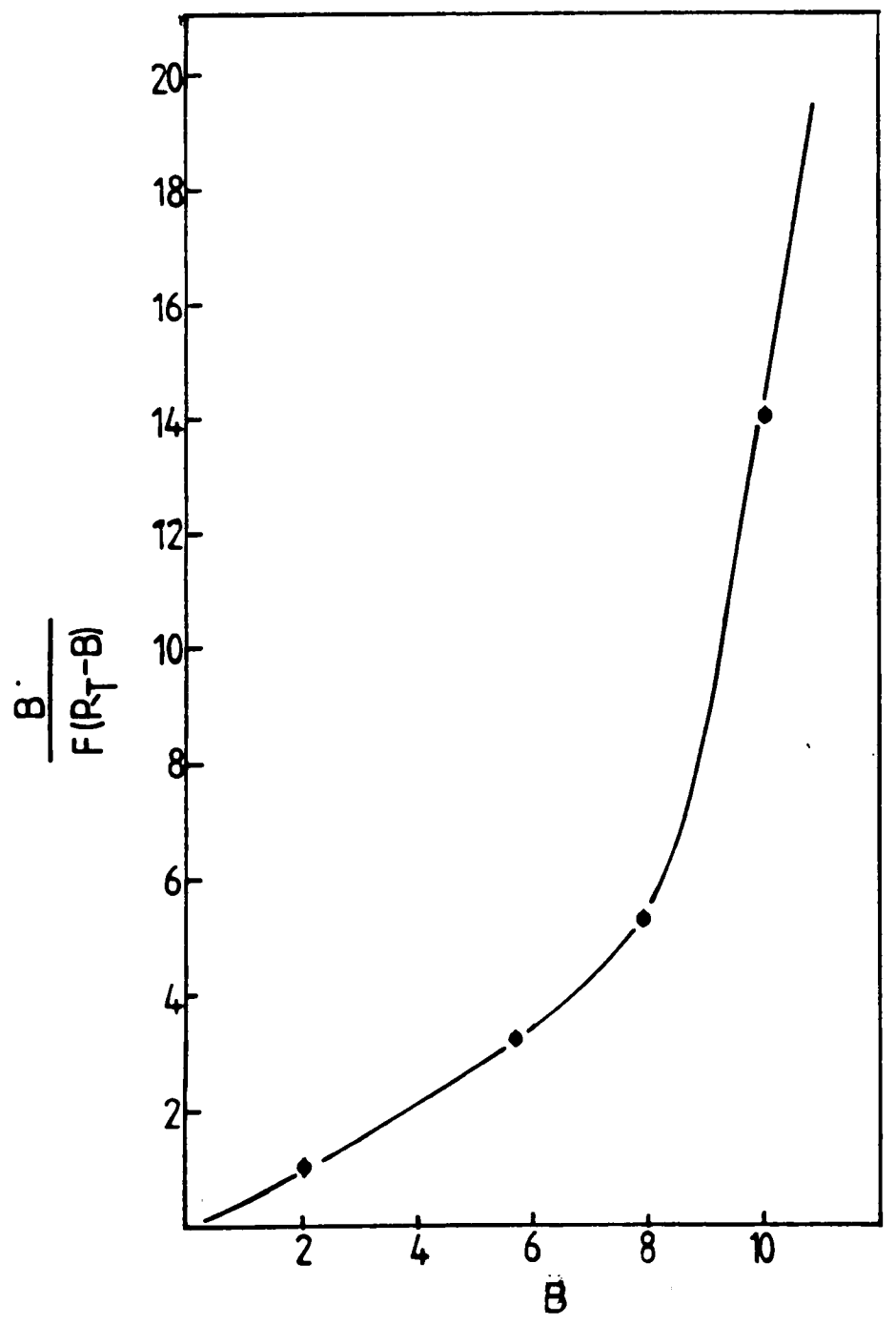


Fig. 11 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - the average affinity plot of the binding of asialoglycoprotein to phytohemagglutinin in the host cell wall. Age of host plant : 10 days. (continued on facing page)

Fig. 16 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - the immunoprecipitation of de novo synthesised inactive PAL and active PAL and PAT protein. Time of incubation with ^{35}S-methionine, 10 hours. Time of addition of asialoglycoprotein, 0 hours. For doses mentioned, see Fig. 2. Cells were incubated in light (conditions described in the text) except where mentioned. Age of host plant : 10 days. Tracks: A, facsimile of inactive PAL and active PAT protein; B; facsimile of active PAL and active PAT protein; 1. MWM; 2. PAL and PAT standard (see text); 3. dose 0; 4. dose 1; 5. dose 1 + PHA antiserum at 0 hours; 6. dose 1 + cycloheximide at 5 hours; 7. dose 2; 8. dose 3; 9. dose 4; 10. dose 4 + PHA antiserum at 0 hours; 11. dose 4 + cycloheximide at 5 hours; 12. dose 5; 13. MWM; 14. MWM; 15. PAL and PAT standard (see text); 16. dose 0 + L-phenylalanine at 0 hours; 17. dose 4 + actinomycin D at 0 hours; 18. dose 4 + actinomycin D at 5 hours; 19. dose 4 + cycloheximide at 0 hours; 20. dose 4 and incubation in the dark; 21. dose 4 + galactose at 0 hours; 22. dose 4 + UDP at 0 hours; 23. MWM; 24. MWM; 25. PAL and PAT standard; 26. dose 5 + actinomycin D at 0 hours; 27. dose 5 + actinomycin D at 5 hours; 28. dose 5 + cycloheximide at 0 hours; 29. dose 5 and incubation in the dark; 30. dose 5 + N-acetylgalactosamine at 0 hours; 31. dose 5 + incubation at 40^{o}C at -1,5 to 0 hours; 32. dose 5 + UDP at 0 hours; 33. MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

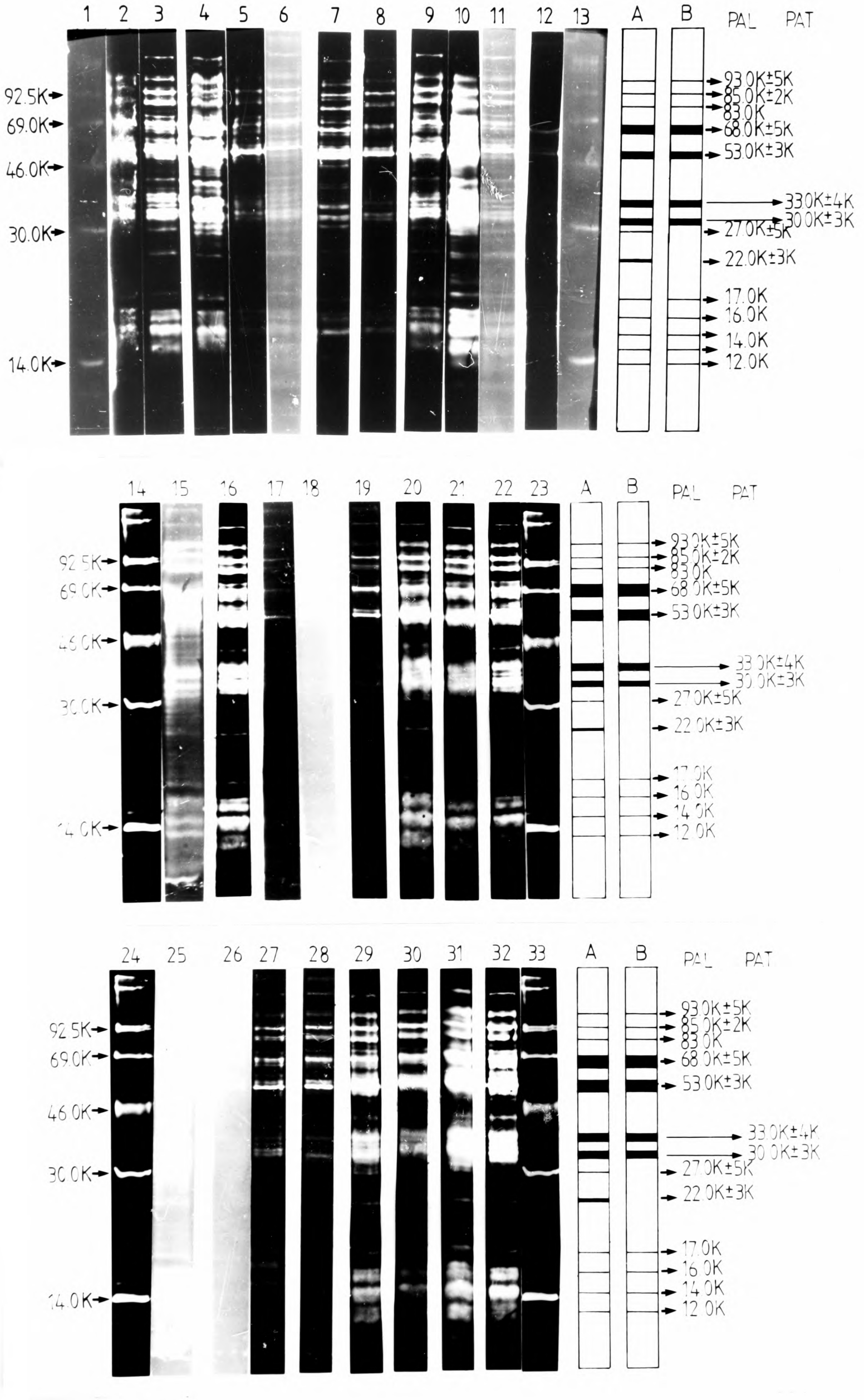
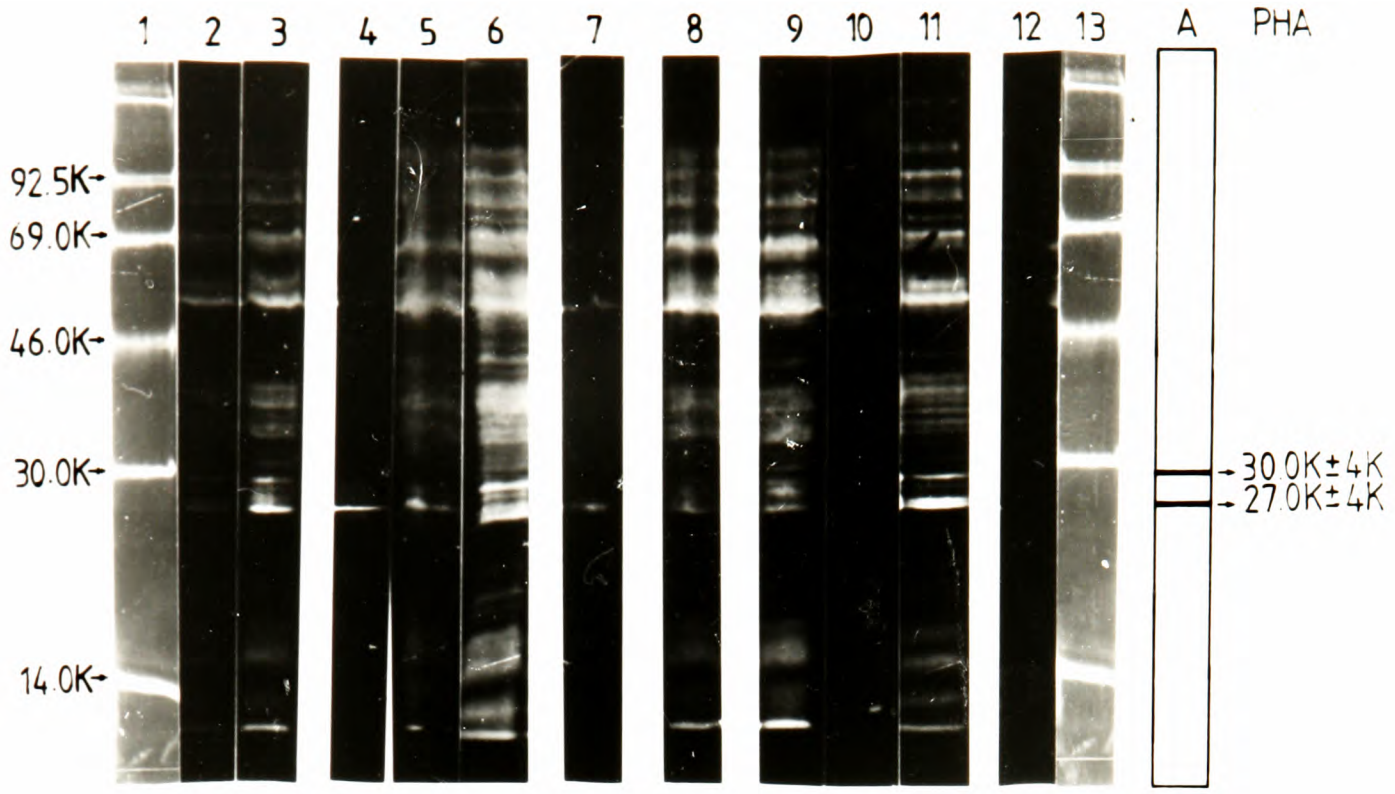

1 2 3 4 5 6 7 8 9 10 11 12 13
A B
PAL PAT
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
33.0K±4K
30.0K±3K
27.0K±5K
22.0K±3K
17.0K
16.0K
14.0K
12.0K
14 15 16 17 18 19 20 21 22 23
A B
PAL PAT
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
33.0K±4K
30.0K±3K
27.0K±5K
22.0K±3K
17.0K
16.0K
14.0K
12.0K
24 25 26 27 28 29 30 31 32 33
A B
PAL PAT
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
33.0K±4K
30.0K±3K
27.0K±5K
22.0K±3K
17.0K
16.0K
14.0K
12.0K

Fig. 16 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - the immunoprecipitation of de novo synthesised and non de novo synthesised phytohemagglutinin (PHA). Time of incubation with ^{35}S-methionine, 10 hours (except where mentioned). Time of addition of asialoglycoprotein, 0 hours. For doses mentioned, see Fig. 2. Cells were incubated in light (conditions described in the text), except where mentioned. Age of host plant : 10 days. Tracks: A, facsimile of PHA; 1. MWM; 2. time, 0 hours, dose 0; 3. dose 0; 4. dose 1; 5. dose 1 + PHA antiserum at 0 hours; 6. dose 1 + cycloheximide at 5 hours; 7. dose 2; 8. dose 3; 9. dose 4; 10. dose 4 + PHA antiserum at 0 hours; 11. dose 4 + cycloheximide at 5 hours; 12. dose 5 13. MWM; 14. MWM; 15. dose 0 + L-phenylalanine at 0 hours; 16. dose 1 + actinomycin D at 0 hours; 17. dose 1 + actinomycin D at 5 hours; 18. dose 1 + cycloheximide at 0 hours; 19. dose and incubation in the dark; 20. dose 2 + galactose at 0 hours; 21. dose 2 + UDP at 0 hours; 22. MWM; 23. MWM; 24. dose 4 + actinomycin D at 0 hours; 25. dose 4 + acinomycin D at 5 hours; 26. dose 4 + cycloheximide at 0 hours; 27. dose 4 and incubation in the dark; 28. dose 4 + N-acetylgalactosamine at 0 hours; 29. dose 4 + incubation at 40^{o}C at -1.5 to 0 hours; 30. dose 4 + UDP at 0 hours; 31. MWM; 32. MWM; 33. 0 hours; 34. 5 hours, dose 0; 35. 5 hours, dose 1; 36. 5 hours, dose 1.5; 37. 5 hours, dose 2; 38. 5 hours, dose 3; 39. 5 hours, dose 4; 40. 5 hours, dose 5; 41. MWM; 42. IGG; 43. 5 hours, dose 0; 44. 5 hours, dose 1; 45. 5 hours, dose 4; 46. 5 hours, dose 0; 47. 5 hours, dose 1; 48. 5 hours, dose 1 + N-acetylgalactosamine at 0 hours; 49. 5 hours, dose 1 + UDP at 0 hours; 50. 10 hours, dose 4; 51. 10 hours, dose 4 + N-acetylgalactosamine at 0 hours; 52. 10 hours, dose 4 + UDP at 0 hours; 53. IgG; 54. IgG.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.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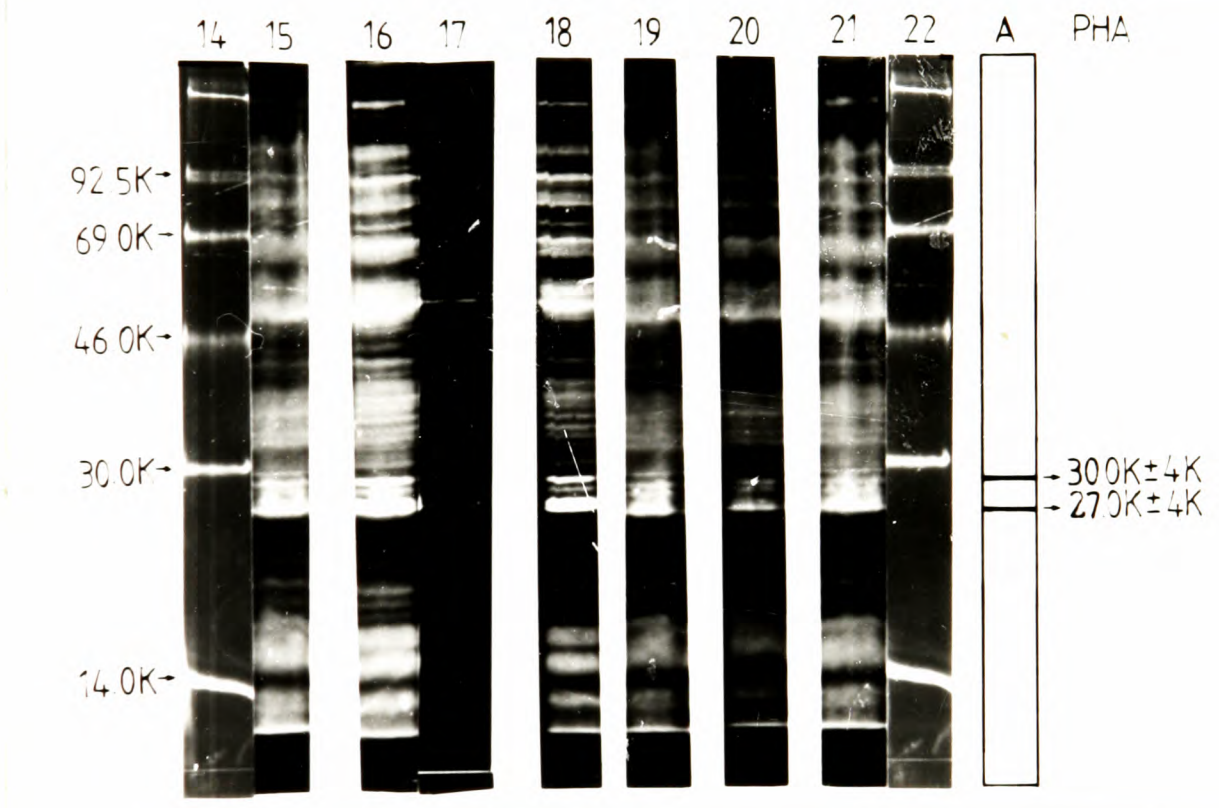
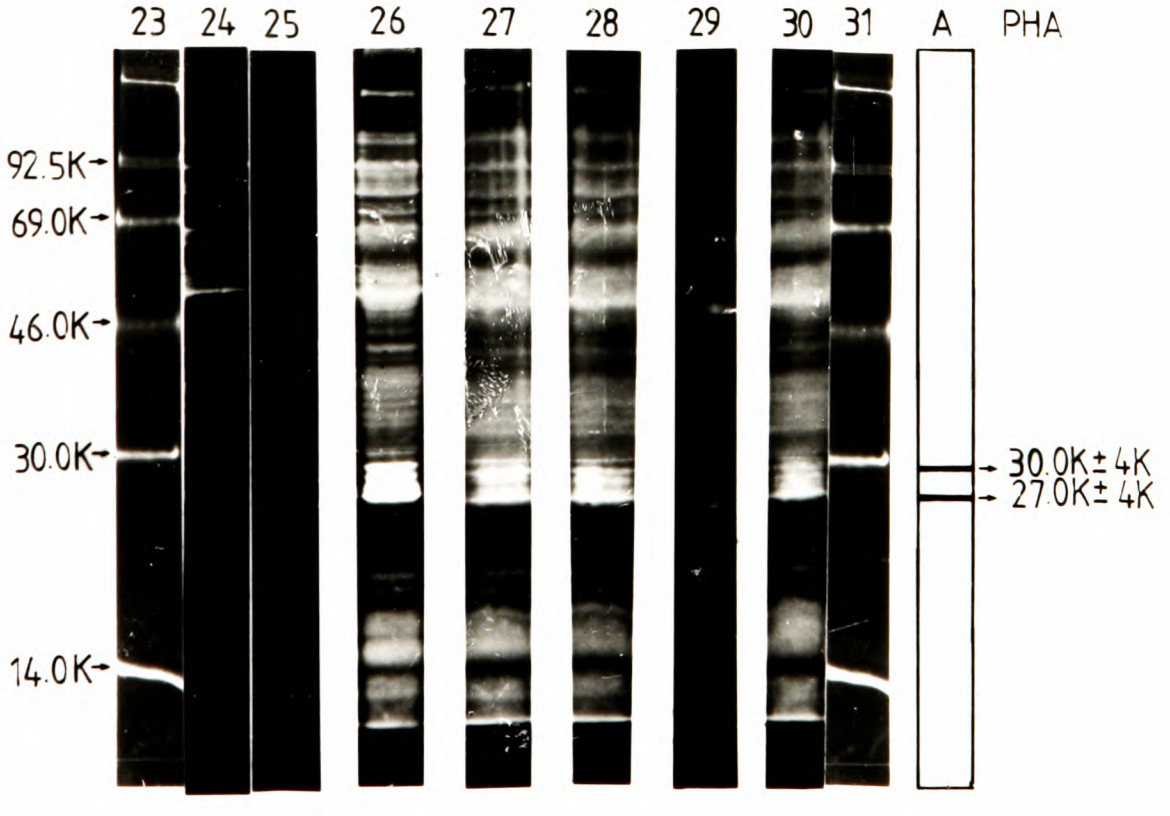
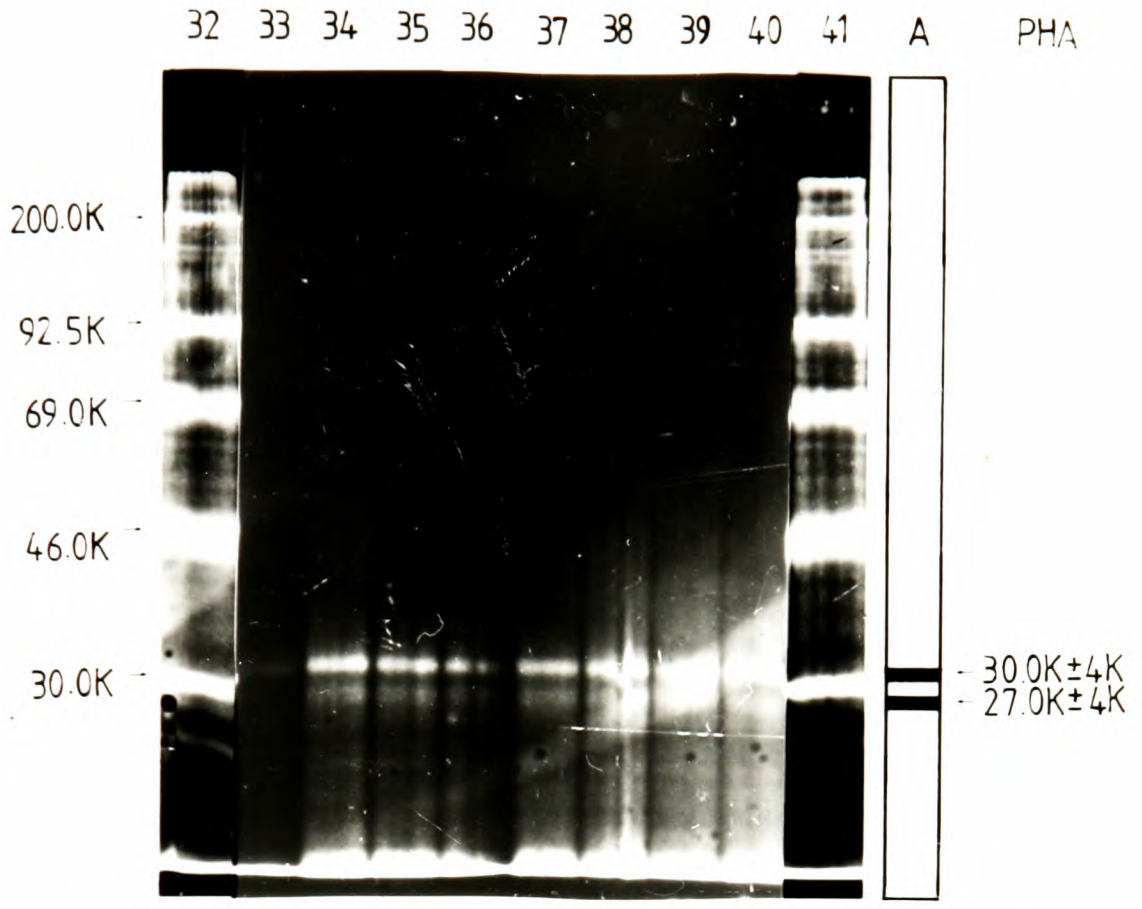
69.0K
46.0K
30.0K
14.0K
30.0K±4K
27.0K±4K


14 15 16 17 18 19 20 21 22 A PHA
92.5K
69.0K
46.0K
30.0K
14.0K
30.0K±4K
27.0K±4K


23 24 25 26 27 28 29 30 31 A PHA
92.5K
69.0K
46.0K
30.0K
14.0K
30.0K±4K
27.0K±4K


32 33 34 35 36 37 38 39 40 41 A PHA
200.0K
92.5K
69.0K
46.0K
30.0K
30.0K±4K
27.0K±4K

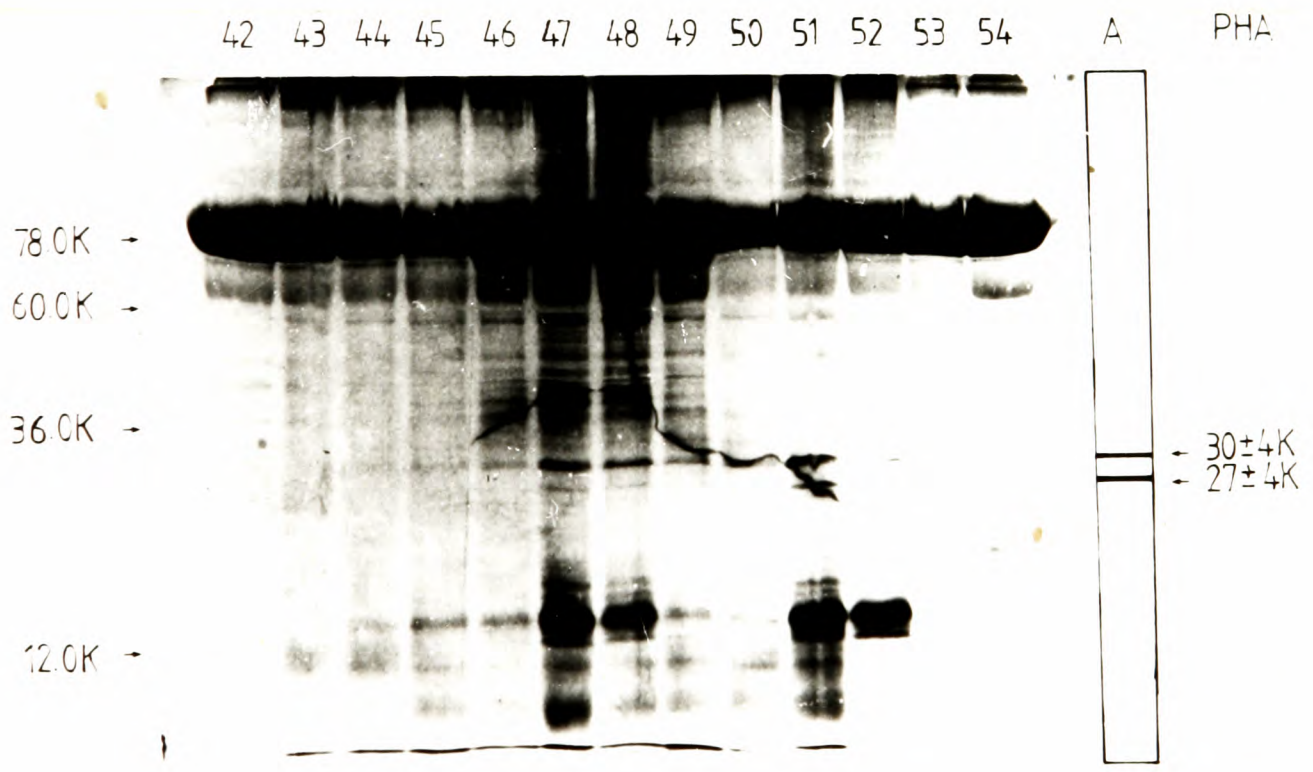
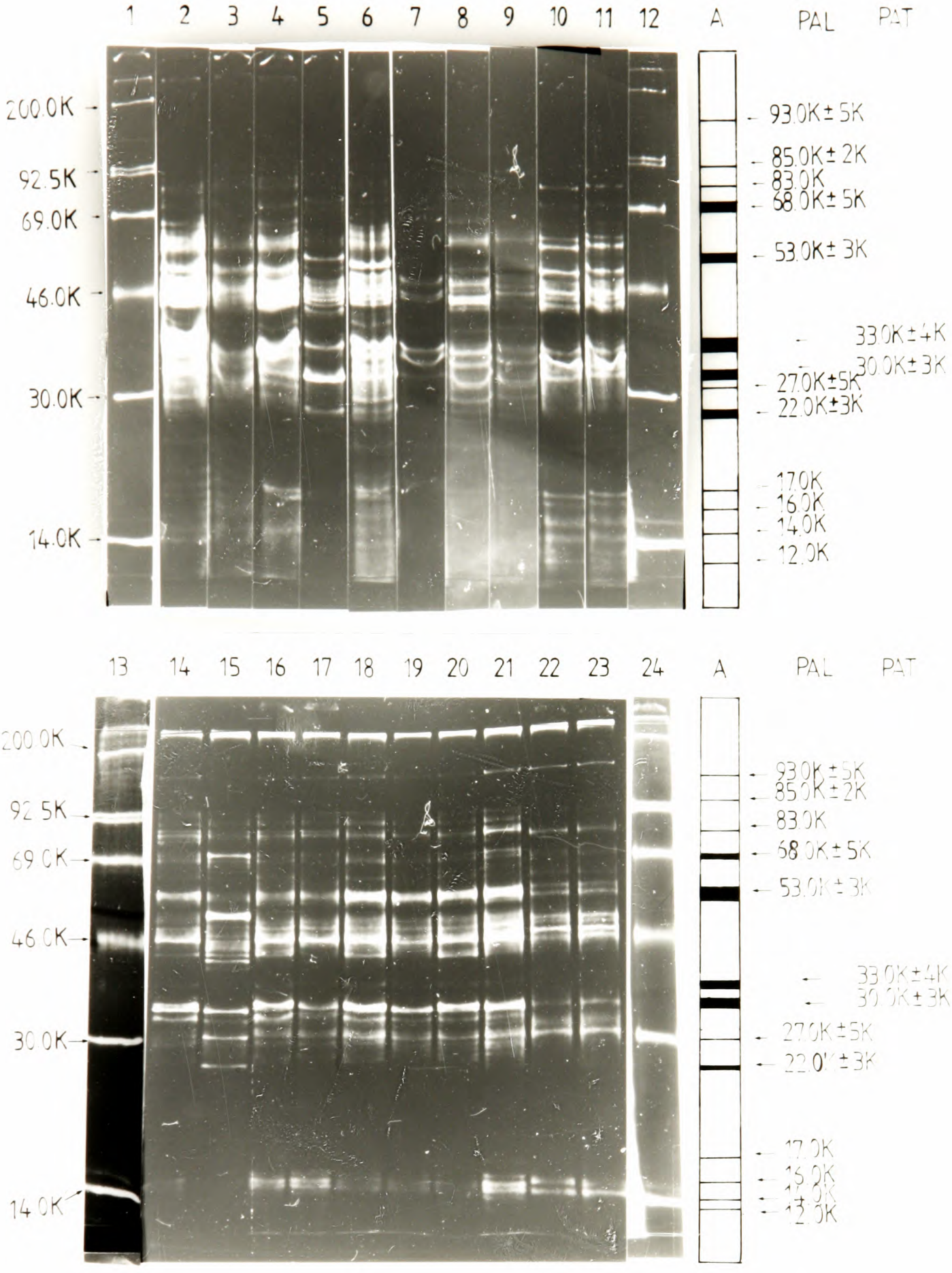

42 43 44 45 46 47 48 49 50 51 52 53 54 A PHA
78.0K
60.0K
36.0K
12.0K
30±4K
27±4K

Fig. 16 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - de novo synthesis of inactive PAL and active PAL and PAT protein. Time of incubation with ^{35}S-methionine, 0.5 hour pulses from time mentioned. Time of addition of dose 2 (see Fig. 2) asialoglycoprotein, 0 hours. Age of host plant : 10 days. For fascsimile of active PAL and active PAT protein, see Fig. 14, B. Tracks: A. facsimile of inactive PAL and active PAT protein; 1. MWM; 2 to 12: soluble protein: 2. -0.5 hours; 3. 0 hours; 4. 0.5 hours; 5. 1 hour; 6. 1.5 hours; 7. 2 hours; 8. 2.5 hours; 9. 3 hours; 10 3.5 hours; 11. 4 hours; 12. 4.5 hours; 13. MWM; 14. MWM; 15 to 25: membrane protein; 15. -0.5 hours; 16. 0 hours; 17. 0.5 hours; 18. 1 hour; 19. 1.5 hours; 20. 2 hours; 21. 2.5 hours; 22. 3 hours; 23. 3.5 hours; 24. 4 hours; 25. 4.5 hours; 26. MWM; 27 to 37: extracellular protein: 27. -0.5 hours; 28. 0 hours; 29. 0.5 hours; 30. 1 hour; 31. 1.5 hours; 32. 2 hours; 33. 2.5 hours; 34. 3 hours; 35. 3.5 hours; 36. 4 hours; 37. 4.5 hours;

Cells were treated with ^{35}S-methionine in presence of different concentrations of pathogen cell wall asialoglycoprotein shown in Fig. 3. Protein present in the membrane, soluble and extracellular fractions were subjected to SDS-PAGE. Peptides corresponding to those obtained for purified preparations of PAT and PAL protein were quantified. For further details see 'methods'. Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run.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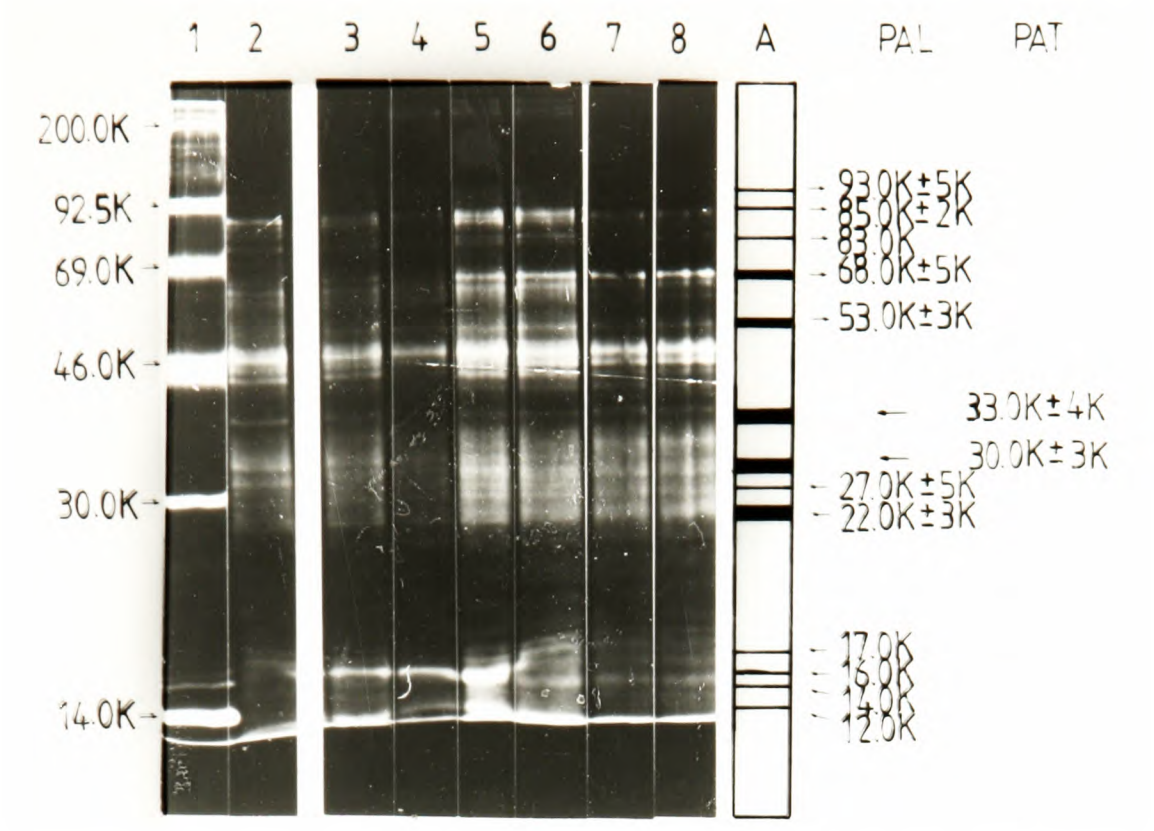
PAL
PAT
200.0K
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K ± 5K
85.0K ± 2K
83.0K
68.0K ± 5K
53.0K ± 3K
33.0K ± 4K
30.0K ± 3K
27.0K ± 5K
22.0K ± 3K
17.0K
16.0K
14.0K
12.0K

13 14 15 16 17 18 19 20 21 22 23 24
A
PAL
PAT
200.0K
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K ± 5K
85.0K ± 2K
83.0K
68.0K ± 5K
53.0K ± 3K
33.0K ± 4K
30.0K ± 3K
27.0K ± 5K
22.0K ± 3K
17.0K
16.0K
14.0K
12.0K

25 26 27 28 29 30 31 32 33 34 35 36
A
PAL
PAT
200.0K
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K ± 5K
85.0K ± 2K
83.0K
68.0K ± 5K
53.0K ± 3K
33.0K ± 4K
30.0K ± 3K
27.0K ± 5K
22.0K ± 3K
17.0K
16.0K
14.0K
12.0K

Fig. 19 Protein synthesis in host cells (devoid of middle lamella). Time of incubation with ^{35}S-methionine, 1 hour pulses from time mentioned. For facsimile of active PAL and active PAT protein, see Fig. 14. B. Age of host plant : 10 days. Tracks: A. facsimile of inactive PAL and active PAT protein; 1. MWM; 2 to 8: soluble protein: 2. 0 hours; 3. 3 hours; 4. 4 hours; 5. 5 hours; 6. 6 hours; 7. 8 hours; 8. 11.5 hours; 8. MWM; 8 to 16: membrane protein: 10. 0 hours; 11. 3 hours; 12. 4 hours; 13. 5 hours; 14. 6 hours; 15. 8 hours; 16. 11.5 hours; 17. MWM; 18 to 24: extracellular protein: 18. 0 hours; 19. 3 hours; 20. 4 hours; 21. 5 hours; 22. 6 hours; 23. 8 hours; 24. 11.5 hours.

Cells were treated with ^{35}S-methionine in presence of different concentrations of pathogen cell wall asialoglycoprotein shown in Fig. 3. Protein present in the membrane, soluble and extracellular fractions were subjected to SDS-PAGE. Peptides corresponding to those obtained for purified preparations of PAT and PAL protein were quantitted. For further letails see 'methods'. Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run.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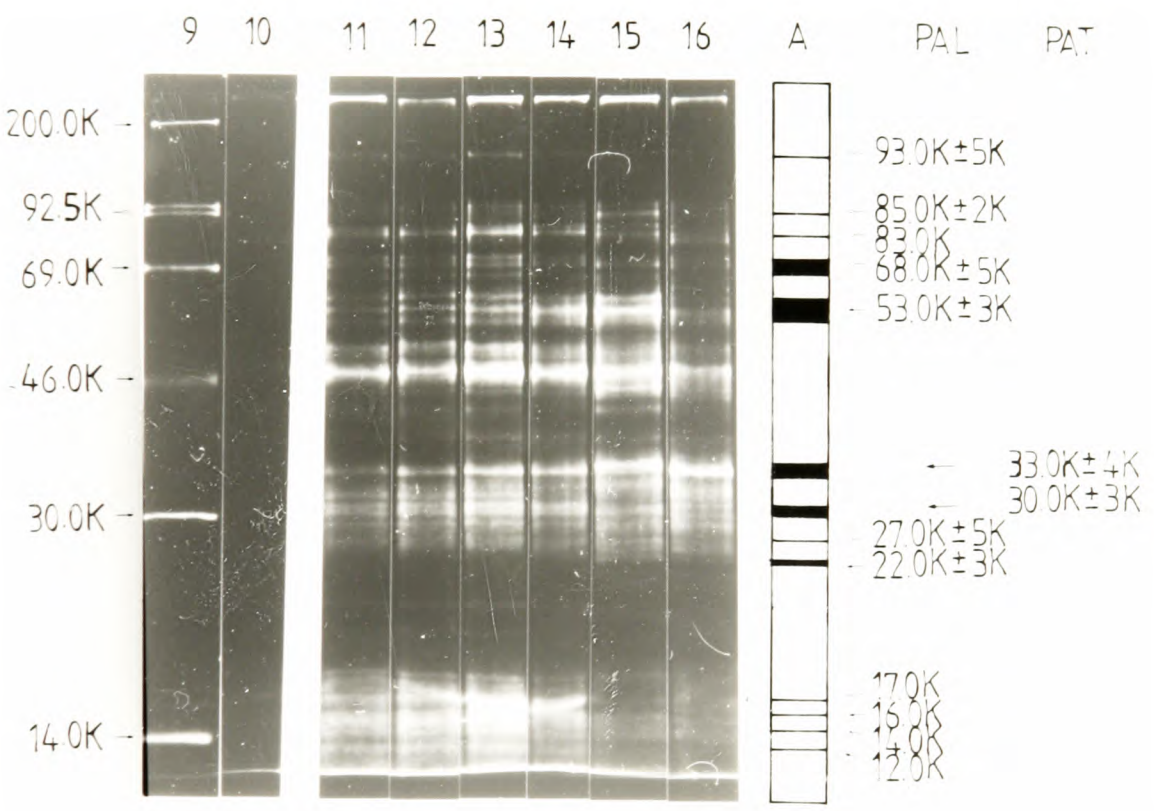
69.0K
46.0K
30.0K
14.0K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
33.0K±4K
30.0K±3K
27.0K±5K
22.0K±3K
17.0K
16.0K
12.0K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92.5K
69.0K
46.0K
30.0K
14.0K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
33.0K±4K
30.0K±3K
27.0K±5K
22.0K±3K
17.0K
16.0K
12.0K

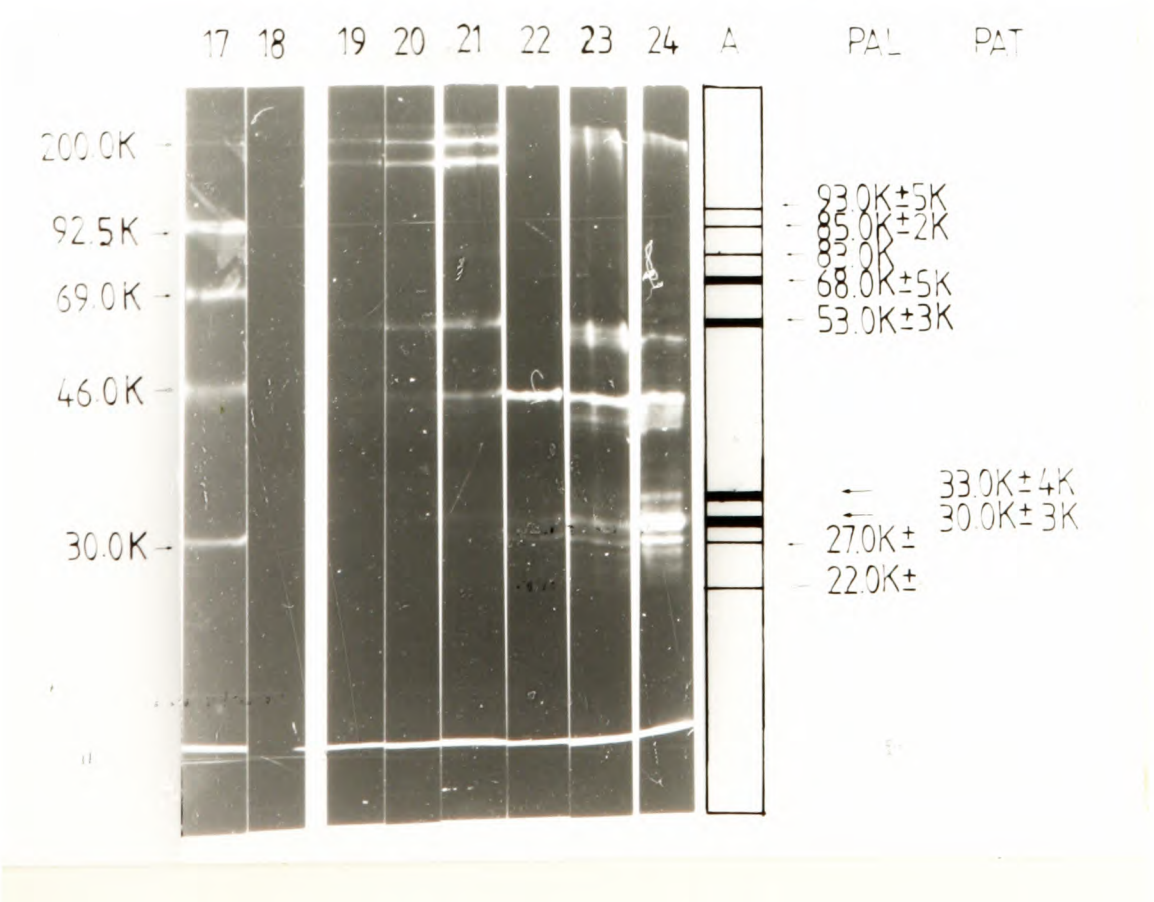
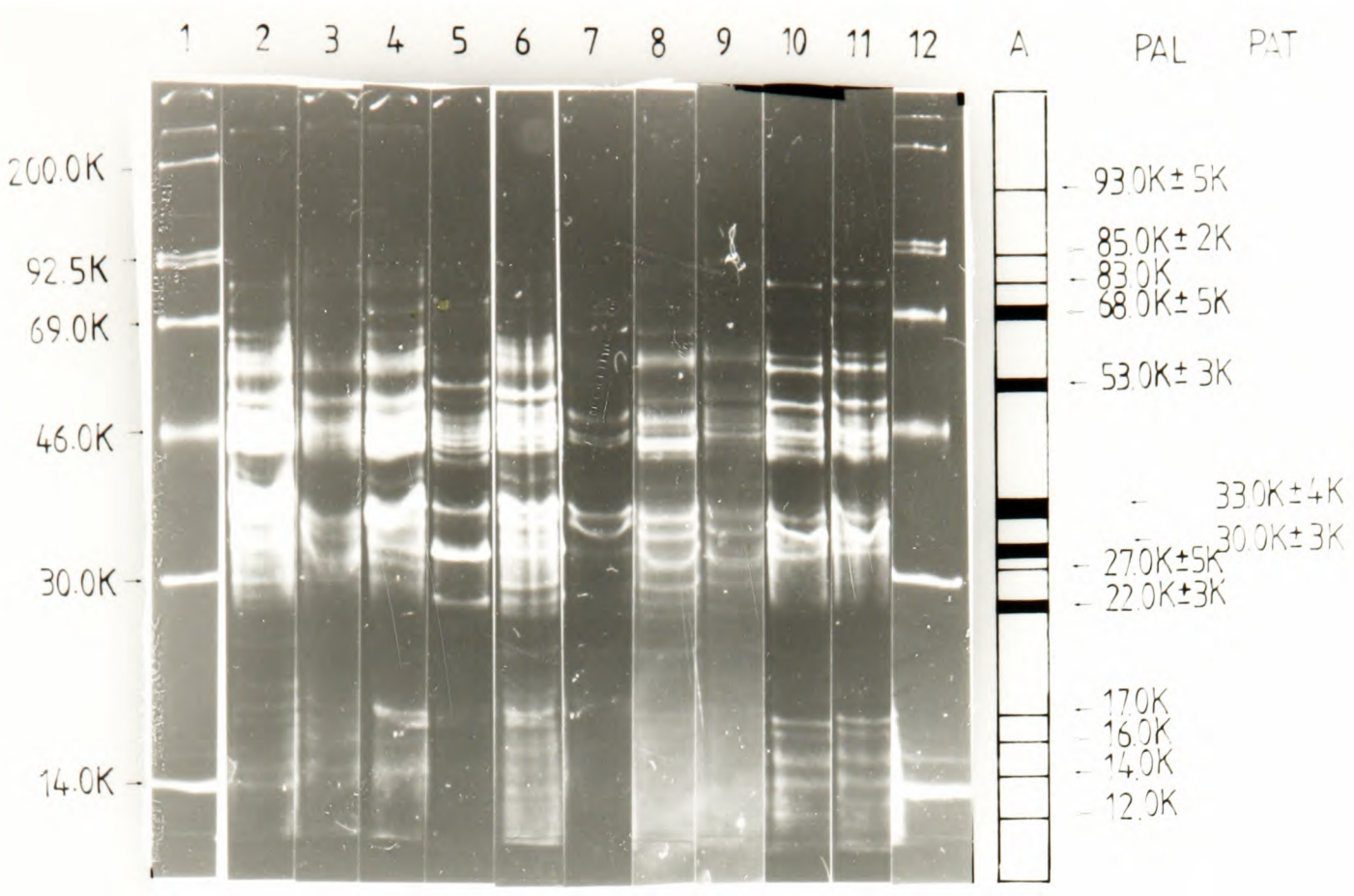

17 18 19 20 21 22 23 24 A PAL PAT
200.0K
92.5K
69.0K
46.0K
30.0K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
33.0K±4K
30.0K±3K
27.0K±
22.0K±

Fig. 20 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - the de novo synthesis of inactive PAL and active PAL and PAT protein in host cells of various ages. Time of incubation with ^{35}S-methionine, 10 hours. Time of addition of dose 2 (see Fig. 2), 0 hours. For facsimile of active PAL and active PAT protein, see Fig. 14, B. Tracks: A. facsimile of inactive PAL and active PAT protein; 1. MWM; 2 to 11: soluble protein: 2. 10 days (control); 3. 10 days (treated); 4. 14 days (control); 5. 14 days, (treated); 6. 17 days, (control); 7. 17 days, (treated); 8. 21 days, (control); 9. 21 days, (treated); 10. 30 days, (control); 11. 10 days, (treated); 12. MWM; 13. MWM; 14 to 23: membrane protein: 14. 10 days, (control); 15. 10 days, (treated); 16. 14 days, (control); 17. 14 days, (treated); 18. 17 days, (control); 19. 17 days, (treated); 20. 21 days, (control); 21. 21 days, (treated); 22. 30 days, (control); 23. 30 days, (treated); 24. MWM; 25. MWM; 26 to 35: extracellular protein: 26. 10 days, (control); 27. 10 days, (treated); 28. 14 days, (control); 29. 14 days, (treated); 30. 17 days, (control); 31. 17 days, (treated); 32. 21 days, (control); 33. 21 days, (treated); 34. 30 days, (control); 35. 30 days, (treated).

Cells were treated with ^{35}S-methionine in presence of different concentrations of pathogen cell wall asialoglycoprotein shown in Fig. 3. Protein present in the membrane, soluble and extracellular fractions were subjected to SDS-PAGE. Peptides corresponding to those obtained for purified preparations of PAT and PAL protein were quantified. For further details see 'methods'. Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run.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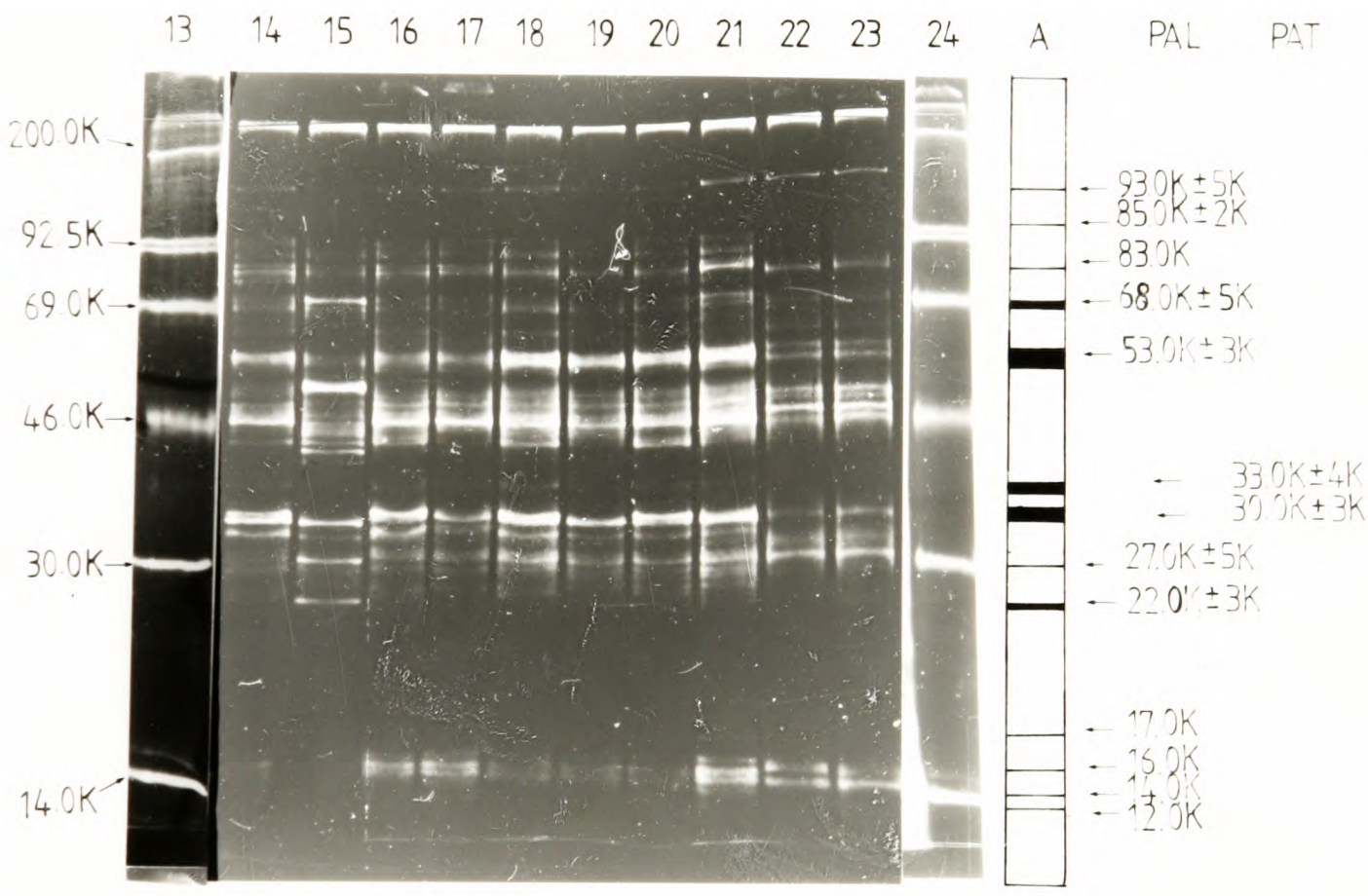
A
PAL
PAT
200.0K
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K ± 5K
85.0K ± 2K
83.0K
68.0K ± 5K
53.0K ± 3K
33.0K ± 4K
30.0K ± 3K
27.0K ± 5K
22.0K ± 3K
17.0K
16.0K
14.0K
12.0K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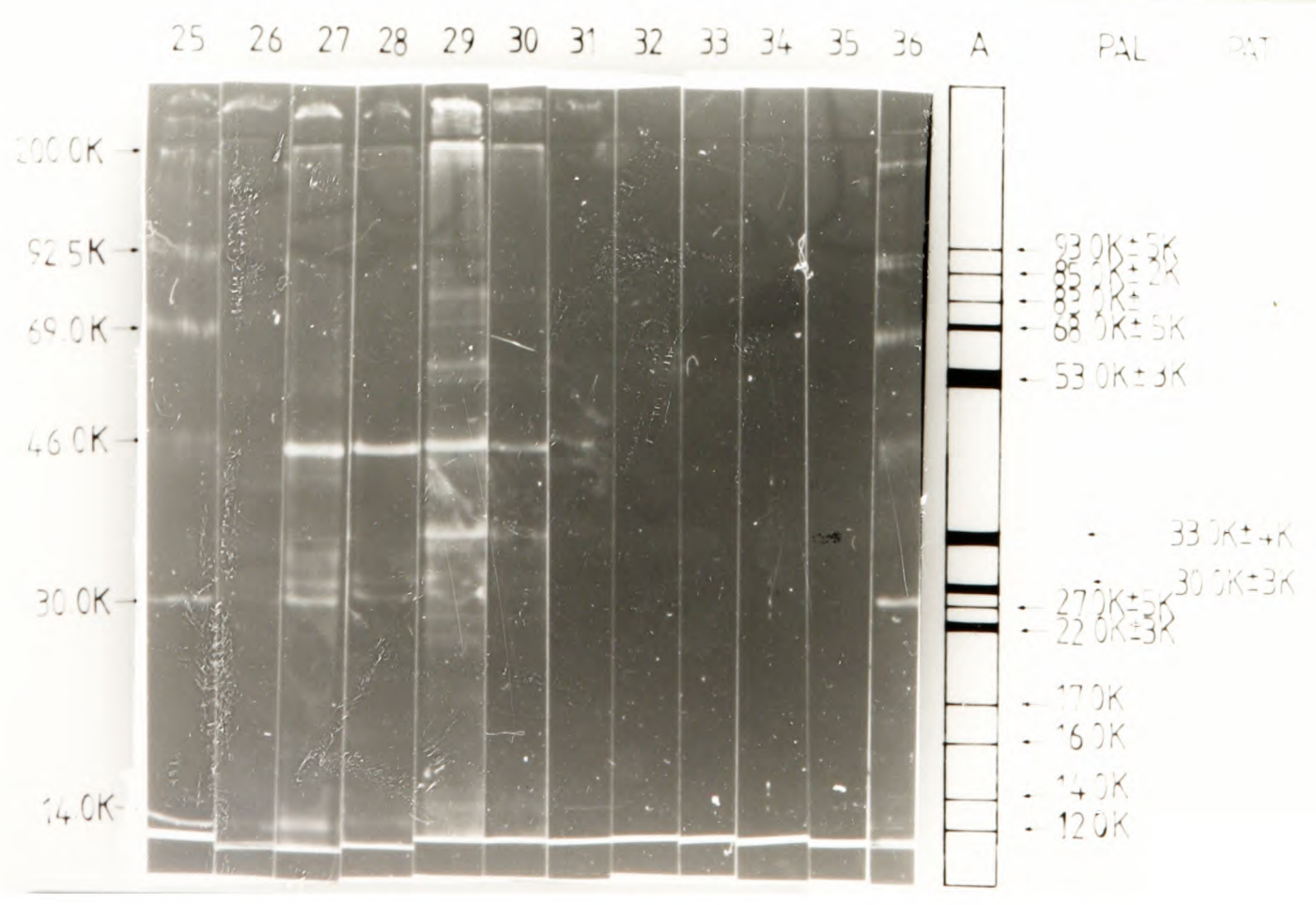
A
PAL
PAT
200.0K
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K ± 5K
85.0K ± 2K
83.0K
68.0K ± 5K
53.0K ± 3K
33.0K ± 4K
30.0K ± 3K
27.0K ± 5K
22.0K ± 3K
17.0K
16.0K
14.0K
12.0K


25 26 27 28 29 30 31 32 33 34 35 36
A
PAL
PAT
200.0K
92.5K
69.0K
46.0K
30.0K
14.0K
93.0K ± 5K
68.0K ± 5K
53.0K ± 3K
33.0K ± 4K
30.0K ± 3K
27.0K ± 5K
22.0K ± 3K
17.0K
16.0K
14.0K
12.0K

the finding that the synthesis of isoflavonoid phytoalexins is controlled by the first enzyme in the pathway of phenylpropanoid-derived phytoalexin synthesis, L-phenylalanine ammonia-lyase (PAL) [Chapter 1, section 1.3]. 4.1.3.5

It was therefore concluded that increased PAL activity depended on the de novo synthesis of PAL protein.

2.3.2.3. Mechanism of regulation of PAL activity:

Using a polyclonal antiserum raised to PAL (described in detail in chapter 7) and using homogenates of the whole cell which had been infiltrated with the pathogen, experiments were carrried out to study the method of PAL regulation by end product inhibition, substrate supply and substrate availability.

2.3.2.3.1. Regulation of PAL activity by end product inhibition has been suggested (Shields et al., 1982). However, the end product, t-cinnamic acid did not inhibit PAL, but did inhibit PAT, the enzyme responsible for the synthesis of L-phenylalanine, the substrate for PAL. This inhibition was only at a very high concentration, far higher than that found in the cell (described in detail in chapter 5).

It was therefore concluded that PAL activity could not be regulated by end product inhibition.

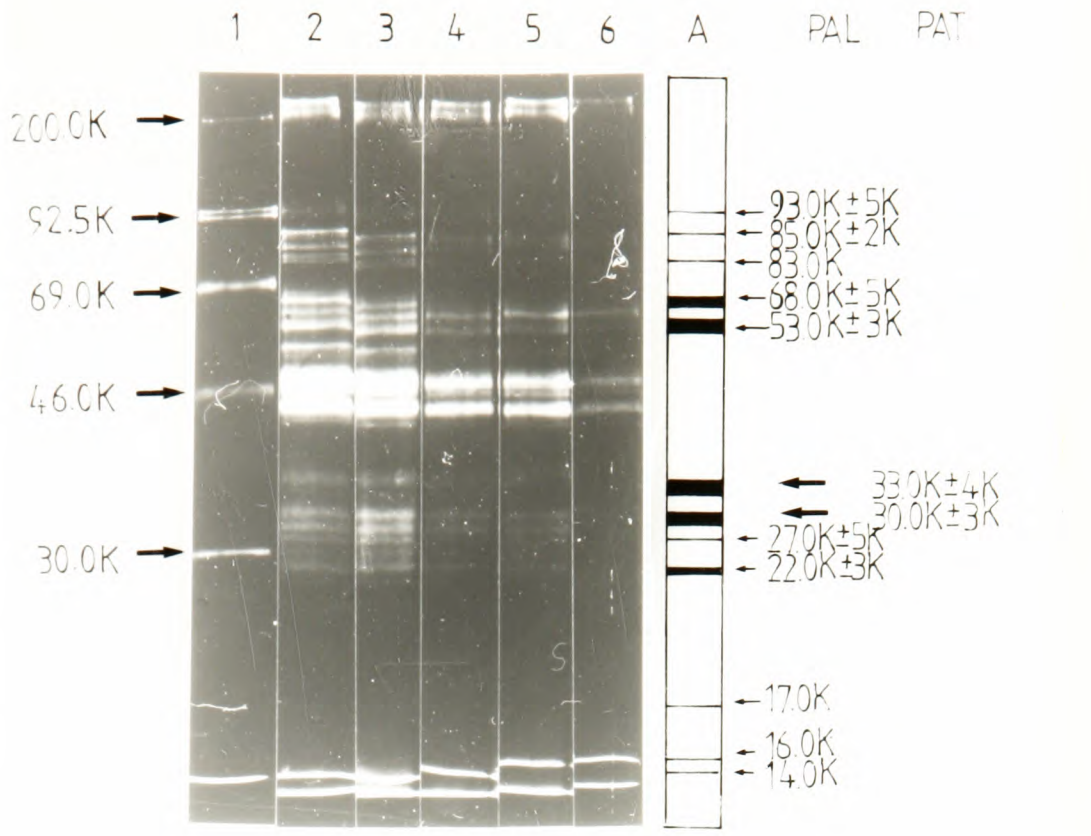
The possibility that PAL was regulated by the activity of PAT, which

is responsible for the synthesis of L-phenylalanine was considered. PAT like PAL was not affected by its end product, L-phenylpyruvic acid. PAL was inhibited by the end-product of PAT, L-phenylpyruvic acid (also reported by Henson & Havir, 1981), but at concentrations far higher than that found in the cell (described more fully in chapter 5).

2.3.2.3.2. Regulation of PAL activity by substrate supply: An enzyme PAT is responsible for the supply of L-phenylalanine. Furthermore, the activities of PAT protein during host-cell pathogen incubation resulted in an important finding, that of the induction of de novo synthesis and increased activity of PAT protein (measured in the direction L-phenylpyruvate ➡ L-phenylalanine) prior to induction of PAL activity and de novo synthesis of active PAL protein at higher concentrations of pathogen asialoglycoprotein (Fig. 8; Fig. 10; Fig. 16, tracks 3,4,7,8, and 9; Fig. 18). Controls showed no induction of de novo synthesised active PAT protein (Fig. 8; Fig. 10; Fig. 16, track 3; Fig. 18; Fig. 21). Further, the induction of de novo synthesis and increased activity of PAT protein was dependent on the age of the host leaf from which the cells were isolated. Host cells isolated from leaves older than 2 weeks showed neither induction of PAT activity nor de novo synthesis of activated PAT protein (Fig. 20). These results coincided with results obtained on interaction of live pathogen mycelia with intact host plants described above. However, when host cells isolated from leaves younger than 2 weeks were incubated with pathogen-derived asialoglycoprotein in the dark, de novo synthesis of PAT protein and induction of PAT activity did not occur (Fig. 16, tracks 20 and 29). These results coincided with

Fig. 19 The response of host cells (devoid of middle lamella) to fungal pathogen cell wall asialoglycoprotein - the de novo synthesis of inactive PAL and active PAL and PAT protein in aged host cells. Time of incubation with ^{35}S-methionine, 10 hours. Time of addition of asialoglycoprotein, 0 hours. Age of host plant: 27 days. For doses mentioned, see Fig.2. For facsimile of active PAL and active PAT protein, see Fig.14b. Tracks: A. facsimile of inactive PAL and active PAT protein; 1. MWM; 2 to 6: soluble protein: 2. dose 0; 3. dose 1; 4. dose 2; 5. dose 3; 6. dose 5; 7. MWM; 8 to 13: membrane protein: 8. dose 0; 9. dose 1; 10. dose 2; 11. dose 3; 12. dose 4; 13. dose 5; 14. MWM; 15 to 20: extracellular protein: 15. dose 0; 16. dose 1; 17. dose 2; 18. dose 3; 19. dose 4; 20. dose 5; 21. MWM.
All tracks contain an equal concentration of protein (2 µg).

Cells were treated with ^{35}S-methionine in presence of different concentrations of pathogen cell wall asialoglycoprotein shown in Fig. 3. Protein present in the membrane, soluble and extracellular fractions were subjected to SDS-PAGE. Peptides corresponding to those obtained for purified preparations of PAT and PAL protein were quantified. For further details see 'methods'. Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run.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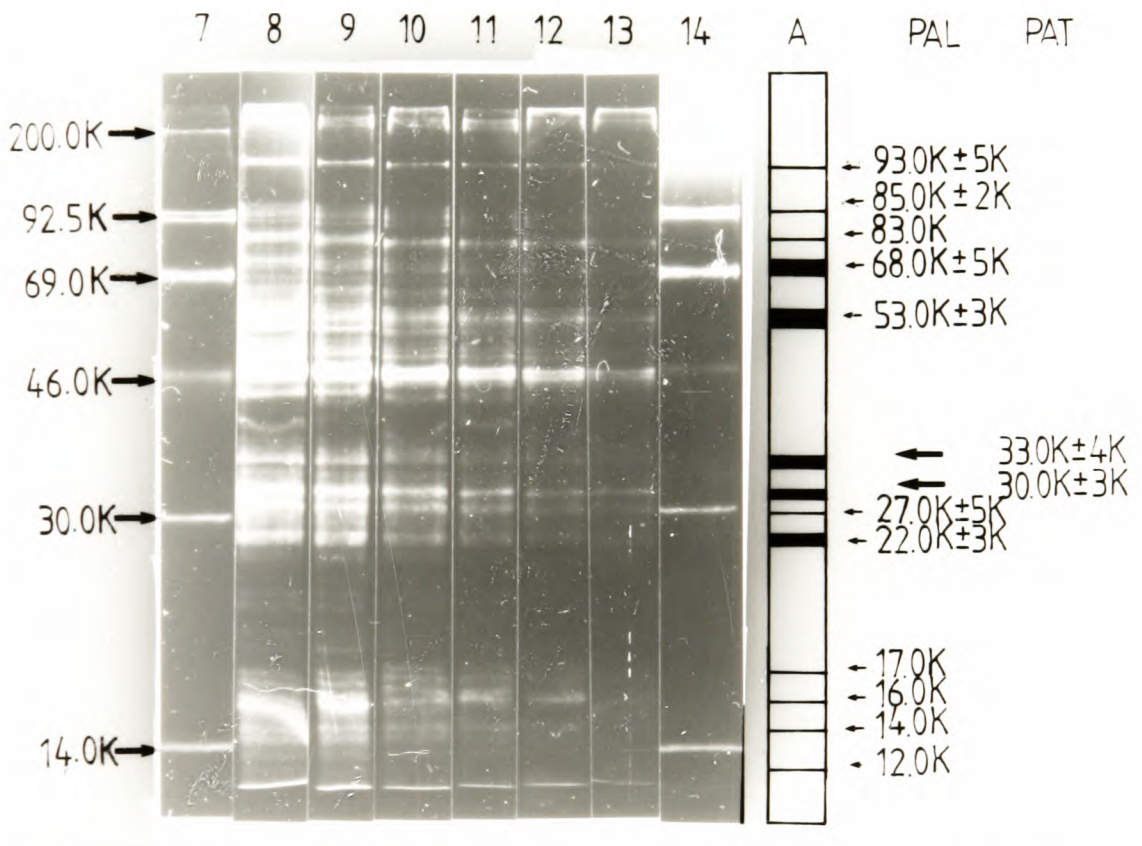
200.0K
92.5K
69.0K
46.0K
30.0K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
33.0K±4K
30.0K±3K
27.0K±5K
22.0K±3K
17.0K
16.0K
14.0K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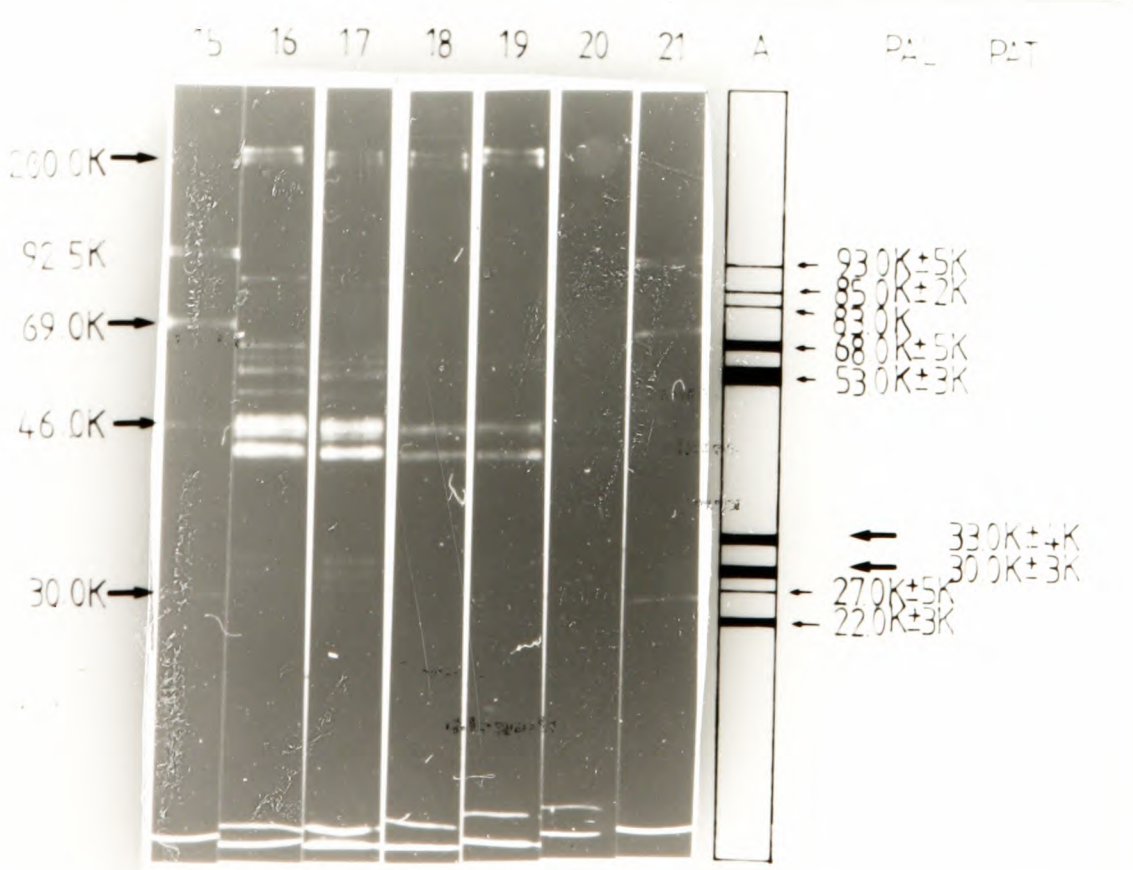
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results mentioned above, on interaction of live pathogen mycelia and cell wall asialoglycoprotein with whole plant and isolated cells respectively. The fact that these results were obtained 5 to 10 hours after removal of host cell wall and in presence of abiotic pathogen cell wall constituents illustrated, indirectly, a mechanism of regulation of PAL ativity, namely substrate availability. The presence of PAT protein in the extracellular medium of the cells suggests the possible regulation of substrate availability of surrounding cells. This finding was further tested as described below:

If the effect of increased PAT activity is to provide L-phenylalanine, prior to induction of de novo synthesis and activation of PAL protein, then it is conceivable that the host would respond to added L-phenylalanine by promoting de novo synthesis and activation of PAL in absence of pathogen cell wall asialoglycoprotein. However, addition of L-phenylalanine (assuming all L-phenylalanine is taken up by the cell) to isolated host cells in absence of pathogen cell wall asialoglycoprotein resulted in de novo synthesis and induction of activity of PAT and inactive PAL protein (Fig. 18; Fig. 16, track 16, compared to controls Fig. 16, track 3). This can be interpreted to mean that exogenous L-phenylalanine was not reaching the active site of PAL. Further observation of absence of increase in protein synthesis, and further, glycoprotein synthesis (eg. phytohemagglutinin) synthesis on addition of exogenous L-phenylalanine (Fig. 17, track 15) leads to the conclusion that exogenous L-phenylalanine was not made accessible to PAL because of its use in protein synthesis.

If the *de novo* synthesis and induction of PAT activity (L-phenylpyruvate ➡ L-phenylalanine) is determined by the flux of L-phenylalanine protein synthesis and protein synthesis is optimal in the uninfected host cell and further, if the Km of PAL (1.12mM) is higher than that of PAT (0.05mM) (described more fully in chapter 5) then, it is apparent that a large concentration of L-phenylalanine is necessary for PAL to function at half maximal velocity. If the supply of L-phenylpyruvate is kept constant by the controlling activity of prephenate dehydratase, and if the reaction catalysed by PAT is maintained in equilibrium, then the flux of L-phenylalanine into PAL (hence phenylpropanoid metabolism) would be greatly facilitated if protein synthesis were not functioning optimally. This would make it necessary for general protein synthesis to decrease prior to getting increased availability of L-phenylalanine for PAL activity.

A decline in protein synthesis concomitant with increased PAL activity was observed during host-pathogen interaction. Cells isolated from the young host plant responded to the pathogen cell wall asialoglycoprotein by inhibition of general protein synthesis in a dose-dependent manner (Fig. 11). It should be noted that the doses at which a decline in protein synthesis was observed, corresponded with doses at which an increase in active PAL protein was seen. On the other hand, it should also be noted that the doses at which no decline in protein synthesis was observed, corresponded with doses at which an increase in *de novo* synthesis of both active PAT and inactive PAL protein were seen. This response was not due to wounding, as no significant leakage (>10%) of *de novo* synthesised protein occurred

during the incubation period (further discussed in chapter 3).

However, the decline in protein synthesis was not associated with the phytoalexin response and hypersensitive necrosis in an age and light-dependent manner. Cells isolated from leaves older than 2 weeks also showed an inhibition of protein synthesis, concomitant with no induction of PAL activity on incubation with asialoglycoprotein (Fig. 21). A likely explanation for this result was the lack of induction of PAT activity observed in older plant leaves (as mentioned above) thus making inhibition of protein synthesis possible. These results suggest that the precursor of L-phenylalanine, L-phenylpyruvate is not produced by old plants. This may be because of diversion of pre-shikimate products into lignin synthesis in older plant cells.

Biotic and abiotic components that inhibited protein synthesis (as opposed to components which did not inhibit protein synthesis) induced PAL and PAT activities in isolated cells (Table 2),

Table 2. The effect of biotic and abiotic compounds, ribonuclease A and cupric chloride on protein synthesis and percentage PAL and PAT activities compared with control levels:

Treatment	protein synthesis %	PAT %	PAL %
biotic compound:			
Ribonuclease A (4.6_{-1} to 9.2µg. gFW^{-1} leaf tissue)	75 ± 1	33 ± 1	20 ± 10
abiotic compound:			
Cupric chloride (10^{-6} to 10^{-3} M)	25 ± 1	49 ± 10	6 ± 1

Values obtained for ribonuclease induction of PAL and PAT activities were 61 and 10% lower that those obtained for interaction of the host cell with pathogen asialoglycoprotein. It is conceivable that L-phenylalanine under these conditions was not allowed to accumulate to satisfy the high Km values of PAL.

A hypothesis which was tested at this stage was the decline in protein synthesis by depletion of compounds such as UDP, a constituent which could be influenced by the presence or absence of pathogen.

Addition of UDP during interaction of young cells of the bean plant (Phaseolus vulgaris L.) with the pathogen cell wall asialoglycoprotein prevented inhibition of protein synthesis in the host by the pathogen. Protein synthesis remained at control levels whilst the pathogen induced de novo synthesis and activity of PAT protein and also induced de novo synthesis of inactive PAL protein (Fig. 16, tracks 22 and 32 compared to controls Fig. 16, tracks 4 and 9). This suggested that the flow of L-phenylalanine to PAL in the host plant was possible but that the induction of de novo synthesis and activation of PAL protein was limited by the flux of L-phenylalanine into protein synthesis.

Following the observations from the previous section, it may be concluded that the level of PAL activity in the host plant in response to pathogen invasion is dependent on the substrate available to the enzyme [. . , Chapter 1, section 1.3.6].

It was conceivable that the decline in endogenous concentrations of UDP was due to its use in the de novo synthesis of a glycoprotein. UDP and sugars are required for the de novo synthesis of phytohemagglutinin (PHA), a glycoprotein thought to be involved a a recognition determinant in the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction. Immunoprecipitation of PHA (as described in methods, section 2.2.1.13) showed the de novo synthesis of PHA in association with the reduction in protein synthesis (Fig. 12; Fig. 17, tracks 3,4,7,8,9, 34 to 39, 47 and 50 compared to controls Fig. 12; Fig. 17, tracks 2,46 and 33). Further, exogenous UDP induced de novo synthesis of PHA (Fig. 17, tracks 21 and 30) to levels above controls (Fig. 17, tracks 4, 9, 49 and 52). Further, cells isolated from leaves of older plants (>2weeks) showed no synthesis of PHA. The incubation of cells isolated from younger leaf tissue (< 2 weeks) with asialoglycoprotein in darkness, resulted in induction of de novo synthesised PHA (Fig. 17, tracks 19 and 27) to levels above control values (Fig. 17, tracks 4 and 9). Mobilisation of starch makes phosphorylated sugars available for glycoprotein synthesis. These findings coincided with results mentioned above, on interaction of live pathogen mycelia and cell wall asialoglycoprotein with the young host plant and host cell (devoid of middle lamella) respectively. These results were indicative of the deviation in flux of L-phenylalanine into protein and glycoprotein (phytohemagglutinin) synthesis, and away from the PAL.

2.3.2.4. The need for PHA as a recognition determinant may need an increase in the PHA synthesis which we observe . The following results

were obtained from experiments designed to study the relationship between the phytoalexin response and hypersensitive necrosis:

PHA in heavy membrane fractions (cell wall and rough ER) of isolated host cells was found to combine with the asialoglycoprotein isolated from cell walls of the fungal pathogen (Fig. 12). Further, the association of PHA with asialoglycoprotein was dependent on the age of the host leaf from which the cells were isolated. Cells isolated from leaves older than 2 weeks of age showed no PHA glycoprotein or associated binding of PHA to asialoglycoprotein. These results coincided with those mentioned above, on interaction of live pathogen mycelia and cell wall asialoglycoprotein with the young, intact host plant and host cell (devoid of middle lamella).

A Scatchard plot (Fig. 14) of the binding process depicts a concave curve, with the concavity facing the right. Using the following equation to achieve best fit (Aducci et al., 1984),

$$\frac{B}{F} = q_{i=1} \frac{n_i P_T}{K_{di} + F} \quad \ldots\ldots \text{ equation 2}$$

where, B = concentration of asialoglycoprotein associated with PHA,
F = concentration of asialoglycoprotein not associated but free;
q = number of site classes corresponding to different affinities;
n_i = number of sites within the phytohemagglutinin (PHA) molecule which combine with the same dissociation constant, K_{di};
and P_T = total protein concentration,

two classes of combining sites corresponding to 2 different

affinities for the asialoglycoprotein were obtained. The Kd values were 10 mg.gFW-1 leaf tissue and 2.5 mg.gFW-1 leaf tissue respectively. These Kd values corresponded to those doses required for maximal activities of induction of PAL activity.

Each PHA glycoprotein has sites which associate with the same dissociation constant or Kd. Therefore every PHA molecule that is newly synthesised has 1 class of association sites with one affinity class. Bound asialoglycoprotein was not reduced appreciably by excess asialoglycoprotein below 10 mg.gFW-1 indicating that the concentrations of pathogen cell wall asialoglycoprotein necessary for inhibition of protein synthesis, induction of de novo synthesis of PAT, PAL and PHA and induction of activity of PAT and PAL protein were below the Kd.

Undissociable association was not decreased (below Kd concentrations) on incubation with asialoglycoprotein in presence or absence of N-acetylgalactosamine or galactose for periods of up to 10 hours, indicating an irreversible association with the membrane following the initial interaction. Further, the asialoglycoprotein-PHA complex was not freely dissociable. The complex could be denatured in presence of sodium dodecyl sulphate.

The average affinity plot (Fig. 15) for the combining process indicated positive cooperativity with increasing concentrations of asialoglycoprotein. The association of 1 asialoglycoprotein molecule to 1 site, enhanced the association of subsequent molecules at the other sites. If every PHA molecule that is synthesised by de novo synthesis has 1

class of combining sites with 1 affinity class, then PHA molecules synthesised de novo during interaction with pathogen asialoglycoprotein probably have combining sites with the higher affinity association class of protein.

The de novo synthesis of PHA was enhanced on addition of N-acetylgalactosamine and galactose (at concentrations of 364 $\mu g.gFW^{-1}$ leaf tissue) during the combining process (Fig. 17, tracks 20 and 28 when compared to controls Fig. 17, tracks 4, 9 and 46). This indicated that the association of asialoglycoprotein with PHA at higher concentrations was not nonspecific, but actually specific. The number of higher affinity PHA combining, sites for N-acetylgalactosamine and the number of lower affinity PHA combining sites, for galactose was therefore increased irrespective of the concentration of either of the sugars endogenous to the asialoglycoprotein.

The combining interaction between PHA and the pathogen asialoglycoprotein and its relation to induction of PAL activity was further tested under condition of inhibited association as follows: Addition of PHA antiserum (at concentrations that would precipitate $0.9g \cdot gFW^{-1}$ PHA) prior to addition of pathogenic asialoglycoprotein, reulted in no association of PHA with pathogenic determinants above control values in host cells (devoid of middle lamella). This also resulted in inhibition of PHA synthesis (Fig. 17, tracks 5 and 10) above control values, when necessary (Fig. 17, tracks 4 and 9). Protein synthesis remained at control levels, while de novo synthesis of PAT occurred (Fig 16, tracks 5 and 10) above control levels, when PHA synthesis was

necessary (Fig. 16, tracks 9 and 4). The lack of inhibition of protein synthesis would theoretically not allow the flux of L-phenylalanine into PAL. This may explain the absence of de novo synthesis and activation of PAL protein (Fig. 16, tracks 5 and 10) above controls, when theoretically required (Fig. 16, tracks 9 and 4).

The host plant responds to the fungal pathogen by recognition of specific determinants and intercellular signals. This we have noted earlier (Chapter 1, section 1.3.2).

2.3.2.5. Protein synthesis was therefore necessary for both the induction of enzyme activities in the metabolic pathway leading to the production of phytoalexins and also molecules involved in recognition of determinants on the host/pathogen cell surface. The following experiments were carried out to test the importance of protein synthesis in the induction of the phytoalexin response:

Protein synthesis was associated with optimal induction of de novo synthesis and activity of PHA, PAL and PAT protein in the host, in response to the pathogen (Fig. 8; Fig. 10; Fig. 11; Fig. 12; Fig. 13). The importance of this effect increases with increasing demands for PHA, PAL and PAT protein. Inhibition of protein synthesis (translation and transcription) (Fig. 11) was associated with loss of asialoglycoprotein bound to PHA (Fig. 13), inhibition of de novo synthesis of PHA (Fig. 12; Fig. 17, track 12) to values below controls (Fig. 17, track 2), and inhibition of de novo synthesis of PAT and PAL protein (Figs. 10; Fig. 16, track 12) to values below controls (Fig.

16, track 3). Partial inhibition of protein synthesis appears to be necessary for induction of de novo synthesis of PHA, PAL and PAT protein and hence, the induction of phenylpropanoid metabolism.

Inhibition of transcription (by addition of $20\,\mu g \cdot ml^{-1}$ actinomycin D) simultaneously with the addition of pathogenic asialoglycoprotein (at concentrations below those usually required for de novo synthesis of PHA) resulted in enhanced de novo synthesis of PHA (Fig. 17, track 16 compared to control values Fig. 17, track 4). However the inhibition of translation (by addition of 0·15mM cycloheximide) simultaneously with addition of pathogenic asialoglycoprotein (at concentrations below those normally required for de novo synthesis of PHA) resulted in inhibition of de novo synthesis of PHA (Fig. 17, track 18) to values below those in the control (Fig. 17, track 4). Initial time points in binding of non de novo synthesised PHA to pathogen cell wall asialoglycoprotein depended on translation (and not transcription) of PHA mRNA. This phenomenon was induced by inhibition of transcription. It is possible that UDP levels (when sugar was sub-optimal in the light) enhanced processing of the mRNA coding for PHA.

The inhibition of transcription or translation (by addition of actinomycin D or cycloheximide) 5 hours after addition of pathogenic asialoglycoprotein resulted in the reverse of the above situation. Inhibition of transcription (at concentrations below those required for de novo synthesis of PHA) resulted in excessive inhibition of de novo PHA synthesis (Fig. 17, tracks 6 and 17) below control values (Fig. 17, track 4). The inhibition of translation (at concentrations

below those required for de novo synthesis of PHA) resulted in induction of de novo synthesis of PHA (Fig. 17, track 6) above controls (Fig. 17, track 4). Hence, later time points in binding non de novo synthesised PHA to pathogen cell wall asialoglycoprotein required transcription of de novo synthesised PHA transcripts and translation of degradatory enzymes of the PHA-asialoglycoprotein complex.

The inhibition of transcription (by addition of actinomycin D) simultaneous with or 5 hours after addition of pathogenic asialoglycoprotein (at concentrations requiring de novo synthesis of PHA) resulted in excessive inhibition of PHA synthesis (Fig. 17, tracks 24 and 25) over controls (Fig. 17, track 9).The inhibition of translation (by addition of cyclohexamide) simultaneous with or 5 hours after addition of pathogenic asialoglycoprotein (at concentrations requiring de novo synthesis of PHA) resulted in induction of PHA synthesis (Fig. 17, tracks 26 and 11) over controls (Fig. 17, track 9). Enhanced binding of PHA to asialoglycoprotein required transcription (and not translation) of PHA transcripts together with translation of mRNA of enzymes that degrade the PHA-asialoglycoprotein complex.

The above results showed protein synthesis be to an important requirement for the response.

2.3.2.6. Experiments were carried out to test the ability of the host cell to respond to the pathogen asialoglycoprotein at higher temperatures:

Incubation of young host cells at 40°C prior to addition of pathogen cell wall asialoglycoprotein (at concentration optimal for protein synthesis and induction of de novo synthesis of PHA, PAT and PAL protein) resulted in the inability of the host to respond to the pathogen by lack of de novo synthesis of PHA glycoprotein (Fig. 17, track 29) over controls (Fig. 17, track 3), absence of decrease in protein synthesis by an increase in de novo synthesis of PAT protein (Fig. 16, track 31) over controls (Fig. 16, track 3) and inhibition of de novo synthesis of PAL protein (Fig. 16, track 31) over controls (Fig. 16, track 3). It may be considered that the flux of L-phenylalanine into protein synthesis was theoretically greater than that which would allow L-phenylalanine to increase to Km levels of PAL. Hence it may be concluded that higher temperatures prevent both the synthesis of enzymes necessary for the production of phytoalexins and the biosynthesis (rate of turnover) of molecules involved in the interaction between the pathogen and host cell surface. This may explain the ability of the host plant to respond to the pathogen by expressing disease resistance at low temperatures and disease susceptibility at higher temperatures.

The following results were obtained from experiments designed to test the importance of the cell wall of the host during interaction with the pathogen:

2.3.3. Isolated leaf protoplast system - abiotic pathogen mycelial component :

The removal of the host cell wall by protoplast formation resulted in the inability of the host to respond to the pathogen cell wall asialoglycoprotein by de novo synthesis of PHA, PAT and PAL protein and activation of PAT and PAL protein compared to controls (Fig. 22).

It may be concluded that the host cell wall is important in the determination of the phytoalexin response in the Phaseolus vulgaris - Colletotrichum lindemuthianum host pathogen interaction.

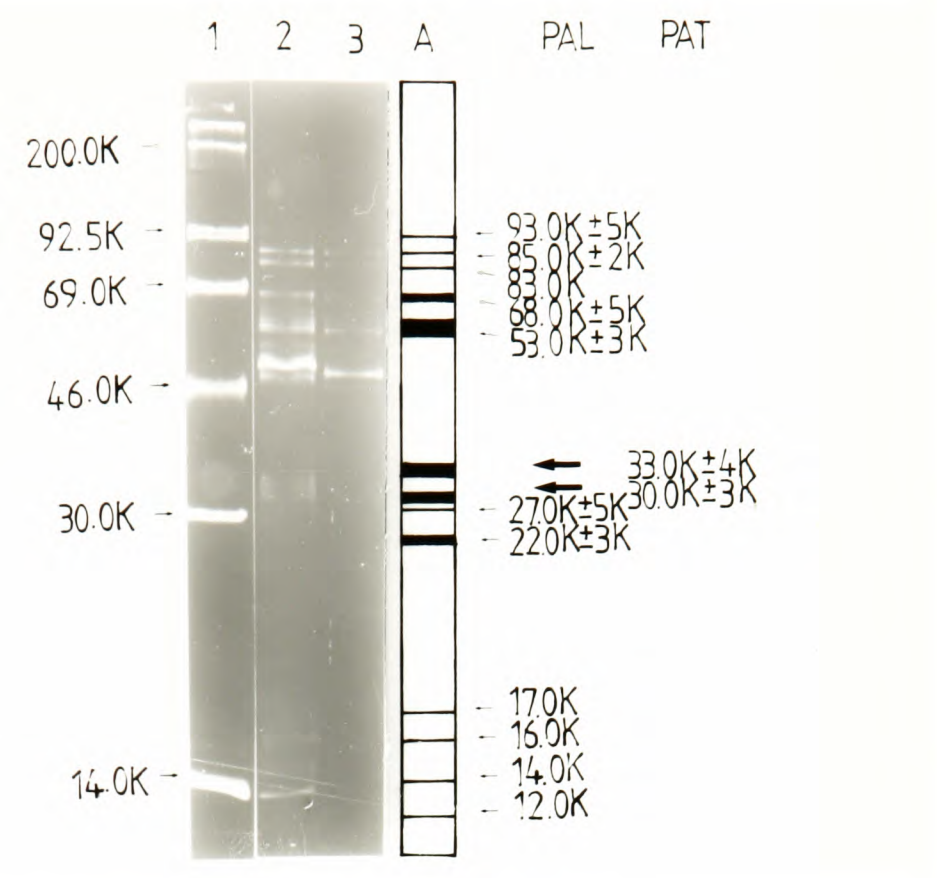
2.4. DISCUSSION:

The leaf tissue of Phaseolus vulgaris was found to exhibit dark green areas containing dead cells with pathogen mycelia (necrosis) 7 days after infiltration with live mycelia of the fungal pathogen Colletotrichum lindemuthianum. This observation is similar to that reported for the induction of the necrosis of the bean plant grown in the field and in the green house (Barrus, 1915; McKostie, 1919).

The appearance of necrosis, 7 days after pathogen infiltration, suggested necrosis to be a result of post-invasion by the fungal pathogen. This is understandable, as the pathogen Colletotrichum lindemuthianum is a hemibiotroph. Invasion of the host cell by a hemibiotrophic pathogen is critical to the establishment of a biotrophic relationship with the host.

Fig. 22 The response of host protoplasts (devoid of cell wall and middle lamella) to fungal pathogen cell wall asialoglycoprotein - the de novo synthesis of inactive PAL and active PAL and PAT protein and the de novo synthesis of PHA. Time of incubation with ^{35}S-methionine, 10 hours. Time of addition of dose 2 (see Fig. 2) asialoglycoprotein, 0 hours. For facsimile of active PAL and active PAT protein, see Fig. 14. B. Tracks: A. facsimile of inactive PAL and active PAT protein. 1. MWM; 2. soluble protein (control); 3. soluble protein (treated); 4. MWM 5. membrane protein (control); 6. membrane protein (treated); 7. MWM 8. extracellular protein (control); 9. extracellular protein (treated).

Cells were treated with ^{35}S-methionine in presence of different concentrations of pathogen cell wall asialoglycoprotein shown in Fig. 3. Protein present in the membrane, soluble and extracellular fractions were subjected to SDS-PAGE. Peptides corresponding to those obtained for purified preparations of PAT and PAL protein were quantified. For further details see 'methods'. Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run.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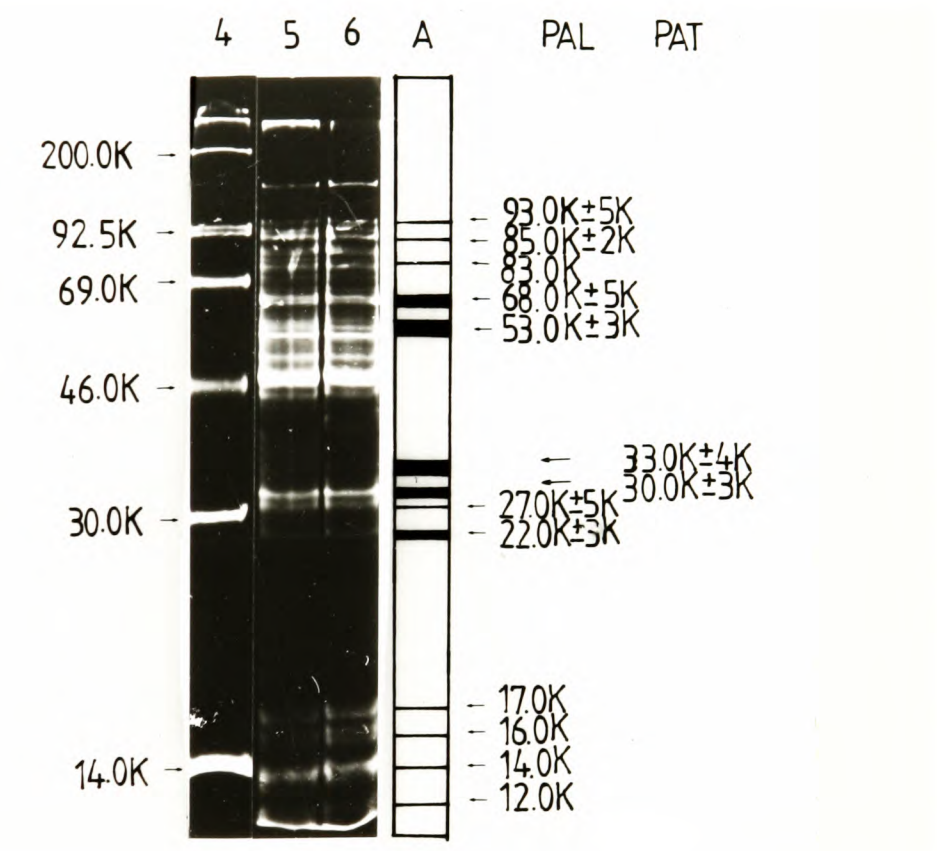
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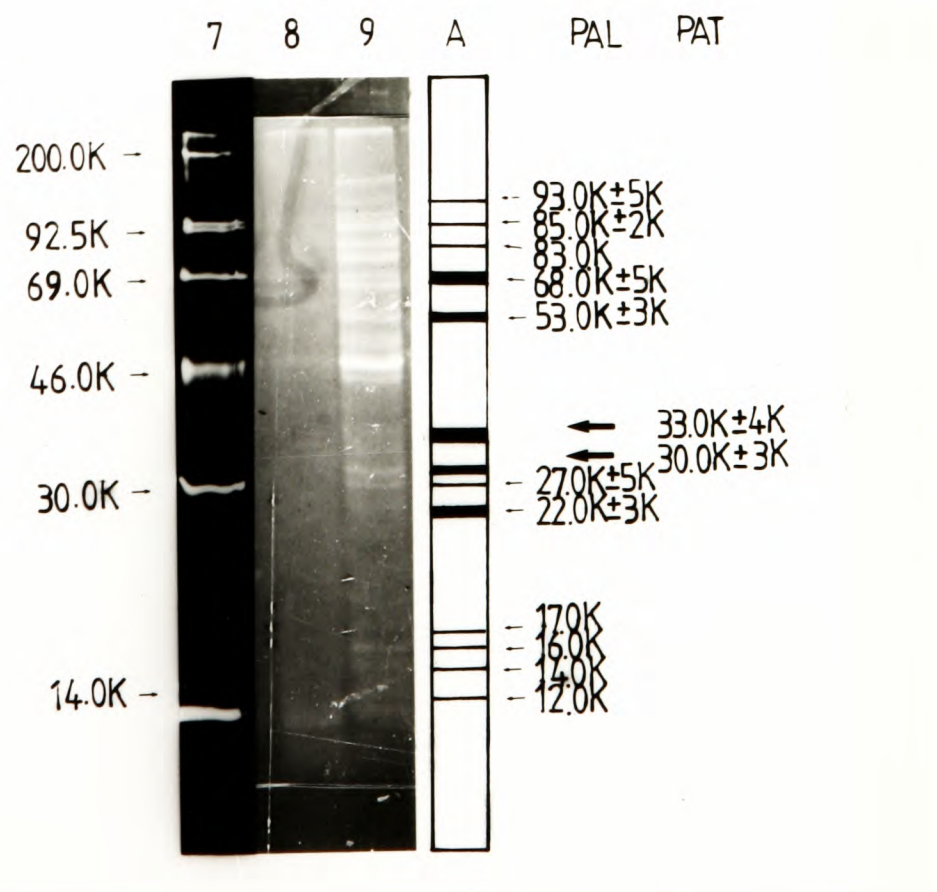
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The absence of pathogen mycelium, hypersensitive necrosis and any other visible form of reaction to the pathogen in older host plants (as opposed to younger host plants) suggested a failure of pathogen invasion in older host plants. Failure of pathogen invasion in older host plants could be explained by physical resistance. For example, older bean plant cell walls have been found to contain calcium pectate (Bateman & Lumsden, 1965) a compound which cannot be degraded by enzymes such as polygalacturonases, secreted by live pathogen mycelium (including those of Colletotrichum lindemuthianum) (Bateman & Lumsden, 1965).

On the other hand younger bean plant cell walls are characterised by the absence of calcium pectate but the presence of cellulose and pectin. Both cellulose and pectin may be degraded by enzymes secreted during growth by live pathogen mycelium (including those of Colletotrichum lindemuthianum), such as pectinase and cellulase. Younger bean plant cells exhibited the presence of pathogen mycelium restricted to hypersensitive necrotic areas of the leaf. This suggested hypersensitive necrosis to be an expression of resistance to further pathogen invasion. This event may be depicted as reaction A in Fig. 23.

During the growth of Colletotrichum lindemuthianum on isolated cell walls of Phaseolus vulgaris, the pathogen first secretes pectinase and α-arabinosidase, followed by β-xylosidase and cellulase, then β-glucosidase and finally α-galactosidase (English et al., 1971).

Further examination, showed younger (as opposed to older) bean plant cells to exhibit an increased synthesis of phaseollin, 7 to 10 days after pathogen invasion. This confirms the results obtained for this and several other

[e.g. in the Pisum sativum - Penicillium expansum interaction, pisatin production is inversely related to age of the host plant (Bailey, 1969), and in the Glycine max - Phytophthora megasperma var. sojae interaction, glyceollin production is inversely related to the age of the host plant (Lazarovits et al., 1981; Ward et al., 1981)].

systems (Bailey , 1982). This is an expression of functional resistance. The correlative increase in phaseollin concentration with (a) the number of necrotic lesions (b) the size of the necrotic lesion together with the absence of phaseollin in control plants and its accumulation to concentrations high enough to be toxic both to the host cell and pathogen suggested this functional resistance to be due the synthesis of antimicrobial compounds after pathogen invasion which restricted further pathogen growth. This response may be termed reaction B in Fig. 23.

The following factors suggest that older plants substitute physical resistance for functional resistance found in younger host plants: (a) absence of phaseollin production in older host plants, (b) the requirement for live pathogen mycelia for phytoalexin formation and hypersensitive necrosis in younger host plants, and (c) the requirement for removal of cellulase and pectin from the host cell wall of young host cells for induction of some enzyme activities needed for the synthesis of isoflavonoid phytoalexins and the production of hypersensitive necrosis (see below) by non-living mycelia and mycelial constituents, and (d) the requirement for wounding of the host cell wall system for induction of phenylpropanoid phytoalexin formation, as has been found for innumerable number of host-pathogen systems (e.g. shown by Bailey (1981) for the French bean (Phaseolus vulgaris) and by Sakai et al., (1979) for the potato (Solanum tuberosum).

The correlative increase in the first enzyme of the phenylpropanoid pathway (PAL) together with induced synthesis of phaseollin (a

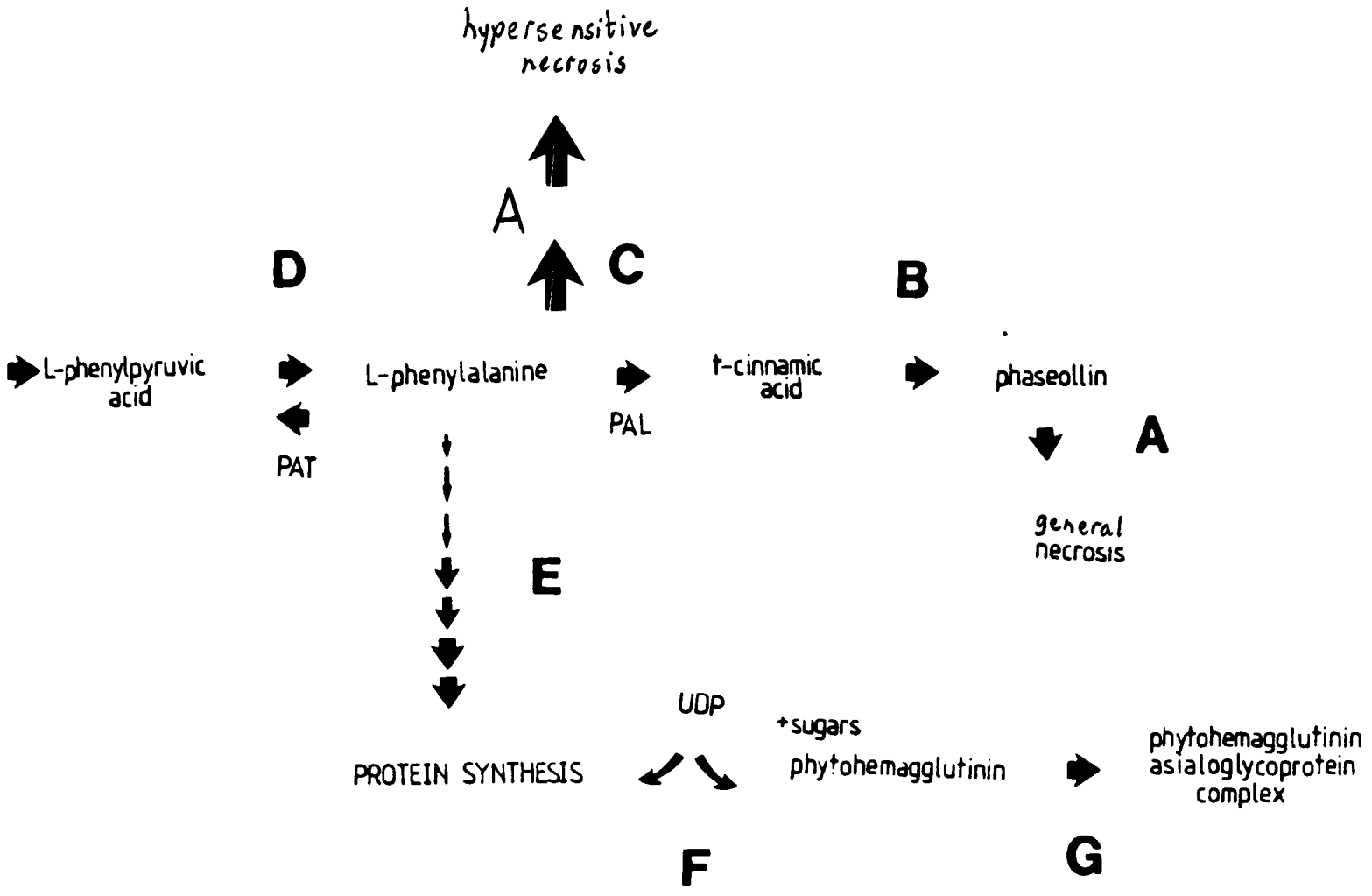


Fig. 23 Induction of general and hypersensitive necrosis and phytoalexin synthesis in the Phaseolus vulgaris L. - Colletotrichum lindemuthianum L. interaction: initial events.

A = induction of general and hypersensitive necrosis

B = induction of phaseollin (phytoalexin) synthesis leads to the induction of general necrosis

C = induction of PAL activity leads to the production of t-cinnamic acid required for phaseollin formation

D = induction of PAT activity leads to the supply of substrate, L-phenyalanine required for PAL activity

E = inhibition of protein synthesis leads to the availability of substrate L-phenylalanine required for PAL activity and possibly hypersensitive necrosis

F = induction of phytohemagglutinin synthesis uses metabolites required for protein synthesis (e.g. UDP)

G = induced synthesis of the phytohemagglutinin-asialoglycoprotein complex.

phenylpropanoid-derived isoflavonoid phytoalexin) suggests that the induction of enzyme activities in the metabolic pathway leading to the production of phytoalexins is a primary response in the host's response to the pathogen. This is further supported by the increased association of increased PAL activity with ability of the host to express both phytoalexin synthesis and necrosis in younger host plants, thus showing functional resistance to further pathogen invasion. PAL has been found to increase concomitantly with synthesis of several other phenylpropanoid-derived phytoalexins and necrosis in the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction by Rathmell (1973) and in the Glycine max - Phytophthora megasperma var sojae interaction by Ebel et al.(1976) and Dixon & Bendall (1978b). This initial event may be termed reaction C in Fig. 23.

The increase in PAL activity was found to be accompanied by an increase in de novo synthesised PAL protein as previously reported by Lamb & Dixon (1978), Dixon & Lamb (1979) and Lawton et al. (1983a).

Increased de novo synthesis of PAL protein has been reported in a number of host-parastite systems*. This de novo synthesised PAL protein was catalytically inactive but subsequently activated.

2 peaks of activation of PAL protein have been observed in cultured cells of Phaseolus vulgaris, in response to the pathogen Colletotrichum lindemuthianum (Lawton et al., 1980).

The method by which this inactive protein was activated was further investigated in the response of Phaseolus vulgaris to Colletotrichum lindemuthianum.

*e.g. Petroselinium hortense - Phytophthora megasperma var. sojae interaction by Hahlbrock et al. (1981) and Ebel et al. (1984).

(i) increased de novo synthesis of PAT protein (active in the direction L-phenylalanine ➡ L-phenylpyruvic acid) together with increased de novo synthesis of inactive PAL protein with low amounts of pathogen and (ii) decreased de novo synthesis of PAT protein (active in the direction L-phenylalanine ➡ L-phenylpyruvic acid) together with increased de novo synthesis of active PAL protein (active in the direction L-phenylalanine ➡ t-cinnamic acid) with high amounts of pathogen. These findings suggest that the appearance of PAL activity was dependent on supply of substrate by PAT activity and may be depicted as reaction D in Fig. 23. It is interesting that the initial phase of the host response appeared to be due to increased PAL and not PAT activity by Lawton et al., (1980). This can be accounted for by errors in the estimation of PAL and PAT activities discussed in Chapter 5. The dependence of PAL activity on 'source' metabolism (light and photosynthesis) provides indirect evidence in favor of substrate regulation of PAL. The absence of de novo synthesised PAL in protoplasts can be accounted for by mannitol which has been found to adversely affect photosynthesis and further supports the idea that active 'source' metabolism is required for phenylpropanoid synthesis.

The results described above also suggest a method of regulation of PAL activity, also suggested by other workers (see chapter 1) for PAL in higher plants. The possibility that PAL activity may be regulated by the de novo synthesis of a sulphydryl enzyme i.e. PAL specific sulphydryl enzyme is suggested by the finding that isolated inactive PAL protein contains a different polypeptide pattern to active PAL

protein, the former relating to the ligand-bound form and the latter to the ligand-free form in presence of SH reduction. These polypeptide patterns and activities have been found to be reversible in the absence of SH reduction in vitro for pure preparations of PAL protein. This regulatory mechanism has been previously suggested by Hanson & Havir (1981).

The induced PAL activity obtained in the Phaseolus vulgaris - Colletotrichum lindemuthianum is transient and depends on the dose of pathogen and the duration of incubation in presence of pathogen. The activity of the PAL induced lasts for 5 hours. This transient increase in activity has also been observed

. This transient nature of PAL activity was evidently due to subsequent inactivation of PAL protein. Two mechanisms for inactivation of PAL protein have been descibed in the literature (see chapter 1): (i) end-product inhibition (indirect evidence for substrate supply) and (ii) the synthesis of an inhibitor.

(i) Results obtained on end-product inhibition were negative. However, feedback inhibition by t-cinnamic acid for PAT activity and feed-forward inhibition by L-phenylpyruvic acid for PAL activity yielded very high Ki values, far higher than the concentrations of these compounds found in the cell (see Chapter 5).

However, the correlation between (a) decrease in de novo synthesis of active PAL protein and (b) absence of de novo synthesised PAT protein and (c) appearance of inactive PAL protein, suggests that inactivation was due to lack of substrate supply and availability. Further, inactive

* by Lawton et al. (1983a) for the Phaseolus vulgaris - Colletotrochum lindemuthianum interaction, by Loschke et al. (1981) for the Pisum sativum - Fusarium solani f.sp. pisi/ f.sp phaseoli interaction and by Hahlbrock et al. (1981) for the Petroselinium hortense - Phytophthora megasperma var. sojae interaction.

PAL protein was formed even under conditions which normally induced active PAL in the light. This has been found to be true for potato (Zucker, 1968) and radish (Faye, 1975).

(ii) Active PAL protein has a different polypeptide pattern from inactive PAL protein. Both the activities and patterns of polypeptide degradation are reversible in presence of SH reducing agents. The presence of an additional de novo synthesised sulphydryl reducing enzyme which causes the inactivation of PAL cannot therefore, be ruled out.

The mechanism of regulation of PAL described above by substrate supplied by the activity of PAT in the isolated cell system appeared to correlate with the ages at which the whole host plant showed both isoflavonoid phytoalexin formation and hypersensitive necrosis.

The addition of exogenous L-phenylalanine did not lead to an increase in active PAL protein. Assuming L-phenylalanine supplied entered the cell, it could only be inferred that it was unavailable for activation of PAL protein. This result was also obtained for several other systems (see Margna, 1977). Other unknown in vivo factors might have prevented the activation of PAL.

As found for other host-pathogen interactions and other systems showing an induction of PAL activity, an inverse correlation was obtained between active PAL protein and protein synthesis in the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction.

Protein synthesis may be envisaged to compete with PAL for L-phenylalanine, depicted as reaction E of Fig. 23. These findings suggest that the mechanism of regulation of PAL activity was due to a combination of both substrate supply and substrate availability.

As found for other host-pathogen interactions, the decrease in protein synthesis was found to be due to a depletion of an important metabolite in protein synthesis, UDP. Further, the addition of UDP to the Phaseolus vulgaris - Colletotrichum lindemuthianum host-pathogen interaction was found to block the induction of PAL activity.

The assumed depletion of UDP during the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction was due to increased synthesis of the glycoprotein: phytohemagglutinin. This initial event may be depicted as reaction F in Fig. 23. Glycoproteins (including PHA) have been described as recognition determinants in several host-pathogen interactions. The correlative increase in de novo synthesis of PHA together with several other proteins such as PAT protein and inactive PAL protein, suggested an interaction with determinants on the pathogen cell wall asialoglycoprotein. The affinity of PHA for N-acetyl-galactosamine and galactose, found by several workers, (see chapter 1) and the presence of N-acetylgalactosamine and galactose in the cell wall asialoglycoprotein of Colletotrichum lindemuthianum and further the binding between the de novo synthesised PHA and asialoglycoprotein lent further support to this finding. This recognition event may be depicted as reaction G in Fig. 23. The endogenous nature of PHA and asialoglycoprotein to the host and

pathogen cell walls respectively, and the absence of de novo synthesis of PHA and subsequent de novo synthesis of active PAT and PAL protein after removal of cell wall further substantiate the above finding.

De novo protein synthesis occurs only at low temperatures. Higher temperatures result in the lack of induction of de novo synthesis of PAT, PAL and PHA protein and other enzymes involved in turnover of the PHA-asialoglycoprotein during the interaction between Phaseolus vulgaris and Colletotrichum lindemuthianum. These inhibitory responses are associated with increased susceptibility of the whole (intact) host to the pathogen and increased growth of Colletotrichum lindemuthianum. The same is true

for the Phaseolus vulgaris - Sclerotinia fructicola interaction (Jerome & Müller, 1958) and the Pisum sativum - Fusarium solani interaction (Hadwiger & Wagoner, 1983).

CHAPTER 3

INDUCTION OF HYPERSENSITIVE NECROSIS AND PHYTOALEXIN SYNTHESIS IN THE PHASEOLUS VULGARIS L. - COLLETOTRICHUM LINDEMUTHIANUM L. INTERACTION : VIABILITY AND INTACTNESS OF THE ISOLATED LEAF CELL AND PROTOPLAST HOST SYSTEM

3.1. INTRODUCTION:

In this chapter are described the methods used to optimise viability and intactness of both the cell and protoplast system of the host plant Phaseolus vulgaris.

Viability of cell and protoplast systems are usually assessed by comparing physiological functions with whole (intact) plant systems. Examples of physiological functions used to assess viability and intactness in cell and protoplast systems include cell division (Pelcher et al., 1974), photosynthesis (Kanai & Edwards, 1973; Morris & Thain, 1980a; Lin 1983) and respiration (Morris & Thain, 1980a).

These methods of assessing viability have yielded results which show discrepancies between the whole (intact or excised) and cultured plant system. These discrepancies arise because of 2 major reasons:

(i) the physiological function of the cell and protoplast system is measured in a metabolically deficient and altered in vitro environment, as compared to that of the whole (intact or excised) host

cell *in situ*. For example: (a) respiration in cell and protoplast systems may require salt and hydrogen ion concentrations markedly different from that of the cell *in situ* (Day *et al.*, 1981; Galun, 1981), (b) cell division may be used to assess viability and intactness of mesophyll cells *in vitro*, at an age when cell division rarely occurs *in vivo*. A typical example is found in protoplasts of *Phaseolus vulgaris* (Pelcher *et al.*, 1974), (c) measurement of enzyme activities: this physiological function exhibits properties not found in the whole (intact) system. For example, either the substrate or activator may be depleted from the cytoplasm during isolation of cell or protoplast and remain inaccessible to the enzyme (Benz *et al.*, 1981; Iversen *et al.*, 1983) or cells and protoplasts may be selective in uptake of substrates and cofactors for unknown reasons (Day *et al.*, 1981), (d) membrane-associated phenomena such as ion-uptake where a leakage of enzymes involved in the membrane-associated phenomena may occur during isolation of cells and protoplasts (Leurs *et al.*, 1982; Lin, 1982 a, b),

and (ii) the physiological function of the whole (intact or excised) and cultured system is measured by non-uniform exposure to conditions required for optimal physiological function, as compared to that of the cell and protoplast host cell *in vitro*. For example, non-uniform exposures of whole (intact or excised) and cultured system, to labelled amino acid are used to compare viability and intactness of isolated protoplasts, resulting in considerable variation in rates of uptake and subsequent protein synthesis (Paszkowski *et al.*, 1980; Rubinstein & Tattar, 1980; Zuily-Fodil & Esnault, 1980).

The aim of this chapter therefore was to reliably estimate viability of a protoplast system by using a physiological function measurable in an altered *in vitro* environment and to compare these values, to cells subjected to uniform exposure of conditions required for optimal physiological function.

The physiological function that was chosen for estimating viability was protein synthesis. Protein synthesis is a major physiological process that does not require much alteration in the environment because a) it is independent of internal compartmentation of amino acids, enzymes (Dureja *et al.*, 1984), hormones (Cooke & Meyer, 1981), intercellular function, morphological cell type or organelle. Further, protein synthesis is a prerequisite for other physiological processes that have been used as assays for physiological function (eg. cell division, Sakai & Takebe, 1970; Wasilewska & Kleczkowski, 1974; Fuchs & Galston, 1976; Premecz *et al.*, 1978; Zelcer & Galun, 1980; Galbraith & Shields, 1982) and membrane-associated phenomena (Chastain *et al.*, 1981).

Intactness of cells and protoplasts may be determined by recently developed techniques such as cell electrophoresis (Onyia *et al.*, 1984) and chlorophyll autofluorescence (Harkins & Galbraith, 1984). These methods do not allow an estimation of possible damage to physiological function.It was thought that if structural damage is directly related to low rates of protein synthesis (a vitally important physiological function) and leakage of proteins so synthesised (Fuchs & Galston, 1976; Ruesink, 1978), then leakage of *de*

novo synthesised proteins should indicate structural damage in relation to impaired function.

In this study, methionine was assumed representative of all other amino acids. It was used because it cannot be transferred to other free amino acids which exist in large pools that fluctuate greatly in size during the synthesis of other amino acids.

3.2. MATERIALS AND METHODS:

3.2.1. METHODS:

Enzymes were purified by the method of Kao et al.(1971). Ficoll floatation methods were from Landgren (1978). Sucrose floatation methods were from Gregory & Cocking (1965). Discontinuous sucrose gradient centrifugation methods were from Lange & Karnosky (1981). Determination of K-stimulated ATPase activity was by the method of Hodges & Leonard (1974). Growth of Phaseolus vulgaris L. plant material was as described in Chapter 2, section 2.2.1.1. Rapid isolation of cells by removal of middle lamella from intact leaf tissue was as described in Chapter 2, section 2.2.1.2. Protoplasts were isolated from leaves of Phaseolus vulgaris by methods described in Chapter 2, section 2.2.1.3A. Purification of protoplasts was by methods described in Chapter 2, section 2.2.1.3B.

Abbreviations: CCD, counter-current distribution; DT_{40}, dextran T40; Evans blue, 6,6´-[(3,3´-dimethyl-4,4´-biphenylene) bis(azo)]-bis(4-amino-5-hydroxy-1,3-naphthalene disulfonic acid), tetrasodium salt; FDA, fluorescein diacetate; PEG, polyethylene glycol.

3.2.1.1. Growth of suspension cells of hypocotyl callus tissue of Phaseolus vulgaris L. was in the following medium (pH 5.7 to 5.9): KNO_3, 25mM; $NH_4H_2PO_4$, 2.5mM; $MgSO_4$, 1.5mM; $CaCl_2$, 1.5mM; $MnSO_4$, 0.07mM; H_3BO_3, 9.0µM; $ZnSO_4$, 0.5µM; KI, 0.5µM; $CuSO_4$, 0.13µM; $NaMoO_4$, 0.5µM; $FeSO_4$, 0.01mM; EDTA, 0.12mM; thiamine-HCl, 0.05mM; nicotinic acid, 0.12mM; pyridoxine-HCl, 7.0µM; 2,4, dichlorophenoxyacetic acid, 0.02mM; p-chlorophenoxyacetic acid, 0.1mM; kinetin, 0.05mM; sucrose, 0.1M; inositol, 5.5mM. Cultures were maintained at 25°C in an orbital incubator at 120 $rev.min^{-1}$ under a 16 hour daylength illumination of 30 $w.m^{-2}$ with warm white fluorescent tubes (F30 T12/WW/RS, Sylvania, W. Germany).

3.2.1.2. Determination of yield and viability of cells and protoplasts:

Cells / protoplasts were counted using a double chamber Fuchs-Rosenthal haemocytometer. Isolation efficiency was determined as the number of cells / protoplasts isolated. gFW^{-1} leaf tissue. Viability of cells / protoplasts was determined by making a cell / protoplast suspension to 0.1% (w/v) with fluorescein diacetate (FDA) (in acetone) (Widholm, 1972) and fluorescence visualised through a fluorescence microscope fitted with an HBO 200 W/4 super pressure mercury vapor lamp shining through a BG 12 (blue/violet) exciter filter. Cells / protoplasts were observed through a model 53 barrier filter.

3.2.1.3. Determination of intactness of cells / protoplasts:

Intactness of cells / protoplasts was determined by comparing incorporation of labelled amino acid in to de novo synthesised protein in extracellular / extraprotoplast fractions with cellular / protoplast fractions as described in sections 2.2.1.10.1. and 2.2.1.10.2.

3.2.2.MATERIALS:

Fluorescence microscope from Carl Zeiss, Oberkochen/Wuertt. W.Germany; haemocytometer from Hawksley Ltd., Lancing, Sussex and orbital incubator from A. Gallenkamp & Co., London.

All glassware was washed in sterile 1M NaOH, sterile 2% Decon-90, and sterile distilled water, siliconised with `Repelcote´ and autoclaved at 121°C for 20 minutes. This procedure was used to remove any ribonuclease present.

Chemicals used were of the analar grade.

`Repelcote´ was from Hopkins and Williams, Chadwell Heath, Essex; Cellulysin from Calbiochem-Behring Corp., C.P. Labs. Ltd., Hertfordshire; Cellulase R 10 and Macerase R 10, from Kinki Yakult. Manuf. Co.Ltd., Nishinomiya, Japan.

All solutions and equipment were autoclaved at 121°C for 20 minutes prior to use. When heat-sterilisation was not possible, solutions were filter-sterilised using an Acrodisc filter assembly (0.2µm) (Gelman

Sciences Ltd., Northampton).

3.3. RESULTS:

3.3.1. Isolation of cells from leaf tissue by enzymatic digestion of middle lamella:

The method of Servaites & Ogren (1977) produced yields of 10^5 cells.gFW^{-1} leaf tissue. Attempts were made to obtain yields of 10^7 cells.gFW^{-1} leaf tissue using conditions favorable to other cell and protoplast systems.

3.3.1.1. Growth conditions: Production of cells was enhanced (+ 20%) when seedlings were grown in soilless potting compost, compared to that containing soil (John Innes No. 1 potting compost).

3.3.1.2. Age of plant: Release of cells from leaves was not found to vary significantly with age of leaf tissue.

3.3.1.3. Osmotic requirements: The concentration of osmoticum was important. Slowly permeating sorbitol stabilised cells during isolation. A concentration of 0.3M sorbitol proved optimal for bean leaves.

3.3.1.4. Amount of leaf tissue exposed to enzyme: The greater the quantity of leaf tissue incubated in presence of enzyme, the greater was the yield of cells. However, FDA-viability decreased if higher

concentrations of leaf tissue were exposed to enzyme (Fig. 25).

3.3.2. Purification of cells:

The avoidance of air bubble formation (maintenance of continuous flow of solution) in the apparatus used during isolation (Fig. 24) and complete removal of enzyme by centrifugation, resulted in FDA-viabilities of 89 to 90%, indicating structural damage to some cells. To further increase the FDA-viability, osmotic requirements were optimised. Suspending bean cells in 0.2M sorbitol proved optimal for 95 to 96% viability (Fig. 26).

3.3.3. Isolation of protoplasts:

The method of Pelcher et al. (1974) produced yields of 10^3 protoplasts.gFW^{-1} leaf tissue. The following factors were assessed for significance of conditions described for various legumes and few other systems towards obtaining yields of 10^5 to 10^6 protoplasts.gFW^{-1}.

3.3.3.1. Growth conditions: Production of protoplasts was greatly enhanced (+75%) when seedlings were grown in soilless potting compost compared to that containing soil (John Innes No. 1 potting compost). 90% relative humidity, 6 hours prior to isolation was important for prevention of lysis on protoplast release.

3.3.3.2. Age of plant: Maximal release of protoplasts occurred in 2 to 4 week old leaves (Fig. 27).

Fig 24 Contd.

(after Servaites & Ogren, 1977).

Cells released by enzymatic digestion were collected on a millipore filter.

Fig 25 & Fig. 26 contd.

Viability and yield were determined by procedures described in 'methods'. Viability was expressed as that fraction of the total number of cells (percent) which stained with FDA. Each spot represents the mean of results obtained for 3 experiments, 36 determinations obtained for each experiment. The bars indicate the maximum and minimum values in an experiment. The absence of a bar indicates that the variation is contained within each spot.

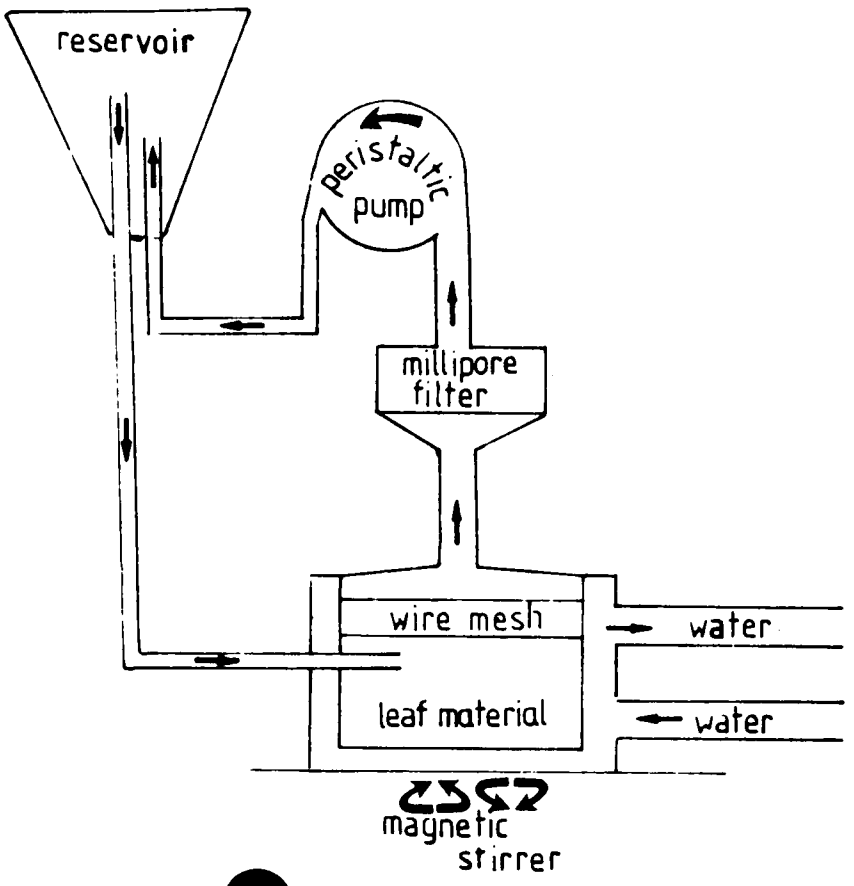


Fig. 24 Apparatus used for rapid isolation of cells by enzymatic digestion of middle lamellae of leaf segments of Phaseolus vulgaris L. (continued on facing page)

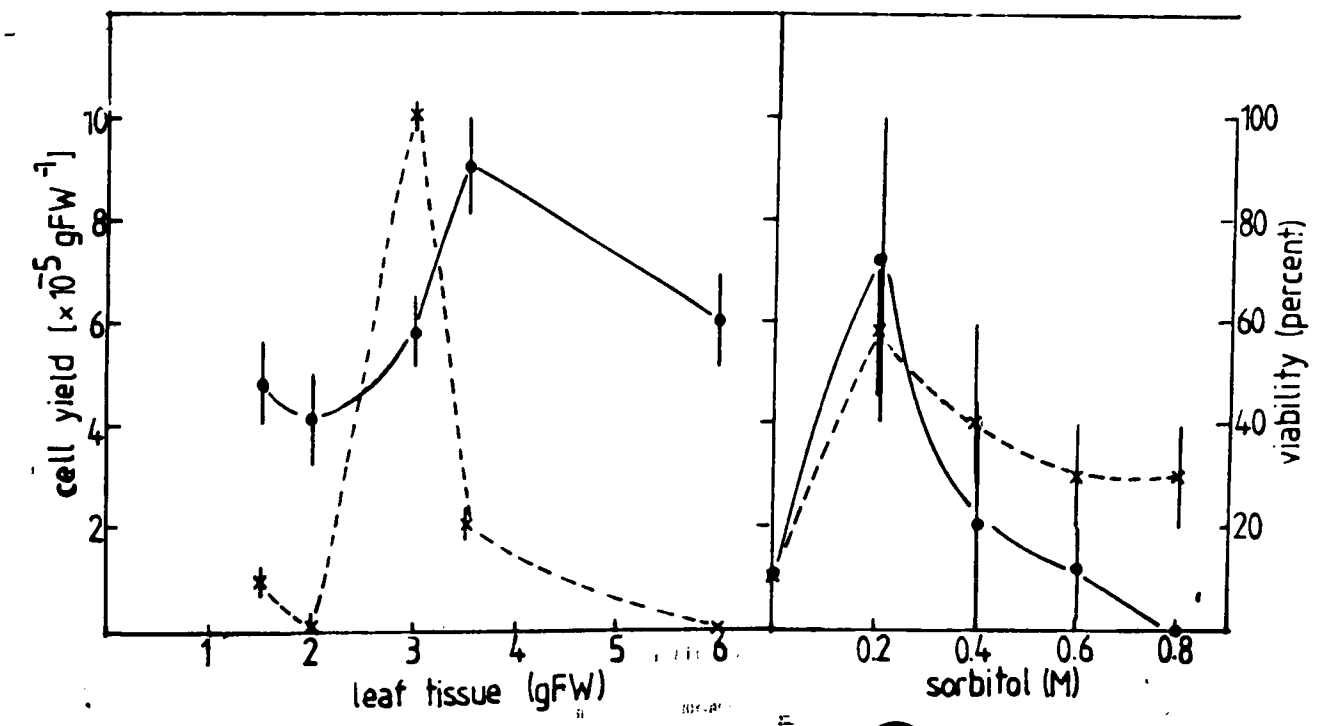


Fig. 25 Cells released by enzymatic digestion of middle lamella : effect of concentration of leaf tissue on yield (●—●—●) and FDA-viability (X---X---X).
(continued on facing page)

Fig. 24 Cells released by enzymatic digestion of middle lamella : effect of osmotic concentrations of sorbitol on yield (●——●——●) and FDA-viability (X---X---X).
(continued on facing page)

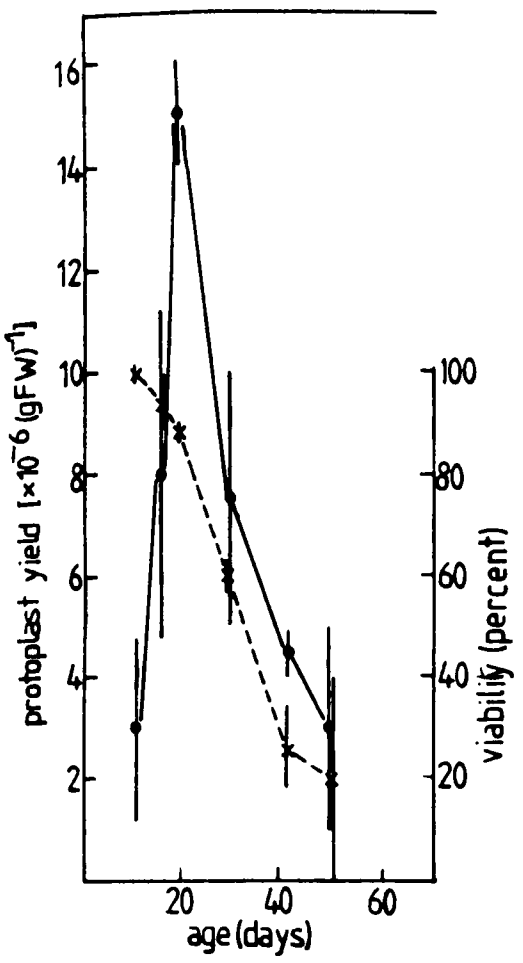
3.3.3.3. Enzyme solution: Low ratios of 1.0 to 2.5 Cellulase : Pectinase proved adequate for protoplast release (Fig. 28). Viability (not yield) decreased with increase in Pectinase, while, yield (not viability) decreased with increase in Cellulase. The substitution of Driselase for Cellulase R 10, or Cellulysin, or substitution of Pectinase for Macerase R 10 did not affect yields obtained. Purification of these enzymes did result in an increase in protoplast yield.

3.3.3.4. Osmotic and other requirements: Osmotically permeable compounds (mannitol and sucrose) failed to enhance protoplast yields (Fig. 29). The addition of 25% (w/v) sucrose, resulted in no protoplast release. Mineral nutrients and other nutrients produced much lysis, and low protoplast yields (Fig. 29). PVP, DTT and $CaCl_2$ greatly facilitated high yields of protoplasts, while BSA failed to increase protoplast yields (Fig. 29).

3.3.3.5. Conditions that shorten the length of the incubation period: The size of the leaf tissue exposed to enzyme digestion was important for yield of 100% FDA-viable protoplasts, lysis being induced if the size of leaf was too small(Fig. 30). Yields were reduced by as much as 97 to 99% by abrading the lower epidermis of the leaf with carborundum powder. Preplasmolysis for a period of 30 to 60 minutes before enzyme incubation did not result in yields greater than 0.14 to 0.2 x 10^4 protoplasts.gFW^{-1} leaf tissue. A faster method of digestion (the stopped digestion method i.e. digestion with repeated changes of enzyme solution, Cellulase, followed by Macerase/Pectinase), failed to

Fig. 29 CONTD

Viability and yield were determined by procedures described in 'methods'. Viability was expressed as the number of protoplasts staining with FDA only. Each spot represents the mean of results obtained for 3 experiments, 36 determinations obtained for each experiment. The bars indicate the maximum and minimum values obtained in an experiment. The absence of a bar indicates that the variation was contained within each spot.



27 Protoplasts released by enzymatic digestion of dle lamella and cell wall : effect of leaf age on yield (—●—●—) and FDA-viability (×---×---×).
(for further details see Fig 26)

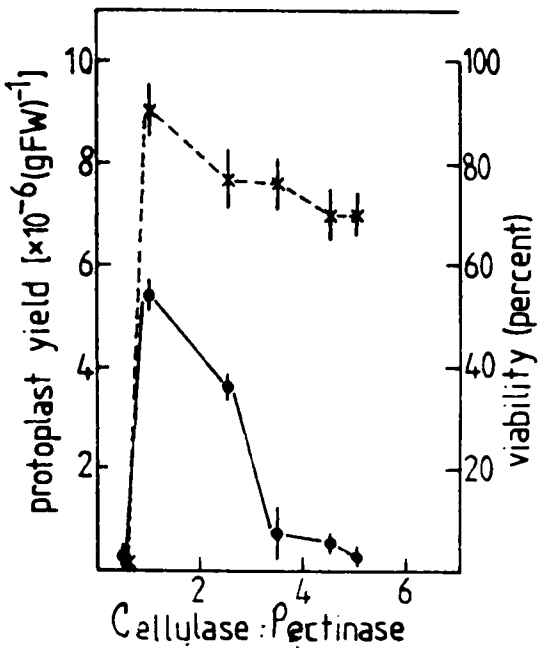
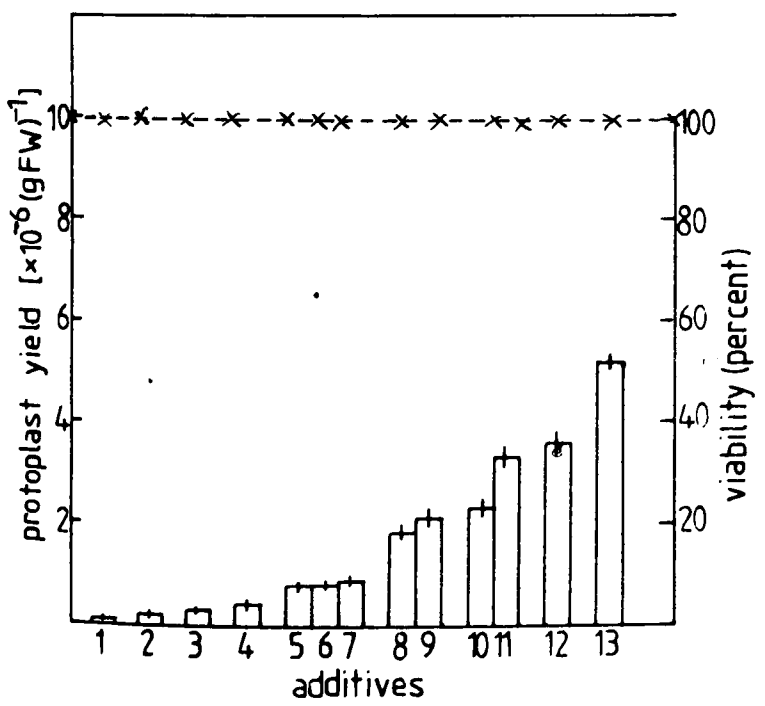


Fig. 28 Protoplasts released by enzymatic digestion of middle lamella and cell wall : effect of enzyme composition on yield (●——●——●) and FDA-viability (X---X---X).
(for further details see Fig. 26)



Yield of viable

24 protoplasts released by enzymatic digestion of middle lamella and cell wall : effect of various osmotic and non-osmotic additives on yield (●——●——●) and FDA-viability (×---×---×). 1. + $CaCl_2$ (1.5mM) + KNO3 (0.25mM) + $NH_4H_2PO_4$ (2.5mM) + $MgSO_4 7H_2O$ (1.5mM) + KI (6.02μM) + $CuSO_4 5H_2O$ (0.8μM) + $MnSO_4$ (0.6μM) + $CoCl_2 6H_2O$ (0.42μM) + $FeSO_4 7H_2O$ (0.54μM) + EDTA (Na_2) (0.54μM) + sucrose (0.88mM + pyridoxine-HCl (2.43μM + nicotinic acid (0.41 μM) + thiamine-HCl (0.15 μM) + m-inositol (5.56mM + 2,4-dichlorophenoxyacetic acid (2μM) + p-chlorophenoxyacetic acid (0.1 μM) + kinetin (0.5μM) (modified from Schenk and Hildebrandt, 1969); 2. + $CaCl_2 2H_2O$ (10.07mM) + KNO_3 (1.0mM) + KH_2PO_4 (0.2mM) + $MgSO_4 7H_2O$ (1.0mM) + KI (1.0μM) + $CuSO_4 5H_2O$ (0.1μM) (Landgren, 1979); 3. + BSA (0.1% w/v) (Oldfield & Coutts, 1980); 4. + mannitol (0.3M) + $CaCl_2 2H_2O$ (2.04mM) + KNO_3 (2.5mM) + NaH_2PO_4 (0.7mM) + NH_4NO_3 (3.123mM) + sorbitol (0.3M) + naphthylacetic acid (5.37μM) + benzylamino purine (0.9μM) (Johston et al. 1981); 5. + mannitol (0.65M); 6. + mannitol (0.5M); 7. + mannitol (0.3M); 8. + PVP 10 (2.0mM); 9. + PVP 10 (0.3mM); 10. + PVP 40 (0.5mM); 11. + PVP 40 (0.125mM); 12. + $CaCl_2 2H_2O$ (6.8mM); 13. + DTT (0.5mM).

(continued on heavy page)

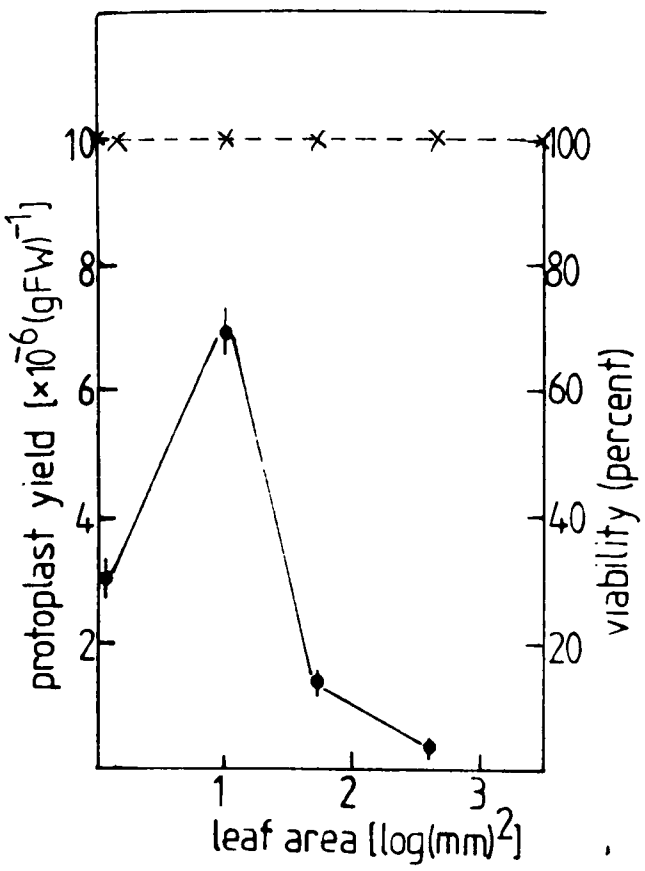


Fig. 30 Yield of viable protoplasts released by enzymatic digestion of middle lamella and cell wall : effect of concentration of leaf tissue on yield (●——●——●) and FDA-viability (x--x--x). (for further details see Fig. 29)

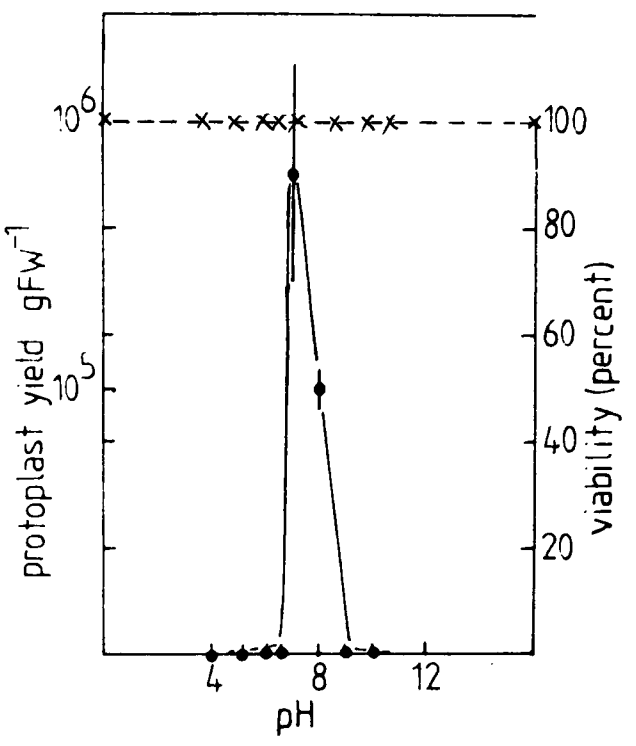


Fig. 31 Yield of viable protoplasts released by enzymatic digestion of middle lamella and cell wall : effect of hydrogen ion concentrations on yield (●——●——●) and FDA-viability (x---x---x). (for further details see Fig. 29)

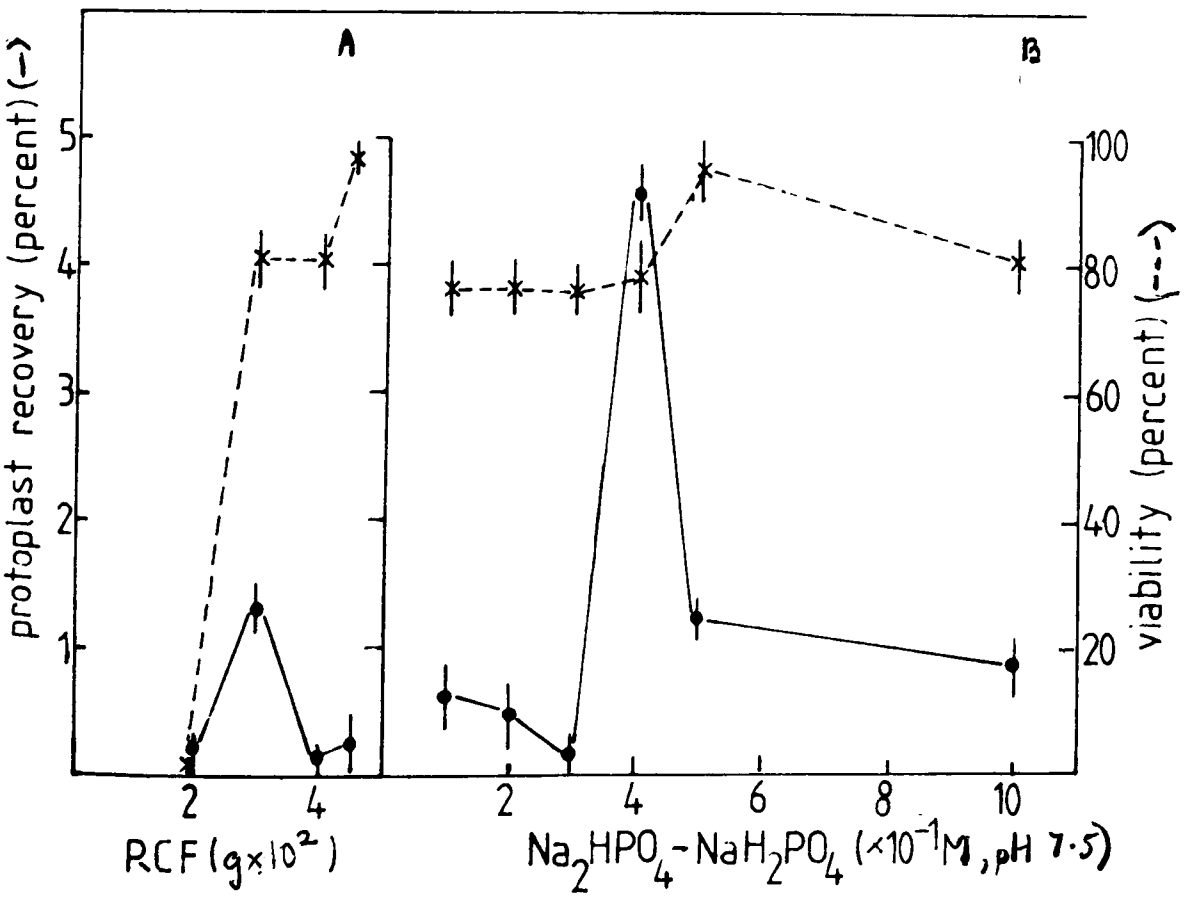


Fig. 32 Purification of protoplasts by CCD: A. effect of speed of centrifugation during CCD; B. effect of concentration of buffer during CCD.
(for further details see Fig. 26)

yield intact protoplasts and greatly promoted lysis. Increased temperatures of 30 to 37°C, vacuum infiltration of enzyme solution and agitation (10 to 50 rpm) during enzyme incubation failed to produce yields greater than 10^3 protoplasts.gFW^{-1} leaf tissue.

Results described so far, (including high humidities prior to isolation) suggest the importance of gradual plasmolysis to avoid the initial osmotic shock on digestion of the cell wall with avoidance of possible release of proteins by degradation or leakage.

3.3.3.6. pH: No protoplasts were produced at pH values of 5.0 to 6.6 even on prolonged incubation in presence of enzyme. Optimal yields of 4 to 11.3 x 10^6 protoplasts. gFW^{-1} were obtained between pH values of 6.8 and 7.5. Higher pHs resulted in a decrease in protoplast to below 10^5 protoplasts.gFW^{-1} leaf tissue (Fig. 31). The importance of pH of enzyme solution was evidenced by inhibition of protoplast release if the pH of the enzyme solution reached 5.6 to 6.5 during enzymatic release. Further, protoplast yields at these low pHs (5.6 to 6.5) could be made to occur by buffering the enzyme solution with 50mM MES (pKa 6.1) or sodium borate (pKa 9.23), though never in excess of 0.6 x 10^4 protoplasts.gFW^{-1}.

Minimal changes were incorporated in the protocol of Pelcher *et al.* (1974) to give yields of 10^5 to 10^6 protoplasts.gFW^1 leaf tissue, with FDA-viabilities of 75 to 87%. This compares closely with yields and FDA-viabiities of cells (4 x 10^6 cells. gFW^1, 89 to 90% of which were FDA-viable). The major limitations of the protocol were the age of

plant material and the necessity for extended plasmolysis during cell wall removal.

3.3.4. Purification of protoplasts:

Despite precautions taken during isolation, FDA-viabilities of 75 to 87% indicated the possibility of structural damage to some protoplasts. Static precipitation and centrifugation at speeds lower than 12.5 x g yielded 0.008 to 0.056% viable protoplasts. Speeds in excess of 12.5 x g (to 100 x g) failed to yield any viable protoplasts. Floatation on ficoll and sucrose in presence and absence of mineral salts, together with discontinuous sucrose gradient centrifugation at 12.5 x g failed to yield viable protoplasts. While osmotica which permeate the cell (e.g. mannitol) did not affect protoplast yield and viability during isolation, slowly permeating sorbitol stabilised protoplasts after release. 200mM sorbitol was optimal, while concentrations above 200mM to 500mM promoted much lysis.

The above results together with minimal amount of exposure to sucrose and sorbitol suggested rupture due to enhanced plasmolysis during enzyme digestion and subsequent purification with release of important components by leakage or degradation. To avoid these effects, temperatures of $4^{o}C$ (not $25^{o}C$) and removal of partially intact protoplasts from intact protoplasts by CCD were used. The CCD system of Kanai & Edwards, (1973) was optimised to avoid detrimental effects of PEG. For this purpose, protoplasts were collected at the interphase

There occurs a linear incorporation of label into protein during initial periods of incubation followed by a decline of incorporation of label during later periods of incubation. The decrease in incorporation of label into protein during later periods of incubation could be due to an absence of increased levels of endogenous methionine or leakage of cellular components containing labelled methionine.

of PEG and DT_{40}. To attain this, speeds of centrifugation and phosphate buffer concentrations were optimised to 300 x g and 0. 5M respectively (Fig. 32A and B). BSA was added to dislodge chloroplasts from the protoplast membrane surface. This method resulted in yields of 2×10^5 protoplasts.gFW^{-1}, with viabilities of 95 to 96%.

3.3.5. Viability of cells / protoplasts using a vital physiological function i.e. uptake and incorporation of labelled amino acid into cellular protein:

Fig. 33 (A to E) shows the incorporation of L-methionine $-^{35}$-S into protein in leaf cells, hypocotyl-derived suspension cells, non-purified protoplasts, and purified protoplasts in hypotonic and isotonic medium respectively.

Hypocotyl-derived cell suspensions exhibit considerable variation in uptake and incorporation of label into protein, as compared to protoplasts and leaf cells (Fig. 33, A to E).

* see facing page

The linear incorporation of label into protein (during the first 4 hours in non-purified protoplasts) and 10 hours (in hypocotyl-derived suspension cells, leaf cells and purified protoplasts) suggests an absence of increased levels of endogenous methionine and cellular components containing methionine as being responsible for the decrease in uptake during later periods of incubation. The appearance of 99% de novo synthesised proteins in the extraprotoplast medium of non purified protoplasts (Fig. 33E; Fig. 34) as compared to <10% in

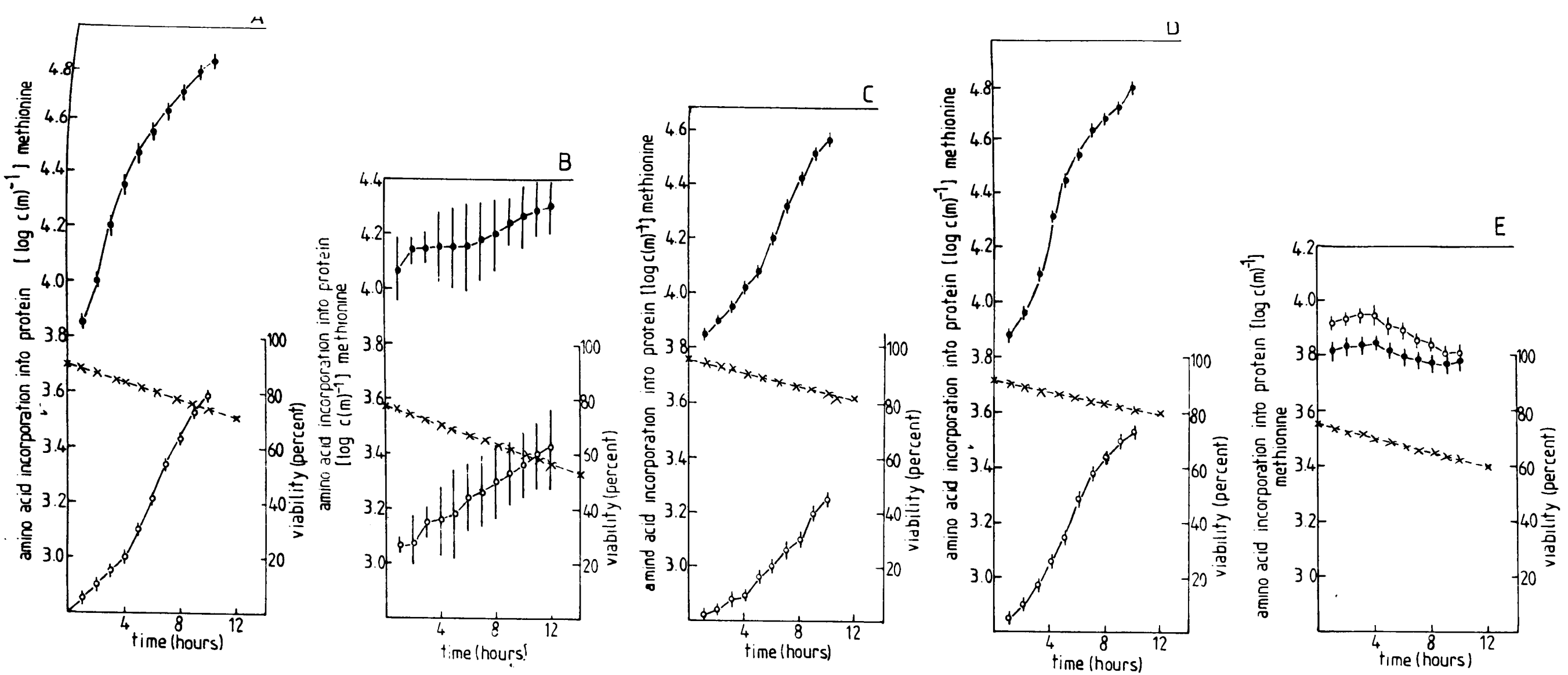


Fig. 33 Viability of cells / protoplasts released by enzymatic digestion of middle lamella : by incorporation of labelled amino acid in to cellular protein (●——●——●) and extracellular protein (o——o——o); and by inclusion of esterases using FDA (×- - -×- - -×). Time of addition of ^{35}S-L-methionine : 0 hours. A. leaf cells; B. hypocotyl-derived suspension cells; C. purified protoplasts in hypotonic medium; D. purified protoplasts in isotonic medium; E. non-purified protoplasts. Time of addition of ^{35}S-L-methionine = 0 hours.

(for further details, see fig. 26).

purified protoplasts, leaf cells and suspension cells, during the linear period of incorporation into protein suggests this decrease as being due to leakage of cellular components containing labelled amino acids of importance to protein synthesis and uptake, together with the possible release of factors (such as proteases) involved in protein degradation. This may explain the necessity described above, for plasmolysis prior to release of protoplasts from non-plasmolysed leaf tissue and removal of partially intact protoplasts during purification.

While protoplasts in isotonic media (as opposed to protoplasts in hypotonic media) showed similar rates of uptake and incorporation of labelled amino acid into cellular protein as leaf cells, FDA-viability did not distinguish between purified protoplasts in isotonic media, hypotonic media, and leaf cells (Fig. 33, A to E; Fig. 34).

While the addition of the metabolic inhibitor ribonuclease I (prior to the initial 3 hour linear period of uptake) to cells and protoplasts resulted in the decline in uptake and incorporation of labelled amino acid into cellular protein to <10% of controls (Fig. 35), FDA-viability did not distinguish between ribonuclease-treated cells, protoplasts and their controls (Fig. 34).

Further, the addition of trypsin to cells and protoplasts at concentrations that cause structural damage to the membrane (prior to the initial 3 hour linear period of uptake) resulted in the decline in uptake and incorporation of labelled amino acid into cellular

Fig. 34. CONTD.

For further details see 'methods'.

Fig. 36. CONTD.

Cells and protoplasts were treated with ^{35}S-methionine. The extraprotoplast proteins were separated from protoplast proteins. Proteins were subjected to SDS-PAGE and fluorography. Fluorographs were scanned to compare proteins present in both fractions for leakiness of de novo synthesised protein as a measure of intactness. For further details see 'methods'.

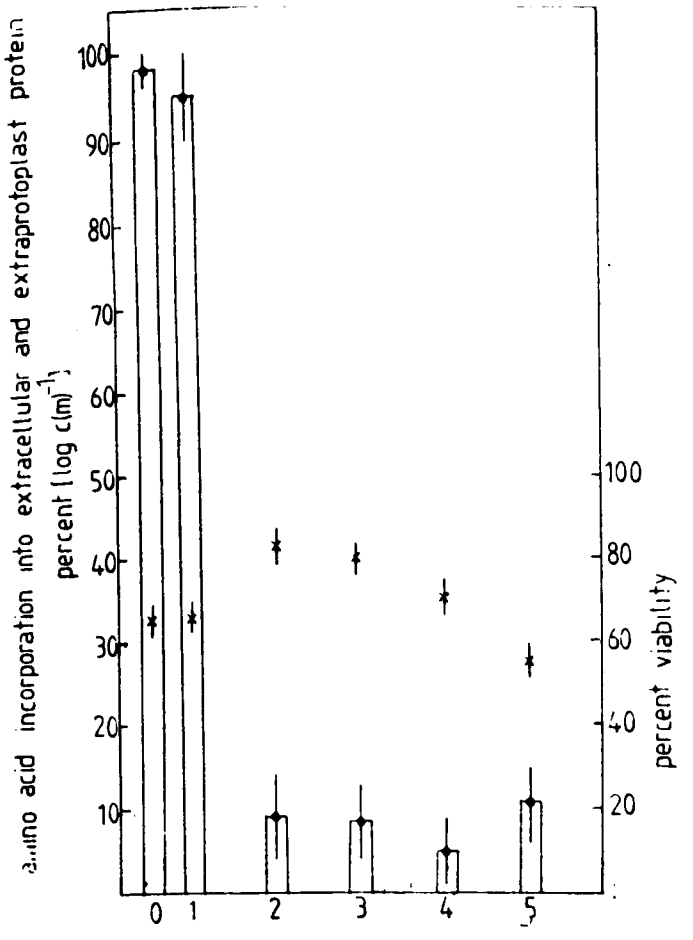


Fig. 34 Intactness of various cell and protoplast systems : by incorporation of labeled amino acid into extracellular and extraprotoplast protein (●); and by inclusion of esterases using FDA (×). Time of incubation with ^{35}S-L-methionine : 10 hours. Time of addition of trypsin and ribonuclease I : 0 hours. 1. trypsin and ribonuclease I treated cells and protoplasts; 2. non-purified protoplasts; 3. purified protoplasts in hypotonic medium; 4. purified protoplasts in isotonic medium; 5. rapidly isolated cells; 6. suspension cells. (continued on facing page ; for further details see Fig. 26)

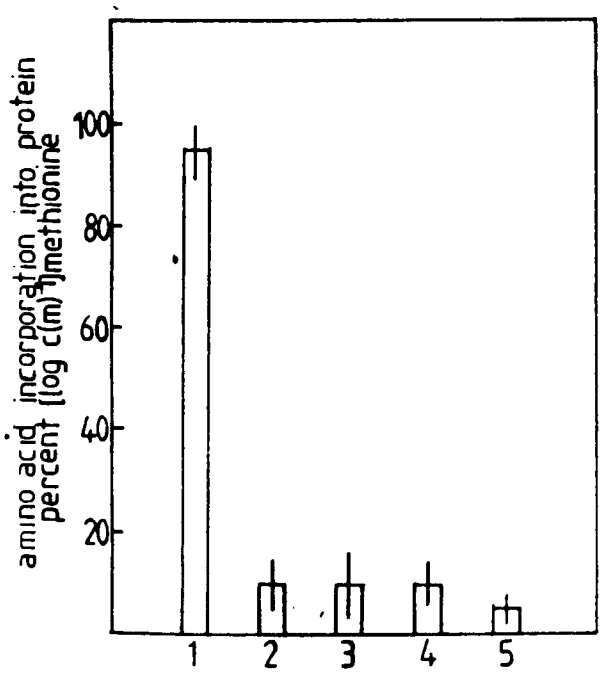


Fig. 35 Viability of various cell and protoplast systems : by incorporation of labelled amino acid in to leaf cells and purified protoplast protein. Time of incubation with ^{35}S-L-methionine : 10 hours. 1. protoplasts and cells; 2. cells + trypsin added at 0 hours; 3. cells + ribonuclease I added at 0 hours; 4. protoplasts + trypsin added at 0 hours; 5. protoplasts + ribonuclease I added at 0 hours.

(for further details see Fig. 26)

Fig. 24 Intactness of protoplasts: by densitometric scans of labelled amino acid in to cell and protoplast protein. Migration of protein : left to right. Time of incubation with ^{35}S-L-methionine : 10 hours. A. soluble protein of purified and non-purified protoplasts; B, extraprotoplast protein of non-purified protoplasts; C, extraprotoplast proteins of purified protoplasts; D, extraprotoplast protein of purified protoplasts treated with trypsin at 0 hours.

(continued on facing page).

protein to <10% of controls (Fig. 35). FDA-viability did not distinguish between trypsin-treated cells, protoplasts and their controls (Fig. 34).

3.3.6. Intactness of cells / protoplasts using a vital physiological function i.e. uptake and incorporation of labelled amino acid in to extracellular protein:

The reliability of various markers for intactness was investigated by comparing intactness of leaf cells and purified protoplasts with damaged cells, damaged purified protoplasts and non-purified protoplasts.

Dye exclusion could not differentiate between intactness of cells, purified protoplasts and non-purified protoplasts. Intactness determined by the exclusion of dyes, such as trypan blue and Evans blue yielded values of 99 to 99% intact ness for cells, purified protoplasts and non-purified protoplasts. The loss of dyes from non-purified protoplasts during washing procedures could not be ruled out.

Determination of esterase inclusion using the dye FDA, could not differentiate between intactness of cells, purified protoplasts and non-purified protoplasts, and protoplasts and cells damaged by the metabolic inhibitor ribonuclease I or protease, trypsin (Fig. 34).

De novo synthesised proteins secreted from the cells / protoplasts

resembled de novo synthesised proteins within the cells / protoplasts secreted in peptide molecular weight (Fig. 36). Among those proteins from the non-purified protoplast during the 10 hour incubation period, was 53% of the activity of K-stimulated ATPase. The estimation of leakage of these de novo synthesised proteins into the extracellular / extraprotoplast medium, served to differentiate intact cells and purified protoplasts from non-purified protoplasts and protoplasts and cells damaged by ribonuclease I and trypsin (Fig. 35). While inclusion of esterases by FDA indicated a decline of 25%, 30%, 10% and 15% intactness in leaf cells, hypocotyl-derived suspension cells, purified protoplasts and non-purified protoplasts respectively, there occurred a leakage of <10% de novo synthesised protein into the extracellular medium in leaf cells, hypocotyl-derived suspension cells and purified protoplasts as opposed to the leakage of >88% de novo synthesised protein in to the extracellular protein of non-purified protoplasts, and protoplasts and cells damaged by ribonuclease I and trypsin (Fig. 33, A to E; Fig. 34; Fig. 35).

Only substantial damage to protoplast structure by centrifugation at 13Kg or vacuum infiltration on negatively charged glass fibre discs indicated a decline in intactness of >70% by FDA, while there occurred a leakage of >99% de novo synthesised protein into the extraprotoplast medium.

Intactness by FDA measures the concentration of the non-permeant fluorescein. Intactness by incorporation of labelled amino acids measures the concentration of de novo synthesised extracellular

protein. Like fluorescein, de novo synthesised protein cannot move out of the membrane without structural damage to the cell / protoplast. The results described above suggest that significant damage amounting to `death´ is necessary for non-inclusion of fluorescein diacetate. On the other hand, slight damage to the membrane (as by trypsin) or to the metabolic activity of the cell (as by ribonuclease) is indicated more readily by exclusion of L-methionine -^{35}-S labelled protein when compared to FDA determinations. This may be because many important integral membrane proteins contain sulphur.

3.4. DISCUSSION:

3.4.1. Isolation of cells / protoplasts from leaf tissue by enzymatic digestion of middle lamella:

Conditions used to isolate cells and protoplasts from leaf tissue of several other systems were used successfully in the Phaseolus vulgaris system. These conditions were sucessful for several known reasons, as described below.

For example, the enhancement of protoplast and cell yields in soilless potting compost compared to that containing soil was probably because of more efficient enzymatic digestion of softer leaves (Cassels & Barlass, 1976). The increased humidity prior to protoplast isolation in Phaseolus vulgaris has been found to be important for prevention of protoplast lysis on release from several systems, but not (Phaseolus vulgaris var. Pinto) by Pelcher et al. (1974).

The decrease in protoplast yields with increased age is a common feature of several systems e.g. tobacco & soybean (Uchimaya & Murashige, 1974; Lin, 1983). Leaves of older plants yield tough and unstable protoplasts (Wood et al., 1980). The ratio of Pectinase to Cellulase has also been found to be an important determinant of higher yields of viable protoplasts. Low ratios of Pectinase to Cellulase found necessary for Phaseolus vulgaris have also been found necessary for several other legume systems (Oldfield & Coutts, 1980; Constabel et al., 1973; Gamborg et al., 1975) and is thought to be indicative of the large amounts of arabinose, galactose and xylose present in cell walls of mesophyll cells of legumes (Kelfer et al., 1970).

Among the additives used during isolation, PVP, DTT and $CaCl_2$ greatly enhanced protoplast yields. The promotive effect of PVP may be due to the adsorption of phenolics released from lysed protoplasts during release, preventing lysis of other intact protoplasts and subsequent brown discoloration. Browning of leaf tissue and lysis of protoplasts has been observed by Pelcher et al. (1974) in Phaseolus vulgaris L. This finding may explain the necessity for removal of lysed protoplasts by CCD for optimal physiological function. The promotive effects of DTT indicate the importance of maintaining sulphydryl groups on the surface of the plama-membrane during release (Stoessl, 1984), and hence the important relation between structure and function of proteins on the membrane surface for maintenance of protoplasts in the intact state. Further DTT does not trigger phytoalexin production (Gustine, 1981; Stoessl, 1984). The promotive effects of $CaCl_2$ may be due to increased membrane stability by decreasing membrane fluidity

(Ruesink, 1978, Boss & Mott, 1980). That instability of some proteins is not the cause of decreased yields is evidenced by the negligible effects of BSA on protoplast yield.

However, some conditions beneficial to other systems (including legume systems), failed to enhance cells and protoplasts from the bean system. Examples include desalted enzymes (Gosch et al., 1975), osmotically permeable compounds eg.mannitol, (Davey, 1974) and conditions that shorten the length of the incubation period such as carborundum powder (Franchesh et al., 1984), preplasmolysis prior to enzyme incubation (Constabel et al., 1973; Oldfield & Coutts, 1980), faster methods of digestion of leaf tissue, for example the stopped digestion method (Otsuki & Takebe, 1969; Wakasa, 1973), increased temperatures of 30 to 37°C, vacuum infiltration of enzyme solution and agitation (10 to 50rpm) (Davey et al., 1973; Gamborg et al., 1975; Gosch et al., 1975; Johnston et al., 1981). The reasons for failure of the above conditions in enhancement of protoplast yields and success of those mentioned above, (including high humidities prior to isolation) suggest the importance of gradual plasmolysis to avoid the initial osmotic shock on digestion of the cell wall (Cassells & Barlass, 1976; Zuily-Fodil & Esnault, 1980) with avoidance of possible release of protein degradation or leakage.

The decline in pH during protoplast release has been reported by other workers for other systems (Pelcher et al., 1974; Gamborg et al., 1975; Roscoe & Bell, 1981), and may be due to rapid extrusion of protons from leaky protoplasts released during

enzyme incubation (Kelly, 1983). The low yields of bean protoplasts at low pHs could have been due to either a decrease in protoplast stability or low pH not being optimal for cellulase or pectinase activity. The absence of lysis in protoplasts released at low pH ruled out the first possibility and is true for other systems (Schenk & Hildebrandt, 1969). The pH optimum for protoplast does not coincide with the pH optimum for cellulase (i.e. 5.5 to 6.0). It is possible that the low yield of protoplasts at low pH (5.5 to 6.0) could have been due to pectinase activity (. Uchimaya & Murashige, 1974).

3.4.2. Purification of cells and protoplasts:

Cells and protoplasts immediate on isolation were in the plasmolysed state. The plasmolysed state of the cell and protoplast was removed during subsequent purification, by use of osmotica.

Purification procedures useful to other systems (including those of legumes) proved unsuccessful for the bean system. Examples used included methods using simple centrifugation to floatation on ficoll and sucrose (. Gosch et al., 1975). The reasons for failure of these methods may have been due to the absence of large vacuoles and presence of a high concentration of cell organelles in protoplasts of the bean leaf.

The results obtained during purification together with minimal amount of exposure to sucrose and sorbitol during isolation and subsequent

purification suggested bean protoplasts to be extremely susceptible to enhanced plasmolysis during both enzyme digestion and subsequent purification, resulting in leakage and degradation of important constituents.

Other workers have used CCD methods of purification to circumvent some of these problems. Further, physiological activities of protoplasts purified by CCD have been found to compare favorably with whole (intact) plant systems (Kanai & Edwards, 1973). CCD methods were therefore used for further purification of bean protoplasts, and special precautions were taken to prevent plasmolysis and its adverse effects. These were the use of CCD methods at temperatures of $4^{o}C$ (not $25^{o}C$ Kanai & Edwards, 1973; Halberg & Larson, 1981), the optimisation of CCD methods for collection of protoplasts at the interphase of PEG and DT_{40} to avoid detrimental effects of PEG, and the addition of BSA to CCD purification to dislodge chloroplasts sticking to the membrane surface (Perlin & Spanswick, 1980).

3.4.3. Viability of cells and protoplasts:

Protoplasts are known to exhibit several abnormalities on isolation, for example subchloroplast formation, pseudocrystalisation of ribulose bis-phosphate carboxylase, abnormal protein and nucleic acid synthesis (Lytlleton & Ts´o, 1958; Murakami, 1972; Milne 1972; Nagata & Yamaki, 1973; Lazar et al., 1973; Dhindsa, 1976; Premecz et al., 1977, 1978). It is therefore likely that isolated protoplasts may have impaired physiological function. Impaired physiological function has been most

commonly estimated by estimation of esterase activity by using the dye fluorescein diacetate. However, the results described above suggest fluorescein diacetate to be an inaccurate estimator of viability.

Impaired physiological function is usually assessed by comparison of physiological function with whole (intact and excised) plant systems. Because these methods yield results which do not compare with the whole (intact or excised) and cultured plant systems for reasons described in section 3.1, it was thought that estimation of protein synthesis would give a reliable estimate of viablity of a protoplast system. Protein synthesis has been used to estimate physiological function in protoplasts of other plant systems. The labelled amino acid used has been ^{14}C-leucine (Oldfield & Coutts, 1980). In these studies, leucine was substituted by ^{35}S-methionine to illustrate optimal physiological function (viability) of protoplasts. The linear incorporation of this amino acid into proteins for a period of up to 10 hours in culture (a survival time limit for a protoplast, Burger & Hackett, 1982), and with a low amount of variation indicated optimal physiological function (viability) of the protoplast.

A comparison of results obtained for estimation of physiological function (hence viability) of protoplasts with results obtained for cultured systems such as callus gave variable results. This was because callus cultures exhibit considerable variation in uptake and incorporation of label into protein. On the other hand, a comparision of results obtained for estimation of physiological function (hence viability) of protoplasts with results obtained for rapidly isolated

cells resulted in reproducible results. This was because cells do not exhibit considerable variation in uptake and incorporation of label into protein.

The above results indicate that uptake of labelled amino acid and its incorporation into protein (a vitally important physiological function) could be used to assess viability. Experiments were designed to test the feasibility of such a method for assessment of viability. Major and minor alterations in the environment were deliberately made and their effects tested on the level of protein synthesis of the protoplast. Minor alterations in the environment of the protoplast consisted of the depletion in osmotic requirements sustaining the cell and protoplast.

The resulting hypotonic medium resulted in lower rates of incorporation of ^{35}S-methionine in to protoplast protein, hence decreased protein synthesis (as opposed to protoplasts in isotonic media). Further, values obtained for protein synthesis of protoplasts (viability) in isotonic media were compared with those obtained for single cells. Such minor osmotic alterations in the protoplast environment have also been reported to result in decreased protein synthesis in several other systems (as opposed to constant levels of protein synthesis in isotonic cells and protoplasts) (Fuchs & Galston, 1976; Kulikowski & Mascarenhas, 1978; Premecz et al., 1978; Ruesink, 1978; Zuily-Fodil & Esnault,1980; Kaiser & Heber, 1983). It must be noted that osmotic disturbances may result in other detrimental effects on the physiology of the cell or protoplast other than protein synthesis,

such as the increase in ribonuclease, rapid efflux of proteins, breakage of chloroplasts, subprotoplast formation, inhibition in ^{14}C-fixation and inhibition of H^+ excretion (Lazar et al., 1973; Cleland, 1975; Dhindsa, 1976; Premecz et al., 1977; Kaiser et al., 1981). Osmotic stress in presence of mannitol in particular, one of the osmotic requirements for the Phaseolus vulgaris protoplast, causes an increase in ethylene formation, induces senescence and inhibits photosynthesis in protoplasts and leaf tissue (Rivo & Yang, 1982; Shabtai et al., 1982). Inhibition of protein synthesis by alteration in osmotic requirements may be a reflection of these other effects mentioned above.

Major alterations in the environment of the protoplast consisted of the addition of compounds detrimental to the cell and protoplast. These compounds were trypsin and ribonuclease I. Both these compounds greatly impaired the uptake and incorporation of label into protoplasts and cells. Trypsin has been known to alter viability and physiological function in several systems by altering membrane asymmetry (Winzler et al., 1967; Buck et al., 1970, 1971; Glick et al., 1973; Kalish et al.,1978). Ribonuclease has been known to disrupt cells and protoplasts even when enzymatically inactive. The reasons for this have been thought to be due to a combination of high basicity and basic nature and increased penetration into the cell or protoplast in presence of 5 to 10mM calcium in the medium (as found in the medium of Phaseolus vulgaris cells and protoplasts, Ruesink, 1971).

3.4.4. Intactness of cells and protoplasts:

Several methods have been used to assess the intactness of cells and protoplasts. The most common ways have been dye staining techniques (Glimelius et al., 1978), cellular activities, respiration (Taiz & Jones, 1971; Nishimura & Beevers, 1978) and photosynthesis (Kanai & Edwards, 1973; Nishimura & Akazawa, 1975). Several workers have found that only substantial damage to protoplast structure by centrifugation at 13000 x g such as that used by Meyer et al. (1984 a, b) or vacuum infiltration on glass fibre discs,such as that of Paszkowski et al. (1980) resulted in loss of intactness (Morris & Thain, 1980b). In many systems, intactness has not been assessed.

The necessity for an efficient method for determination of intactness is evidenced by an enzymic method devised recently. This method uses exclusion of glycolate and its measurement by leaky glycolate oxidase from peroxisomes (Nishimura et al., 1985).

Since the semipermeable nature of the cell membrane is largely unaltered by isolation procedures used for cells, dye exclusion (using Evans blue,for example) is an acceptable way of measuring intactness (Gosch et al., 1975).

In this study dye exclusion failed to distinguish between intact and leaky cells and protoplasts. This was probably because of loss of dyes by structurally damaged protoplasts during washing procedures (Taylor & West, 1980).

However, the uptake and incorporation of label in to de novo synthesised extracellular protein provided an efficient way of estimating intactness of protoplasts and cells. The use of ^{35}S-methionine facilitated these observations, being a common membrane protein amino acid.

Further, major and minor alterations (such as addition of ribonuclease and trypsin and changes in osmotic components) in the environment affected intactness, by inducing leakage of de novo synthesised proteins in both the cell and protoplast systems. Besides these factors also adversely affected the viability of the cell and protoplast.

The main achievement of the studies described in this chapter was the optimisation of protoplast protein synthesis (a vitally important physiological function) to an efficiency equal to that of rapidly isolated cells, subject to minimal alteration in the environment by a) purification to yield structurally intact protoplasts and b) addition of low amounts of sugar to values equivalent to intact cells, to release protoplasts from osmotic stress. Such a method was found to be more sensitive than fluorescein diacetate as a test for viability of impure protoplast preparations.

Further, the establishment of protein synthesis as an efficient marker for viability of protoplasts required yields comparable to those used for studying other physiological phenomena, such as photosynthesis and respiration. An improvement of the method used by Pelcher et al. (1974) increased yields from 10^2 to 10^3 protoplasts.gFW leaf tissue^{-1} to as high as 10^5 to 10^6 protoplasts.gFW leaf tissue^{-1}.

CHAPTER 4

INDUCTION OF HYPERSENSITIVE NECROSIS AND PHYTOALEXIN SYNTHESIS IN THE PHASEOLUS VULGARIS L. - COLLETOTRICHUM LINDEMUTHIANUM L. INTERACTION : ISOLATION AND CHARACTERISATION OF THE PATHOGEN CELL WALL ASIALOGLYCOPROTEIN

4.1. INTRODUCTION:

Several components of the fungal pathogen have been isolated and characterised as inducers of the phytoalexin response and hypersensitive necrosis. Some examples are peptides (Cruickshank & Perrin, 1968; Cruickshank et al., 1974), pathogenic enzymes such as polygalacturonase and pectate lyase (e.g. Lee & West, 1981), unsaturated fatty acids eg. arachidonic acid (Preisig & Kuc, 1985 .), polysaccharides and glycoconjugates (Anderson , 1978 ; Ayers et al., 1974, 1975a, b & c; Anderson & Albersheim, 1975; Wade & Albersheim, 1979; Keen & Legrand, 1980) - Prouty.

These isolated components have been found to have characteristics necessary for the induction of the phytoalexin response. These may be (a) a negatively charged surface helping in the attachment to the positively charged host cell surface (b) an enzymatic nature helping in the digestion of host cell wall, (c) a low molecular weight permitting entry into the host cell via the cell membrane and (d) a carbohydrate moiety capable of recognising glycoprotein or lectin constituents on the host cell surface.

The aim of this work was to isolate and characterise the pathogen

component responsible for the induction of hypersensitive necrosis or the phytoalexin response.

Many studies use culture filtrates of the fungal pathogen to isolate and characterise the pathogen component inducing the phytoalexin response (*). In this study pathogen cell walls were used (as opposed to culture filtrates) because extracellular proteins secreted by the pathogen into the culture medium have been found to change during growth in suspension cultures (Vose & Lamb, unpublished results).

4.2. MATERIALS AND METHODS:

4.2.1. METHODS:

Growth of pathogen material was by methods described in Chapter 2, section 2.2.1.4. Isolation and purification of pathogen cell wall asialoglycoprotein was by methods described in Chapter 2, section 2.2.1.5. Gel electrophoresis and PAS staining was as described in Chapter 2, section 2.2.10 to 2.2.1.13., glycophorin being run as standard.

Abbreviations: BSA, bovine serum albumin; BSTFA, bis(trimethylsilyl) trifluoroacetamide, EDTA, ethylenediamine tetracetic acid; SDS, lauryl sulphate (sodium salt).

* e.g. Anderson, (1980) used culture filtrates of Colletotrichum lindemuthianum, Dow & Callow (1979) used Cladosporium fulvum culture filtrates and Ayers et al. (1976) used Phytophthora megasperma var. sojae culture filtrates.

4.2.1.1. Pronase digestion of asialoglycoprotein: The purified asialoglycoprotein impregnated in polyacrylamide gel was soaked for 30 minutes in 0.125M Tris-HCl pH 6.8, 0.1%(w/v) SDS and 1mM EDTA, and both the solution and gel embedded in sample wells of SDS-PAGE gels filled with the same buffer as follows: Each gel slice was pushed to the bottom of a well with a spatula. Spaces around the slice were filled by overlaying each slice with 10µl of the buffer containing 20%(v/v) glycerol . 10µl of pronase was overlayed in each slot and electrophoresis performed in the normal manner except that the current was turned off for 30 minutes when the bromophenol blue dye reached the bottom of the stacking gel.

4.2.1.2. Characterisation of cell wall constituents:

4.2.1.2.1. Determination of molecular weight:

Molecular weights were determined by molecular exclusion on Sephadex G-25(50-150µm), Sephadex G-50(50-150µm), Biogel P-60, Sepharose 4B-200 and Sephacryl S-1000 gel media. Chromatography was carried out on glass columns (80 x 2.2cm), fitted with a sintered-glass plate. Columns were equilibrated with 0.02M to 0.05M Tris-HCl, pH 7.5 at a flow rate of $15ml.h^{-1}$. Samples were applied to the column as $0.2mg.ml^{-1}$ and 1 ml fractions collected. Aliquots of each fraction were analysed for carbohydrate and protein by methods described below. Void volumes were determined using dextran-2000.

4.2.1.2.2. Determination of carbohydrate:

Carbohydrates were quantified using the method of Fuller (K.W. Fuller, personal communication) using glucose as standard. 0 to 0.1mg .ml^{-1} carbohydrate was adjusted to 1.0ml with distilled water. 2.0ml ∝-naphthol reagent [0.4% (w/v) ∝-naphthol in conc. H_2SO_4] was added, the tubes covered with marbles and heated in a water bath at 100°C for 10 minutes. The tubes were cooled to 18 to 25°C under tap water and absorption read at 550nm in a Perkin-Elmer 551S UV/VIS spectrophotometer. A typical curve is shown in Fig. 37.

4.2.1.2.3. Determination of protein:

Protein was determined by the method of Lowry modified as follows using BSA as standard (Lowry *et al.* 1951). 0 to 0.4mg.ml^{-1} protein was adjusted to 0.2ml with distilled water. 0.06ml 20%(w/v) TCA was added and the solution incubated at 4°C for 10 minutes. Samples were centrifuged at 7520 to 9650 x g for 2.5 minutes at 18 to 25°C. Supernatants were discarded and the pellet dissolved in 0.08ml 1M NaOH followed by the addition of 0.26ml distilled water. Samples were shaken, 0.6ml Folin C [50ml Folin A (2% (w/v) Na_2CO_3 in 0.1M NaOH) and 1ml Folin B (0.5%(w/v) $CuSO_4 5H_2O$ in 1%(w/v) sodium citrate)] was added, the mixture shaken and allowed to stand at 18 to 25°C for 15 minutes. 0.06ml Folin Ciocalteau reagent (diluted 1:1 (v/v) with distilled water) was added, the solution shaken immediately and left to stand at 18 to 25° for 30 minutes. Absorption was read at 700 and 750nm in a Perkin-Elmer 551S UV/VIS spectrophotometer. A typical curve is shown in Fig. 38.

Fig. 37 CONTD

For further details see `methods´. Each spot represents the mean of 12, determinations. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

Fig 38 CONTD

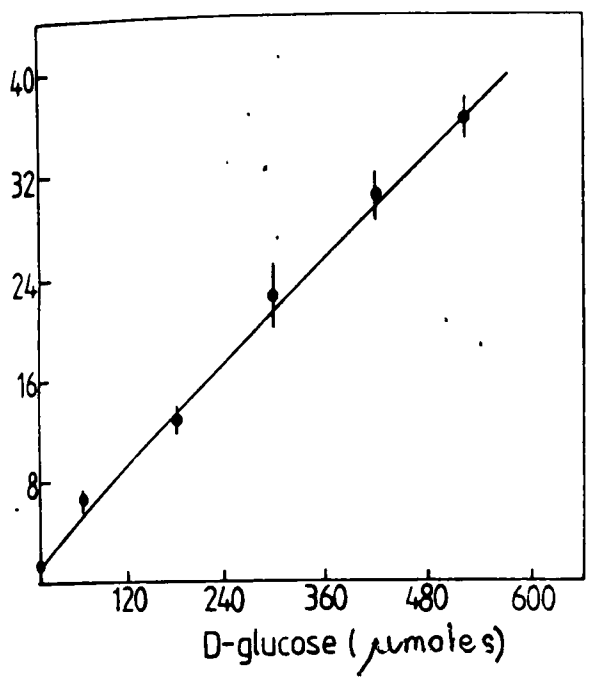
For further details see `methods´. Each spot represents the mean of 12, determinations. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

Fig. 39 CONTD.

For further details see `methods´. Each spot represents the mean of 3 determinations. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

Fig 40. CONTD.

For further details see `methods´. Each spot represents the mean of 3 determinations. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.



Characterisation of pathogen cell wall tuents: standard curve used for determination of ydrates (reducing sugars). (continued on facing page)

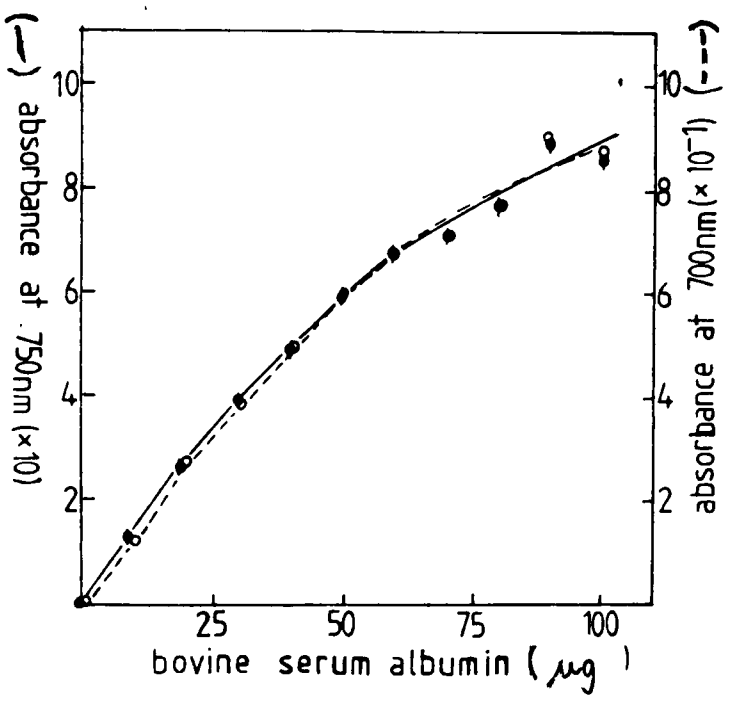
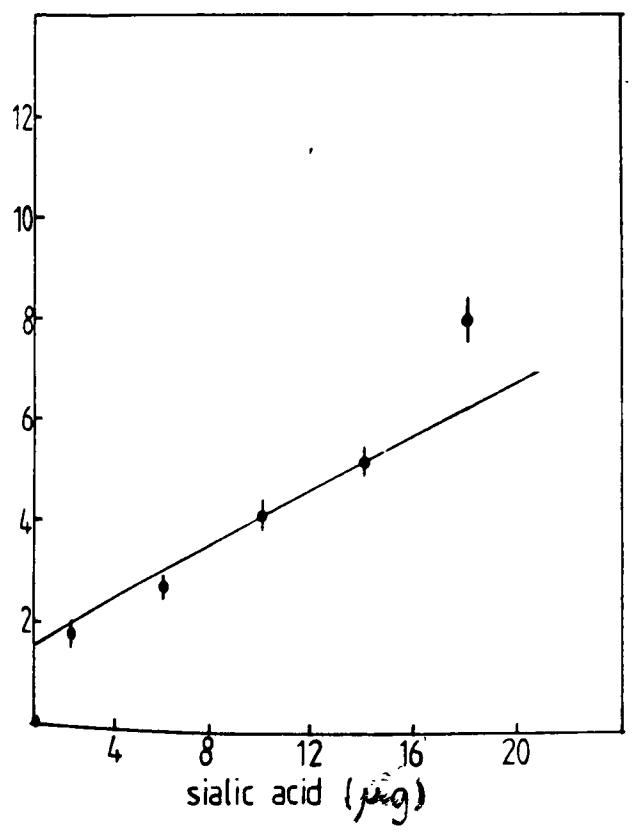


Fig. 24 Characterisation of pathogen cell wall constituents: standard curve used for determination of protein. (continued on facing page)



Characterisation of pathogen cell wall ients: standard curve used for determination of sialic (continued on facing page)

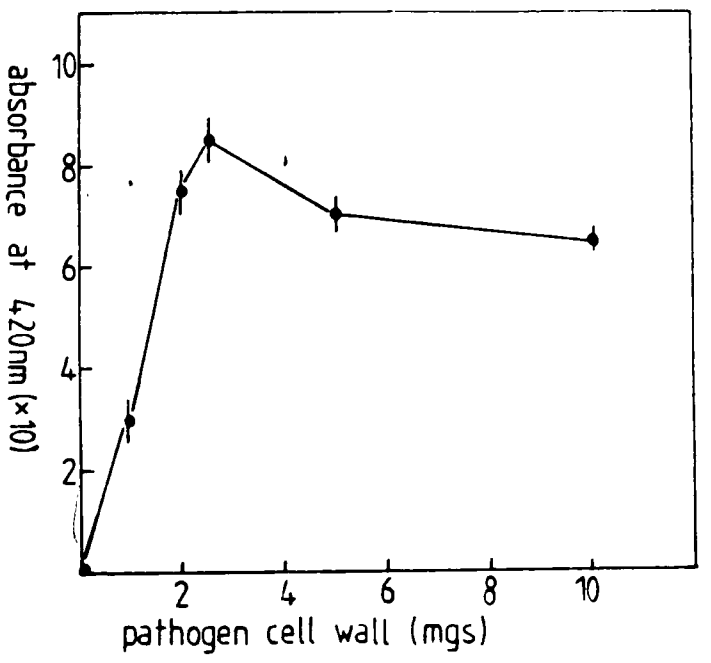


Fig. 40 Purification of pathogen cell wall constituents: Concdnavalin A precipitation.

(continued on facing page)

4.2.1.2.4. Determination of sialic acid (N-acetylneuraminic acid):

Isolation of sialic acid: Samples were subjected to mild acid hydrolysis with 0.025N H_2SO_4 at 80°C, for 1 hour in a glass vial, and passed through a Dowex 1-X8 (100-200 mesh formate form) anion-exchange resin. Sialic acid was eluted with 1M formic acid.

Quantification of sialic acid: Sialic acid was quantified by the method of Spiro, 1966, using commercial sialic acid as a standard. A typical standard curve is shown in Fig. 39.

4.2.1.2.5. Determination of hexosamines:

Isolation of hexosamines: Samples were subjected to acid hydrolysis with 0.05N H_2SO_4 at 100°C for 1 hour in screw-capped tubes followed by separation of hexosamines on a Dowex 50-X12 (50-100 mesh, H^+-form) cation-exchange resin. Hexosamines were eluted with 3N HCl, evaporated to dryness at 50°C in vacuo in a vacuum rotator to remove all HCl, and titrated with 2N NaOH to pH 12 to 14. Samples were titrated to pH 3 to 5 with 0.2N HCl. 0.15ml 12.5% (v/v) cold aqueous solution acetic anhydride was added, followed immediately by addition of 0.1ml 4N Na_2CO_3. The tubes were mixed and allowed to stand at 18 to 25°C for 10 minutes, capped with marbles and heated in a boiling water bath for 5 minutes. Tubes were cooled under tap water. Samples were passed through a Dowex 50-X2 (200-400mesh, H^+-form) cation-exchange resin followed by passage through a Dowex 1-X8 (100-200 mesh, formate form) anion-exchange resin, used in approximately 3 fold excess of ions

present in the mixture. The resin was washed with several times the volume of distilled water and the effluent evaporated to dryness *in vacuo* in a rotary evaporator at 40 to 45°C.

Characterisation of hexosamines: Acetylated hexosamines were analysed by gas-liquid chromatography as follows: Acetylated hexosamines were dissolved in distilled water and transferred to 10 x 7 8mm test tubes, and evaporated to 50 µl under N_2 in a heating block at 105°C. Samples were cooled to 18 to 25°C and 0.1µl internal standard solution [tetradecane, a C_{14} saturated hydrocarbon and hexadecane, a C_{16} saturated hydrocarbon] added to each tube. Samples were evaporated to dryness *in vacuo* in a rotary evaporator at 18 to 25°C and stored in a desiccator. Each sample was heated at 60°C for 1 hour with 25µl bis (trimethylsilyl) trifluoroacetamide (BSTFA) to form BSTFA derivatives and analysed in a Carlo-Erba Series 2150 gas-liquid chromatograph with a flame-ionisation detector. For analysis, 0.1µl of each silylated sample was injected on to a high performance capillary column packed with cross-linked methyl silicone (0.31mm bore x 25cm) and a carrier gas (He) passed through at a flow rate of 12 ml.min^{-1}. The temperature programme was held at 70°C for 3 minutes, increased to 140°C at 3°C min^{-1}, and held at 290°C for 10 minutes. The detector oven temperature was 300°C. The peaks were tentatively identified by co-chromatography with standards.

Quantification of hexosamines: Hexosamines were quantified by the method of Spiro (1966) using commercial N-acetyl-D-galactosamine as a standard. A typical standard curve is shown in Fig. 43.

4.2.1.2.6. Determination of neutral sugars:

Isolation of neutral sugars: Samples were subjected to acid hydrolysis with 0.05N H_2SO_4 at 100°C, for 1 hour in screw-capped tubes followed by separation of hexosamines on a Dowex 50-X12 (50-100 mesh, H^+-form) cation-exchange resin. Samples were then passed through a Dowex 50-X2 (200-400 mesh) (H^+-form,) cation-exchange resin, followed by passage through a Dowex 1-X8 (100-200 mesh) (formate-form) anion-exchange resin. Samples were concentrated in vacuo at 35 to 40°C to remove the formic acid.

Characterisation of neutral sugars: Neutral sugars were dissolved in distilled water, samples transferred to 10 x 78 mm test tubes, and evaporated to 50µl under N_2 in a heating block at 105°C. Samples were cooled to 18 to 25°C and 50µl D-glucoheptose [100nmol.ml^{-1}] was added to each tube as an internal standard. Samples were then evaporated to dryness in vacuo at room temperature, and stored in a desiccator. Each sample was heated at 60°C for 1 to 2 minutes with 25µl Tri-Sil 'Z' to form TMS derivatives, and analysed using a Pye-Unicam Series 104 gas-liquid chromatograph with a flame-ionisation detector. For analysis, 0.5µl of each silylated sample was injected on to a glass column (2mm x 2.7m) packed with 3% OV-1 on Gas Chrom-Q support, with a carrier gas (He) at a flow rate of 2cc.min^{-1}. The temperature programme was to hold for 5 minutes at 80°C, to increase to 300°C at 2°C.min^{-1} and to hold at 300°C for 15 minutes. The detector oven temperature was 300°C. Peaks were tentatively identified by comparison with standards.

Quantification of reducing sugars: Reducing sugars were quantified by adding known quantities of standard sugars (e.g. galactose) to the internal standard D-glucoheptose, and comparing the intensity of the peaks obtained with those obtained for the sample.

4.2.2. MATERIALS:

The spectrophotometer was from Perkin-Elmer & Co., GMBH/Uberlingen, Bundesrepublik Deutschland; gas-liquid chromatographs from Erba Science, Swindon and Pye-Unicam, Cambridge and freeze dryer from Edwards High Vacuum, Crawley, Sussex. Millipore filters were obtained from Millipore Corporation, Bedford, Massachusetts, and teflon liners from Gallenkamp, London.

All glassware was rinsed in EtOH and dried prior to usage.

Chemicals used were of the analar grade.

Biogel P-60 was from Biorad Laboratories, Richmond, California; BSTFA from Lancaster Synthesis Ltd., Lancaster; Tri-Sil 'Z' from Pierce and Warriner (UK) Ltd., Cheshire; dextran-2000 from Pharmacia Fine Chemicals, Uppsala, Sweden; Folin Ciocalteau reagent, BSA, N-acetyl-D-galactosamine, Dowex 50-X2 resin, Dowex 50-X12 resin, Dowex 1-X8 resin, sialic acid, D-glucose, D-mannose and D-galactose, β-dimethylaminobenzaldehyde, cyclohexanone, Sephadex G-25-50, Sephadex G-50-150, Sepharose 4B-200, Sephacryl S-1000, and D-glucoheptose from Sigma Chemical Co. Ltd., Poole, Dorset. Other chemicals were from BDH

Chemicals Ltd., Poole, Dorset.

4.3. RESULTS:

4.3.1. Isolation of the phytoalexin inducing constituent of the fungal pathogen was by comparing activities of live fungal pathogen mycelia in the whole (intact) host plant with activities of abiotic components of the pathogen cell wall in the isolated cell system described in Chapter 2, section 2.3. These responses may be represented quantitatively in Table 3.

4.3.2. Isolation, purification and characterisation of the constituents of the pathogen cell wall:

The mycelial cell wall of the pathogen was found to contain a sugar and protein component that co-migrated on gel filtration columns. Both the sugar and protein components co-precipitated as a single component with concanavalin A (Fig. 40). Further, both sugar and protein components could be separated as a single component from concanavalin A by ion exchange on DEAE-Sephacel in presence of 0.2M to 0.5M methyl-α-D-mannoside (Fig. 41). The constituent was therefore, considered to be single.

The constituent was excluded in the void volume of Sephadex G 25 (50 - 150μm), Sephadex G 50 (50 - 150μm), Sepharose 4B-200 and Sephacryl S-1000. Since these results were obtained in salt solutions of 0.02M to 0.10M ionic strength, this result could not have been due to ionic

Table 3. Purification of the pathogen cell wall asialoglycoprotein:

fraction composition**	activity*	recovery	specific activity*** P***	specific activity*** C***	fold purification P***	fold purification C***
a. pathogen cell wall						
P = 0.025 C = 0.320	90.0 ± 1.0	100.0	3600.0	281.0	–	–
b. acetone and concanavalin A precipitated cell wall						
P = 0.010 C = 0.100 N = 0.332 M = 0.026 G = 0.059	140.0 ± 1.0	155.0	14 000.0	1400.0	3.9	5.0

* determined in 10 day old host plant cells, as PAL activity, nM min^{-1} gFW^{-1} leaf tissue.

**g.gDW^{-1} pathogen cell wall material, where
P = protein
C = carbohydrate (reducing sugar),
N = N-acetyl-D-galactosamine,
M = mannose,
G = galactose.

*** g^{-1} mycelial protein,
g^{-1} mycelial carbohydrate (reducing sugar).

Fig 41 CONTD.

For further details see 'methods'. Each spot represents the mean of 3 determinations. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

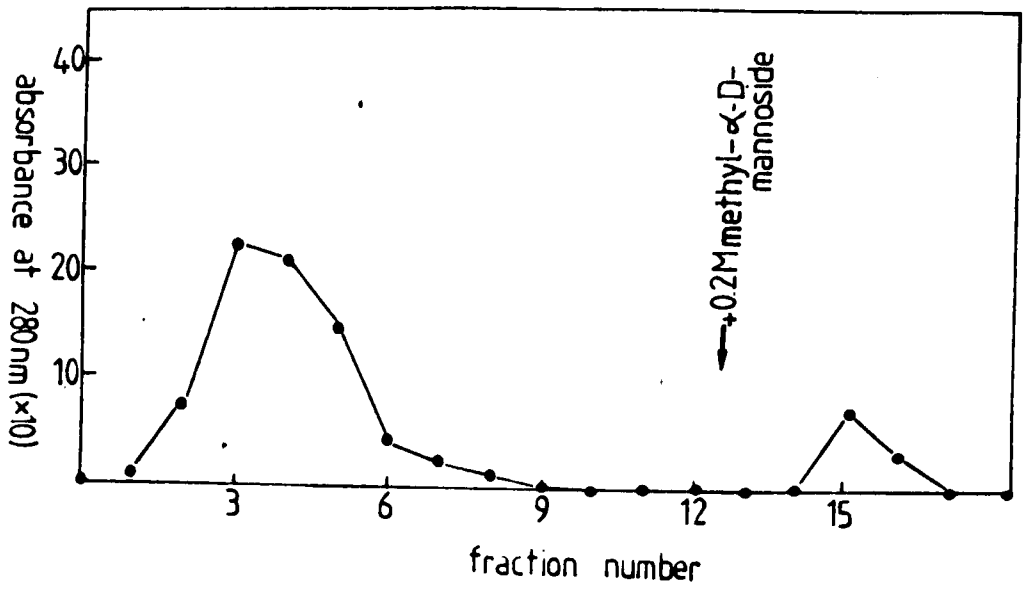


Fig. 24 Purification of pathogen cell wall constituents : separation of concanavalin A from cell wall constituents by methyl-α-D-mannoside. (continued on facing page)

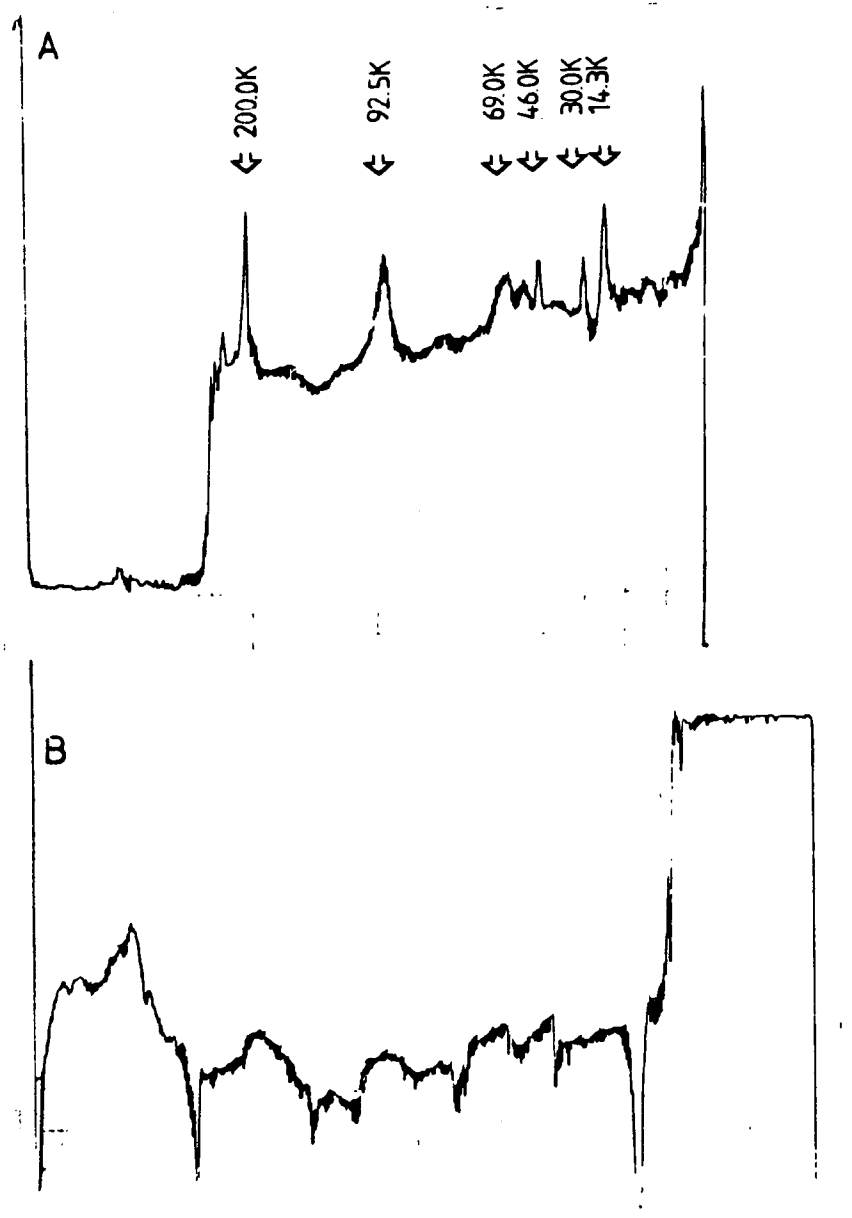


Fig. 40 Characterisation of pathogen cell wall constituents: densitometric scans of discontinuous SDS gels after staining with periodic acid Schiff's reagent where, A, molecular weight markers; B., pathogen cell wall constituents.

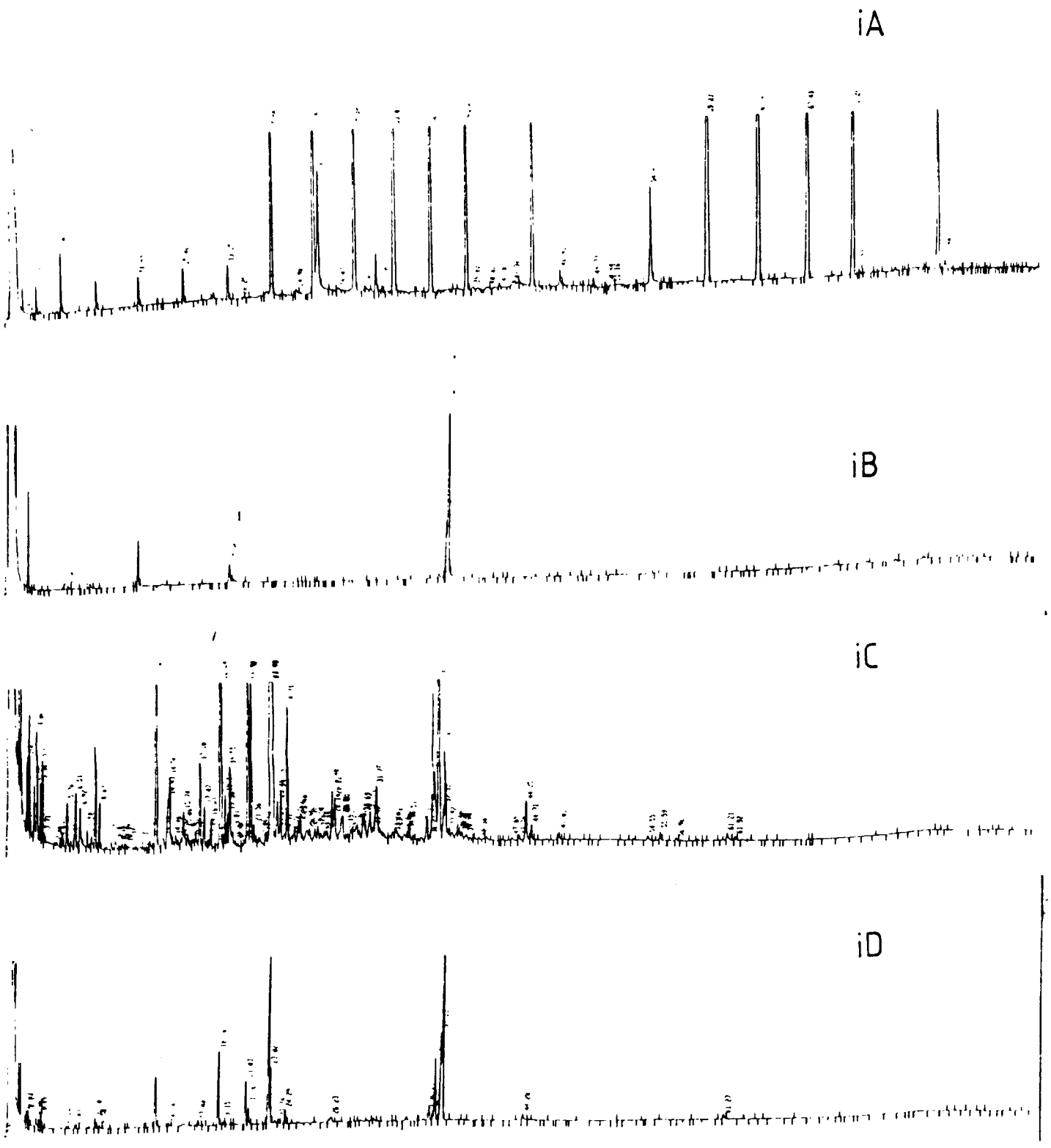
interaction between negatively charged solutes and the matrix of the gels, but probably due to a glycoprotein not being globular and/or >20 x 10^6 kilodaltons in molecular weight. The glycoprotein appeared as a single stained band with periodic acid Schiff's reagent on polyacrylamide gels, glycophorin being used as a standard. This band was retarded at the interface of the separating and stacking gel (Fig. 42), at concentrations of as low as 4%T stacking and 5%T separating gels. In discontinuous buffer systems, this band may be caused by formation of aggregated protein or high molecular weight protein of >20 x 10^1 kD in molecular weight. The glycoprotein did not contain sialic acid, and was therefore called an asialoglycoprotein. Pronase digestion of the band did not result in a shift in electrophoretic mobility.

The glycoprotein was found to contain amino sugars and neutral sugars. In this preliminary study, the peaks in Fig. 43 and Fig. 44 were tentatively identified by co-chromatography with standards. One amino sugar, N-acetylgalactosamine (Fig. 43) and two neutral sugars, mannose and galactose (Fig. 44) co-chromatographed with the sample. Positive identification by gas chromatography/ mass spectrophotometry would be desirable in future studies.

4.3.3. The phytoalexin response inducing properties of the pathogen cell wall asialoglycoprotein during purification were compared with those obtained with mycelium of the pathogen on the intact (whole) host plant. These results are described in Table 3.

Fig 43. CONTD.

For further details see 'methods'. Each spot represents the mean of 3 determinations. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.



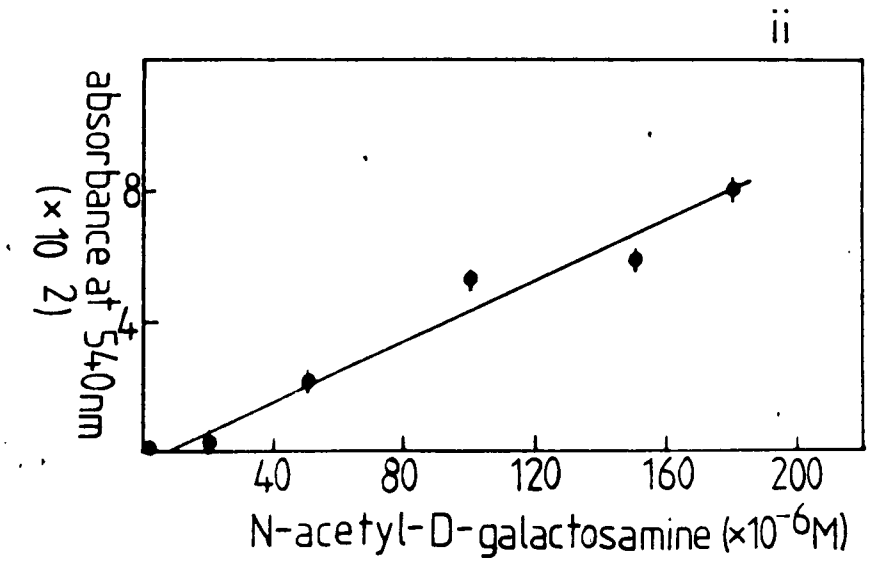


Fig. 40 Characterisation of pathogen cell wall constituents: i. analysis of amino sugars by gas chromatography: A. standards; B. N-acetylgalactosamine standard; C. cell wall constituents; D. N-acetylgalactosamine standard + cell wall constituents. ii. standard curve used for determination of amino sugars. (continued on facing page)

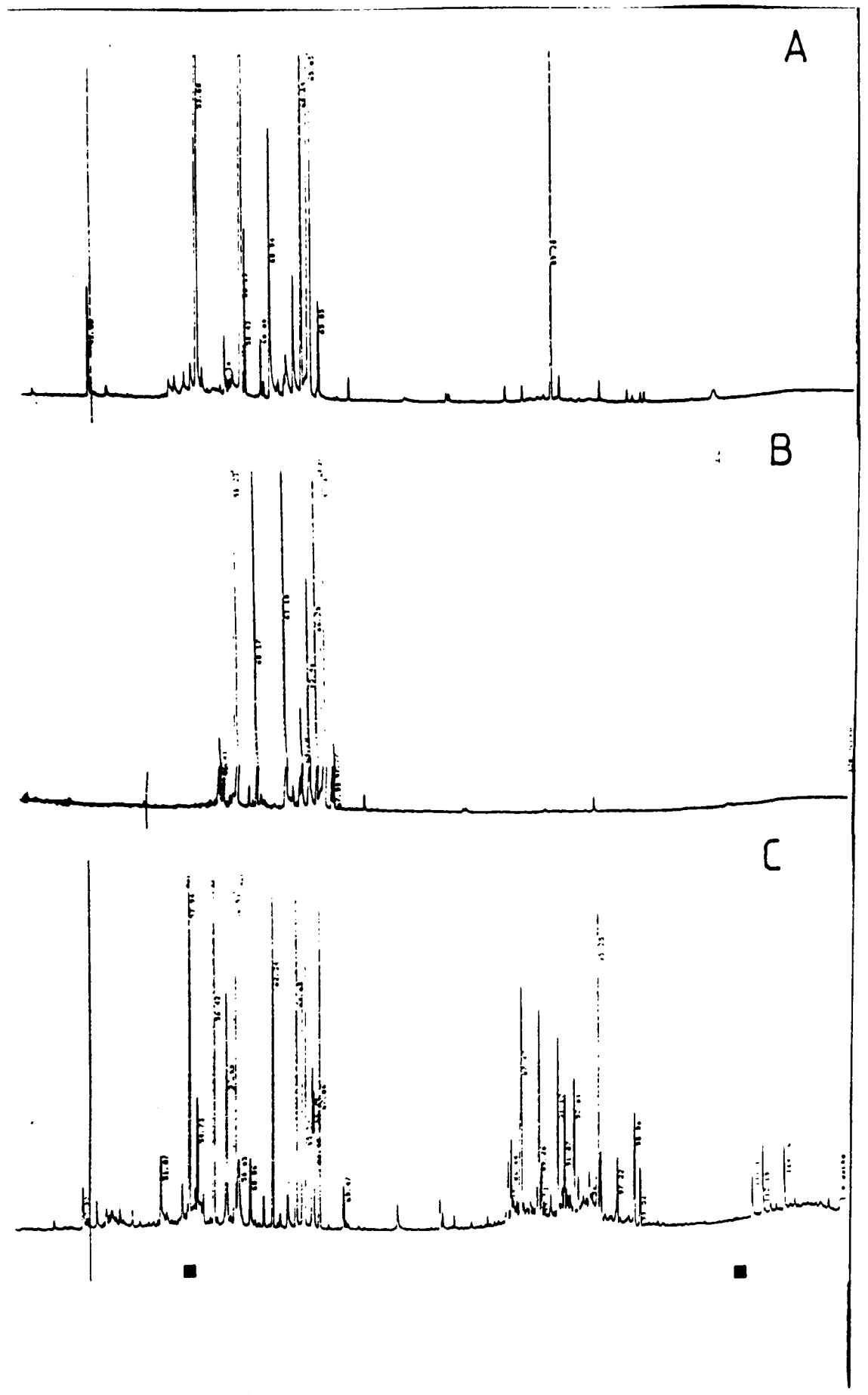


Fig. 40 Characterisation of pathogen cell wall constituents: analysis of neutral sugars by gas chromatography: A. mannose standard; B. galactose standard; C. mannose standard + galactose standard + cell wall constituents.

4.4. DISCUSSION:

During isolation and purification, the asialoglycoprotein inducing the resistant responses (phytoalexin response and [illegible] necrosis) as described above, did not increase more than 10 fold in specific activity. This has been found for other pathogens and is thought to be due to (i) the presence of growth inhibitors in pathogen preparations, (ii) change in primary structure during purification or (iii) masking of active determinants by other moieties (Anderson-Prouty & Albersheim, 1975; Sharp et al., 1984a,b,c). The first possibility is unlikely as concanavalin A precipitation was used for isolation and purification, and linear responses were obtained. The second and third possibility is likely as relatively harsh treatments such as autoclaving and treatment with organic solvents were used to isolate the asialoglycoprotein. The failure to increase specific activity of the asialoglycoprotein has also been found for glucans isolated from Phytophthora megasperma var.sojae (Ebel et al., 1976).

In this study of the Phaseolus vulgaris - Colletotrichum lindemuthianum interaction, no induction of the phytoalexin response or hypersensitive necrosis was obtained by non-living constituents of the pathogen and the whole (intact) plant system. Removal of the middle lamella seemed necessary for the induction of enzymes leading to the synthesis of phenylpropanoid-derived phytoalexins.

Wounding and excision of host tissue has been a necessity for studies on interaction with non-living constituents of the pathogen. This is

consistent with culture filtrate, cell wall extracts and cell wall components of glucan or glycoprotein nature from several pathogens including Colletotrichum lindemuthianum and Phytophthora megasperma var sojae, together with extracts from non-pathogens and several abiotic components where wounding is thought to be necessary for the phytoalexin response (Anderson-Prouty & Albersheim, 1975; Hargreaves, 1979; Bailey, 1981, 1982). However values obtained for wounded and excised systems (as opposed to the cell system) with non-living components of the pathogen have been found to be variable. The reasons for variability in results are thought to be the induction of the phytoalexin response and hypersensitive necrosis by wounding host cells prior to addition of the isolated pathogen component, non-uniform application of the isolated pathogen component, and length of time (several days) taken for induction of the phytoalexin response and induction of hypersensitive necrosis. Variability was reduced in the cell system by testing the induction of the enzymes leading to phytoalexin response in a short time (few hours), in a system subjected to minimal wounding, and subject to uniform application of the isolated pathogen component.

The glycoprotein nature of the component which induces the enzyme responsible for the induction of the phytoalexin response, has also been found for other fungal pathogens, including those of Colletotrichum lindemuthianum (Anderson-Prouty & Albersheim, 1975), Phytopthora megasperma var sojae (Keen & Legrand, 1980) and Cladosporium fulvum (Dow & Callow 1979a; Lazarovits et al., 1979; DeWit & Kodde, 1981).

Besides the glycoprotein nature, the asialoglycoprotein cell wall constituent of Colletotrichum lindemuthianum (the asialoglycoprotein) resembled in various ways other pathogen constituents which induce the phytoalexin response and hypersensitive necrosis (e.g. glucans and glycoproteins). These were the release from pathogen cell walls by harsh treatments in the active state, heat stability, negative charge, presence on the pathogen cell wall (Bailey, 1982a; Dixon et al., 1983; Anderson-Prouty & Albersheim, 1975) and non-dialysable nature (Dixon et al., 1983b). However it differs in certain features. For example it does not have a molecular weight less than 5 x 10^3 kD, and is not heterogeneous (e.g. containing both a glycoprotein and a glucan) (Anderson-Prouty & Albersheim, 1975).

The presence of the asialoglycoprotein on the pathogen cell wall increases the possibility of the interaction with the host cell wall.

The increased release of asialoglycoprotein with heat is suggestive of the increased release of the recognition component with increasing temperature in vivo (Classen & Ward, 1984). The negatively charged nature of the asialoglycoprotein is thought to aid in its interaction with the positively charged host cell surface or moieties on the host cell surface (e.g. polygalacturonic acid) as found for most pathogen components which induce hypersensitive necrosis and the phytoalexin response (Hadwiger & Beckman, 1980). Examples include the terminal glycosyl moieties on the glucan, isolated from cell walls of Colletotrichum lindemuthianum and other fungal pathogens (Ayers et al., 1976c; Keen, 1978; Anderson, 1980; Wade & Albersheim, 1979; Keen & Legrand, 1980; Keen et al., 1983), sugar and amino sugar residues on extracellular

glycoproteins (e.g. Cladosporium fulvum L., Dow & Callow, 1979a) and chitosan (Hadwiger & Beckman, 1980 ; Young et al., 1982).

Such ionic interaction is thought to bring about a phase transition in membrane lipids causing changes in permeability leading to leakage of cellular constituents (Strobel & Hess, 1974). The asialoglycoprotein may interact with cell membrane components.

The activity of glycoproteins involved in the induction of the phytoalexin response and hypersensitive necrosis has been attributed by some to the carbohydrate and not the protein moiety (Anderson, 1978; Keen & Legrand, 1980) and by others to the protein and not the carbohydrate moiety (Dow & Callow, 1979a; Lazarovits et al., 1979; De Wit & Kodde, 1981).

The activity of the asialoglycoprotein of Colletotrichum lindemuthianum was found to be due to the carbohydrate and not the protein moiety, as suggested by no associated induction of PHA synthesis with pronase-digested asialoglycoprotein (results not shown).

CHAPTER 5

INDUCTION OF HYPERSENSITIVE NECROSIS AND PHYTOALEXIN SYNTHESIS IN THE *PHASEOLUS VULGARIS* L. - *COLLETOTRICHUM LINDEMUTHIANUM* L. INTERACTION : ESTIMATION OF L-PHENYLALANINE AMMONIA-LYASE ACTIVITIES

5.1. INTRODUCTION:

PAL is the first enzyme in the pathway leading to isoflavonoid phytoalexin synthesis. It catalyses the conversion of L-phenylalanine to t-cinnamic acid with the elimination of ammonia. Ever since its discovery in 1961, PAL activities have been measured by the determination of its end product t-cinnamic acid. Several methods have been used to estimate t-cinnamic acid, the most common being spectrophotometric, based on the absorption of t-cinnamic acid at 290nm. Some workers estimate t-cinnamic acid isotopically, because several aromatic compounds absorb at 290nm. The latter assay for PAL activity has been found to lead to erroneous estimates, primarily because of isotopic labelling of aromatic compounds other than t-cinnamic acid. Attempts have been made to estimate t-cinnamic acid by gas-chromatography and HPLC (Hanson & Havir, 1981). Unlike the above assays, these methods are both laborious and time-consuming. This chapter describes the way PAL was assayed in crude and partially purified homogenates of the bean leaf, and compares the assay system adopted with other assay systems. The kinetics of PAL in relation to L-phenylalanine supply is also presented.

5.2. MATERIALS AND METHODS:

5.2.1. METHODS:

Growth of Phaseolus vulgaris plant material was by methods described in Chapter 2, section 2.1. Leaves of the plant were used in all experiments. PAL and PAT activities were assayed by methods described in Chapter 2, section 2.2.1.8. Hypocotyl-derived suspension cultures and callus cultures were grown as described in chapter 3, section 3.2.1.1. Protein concentrations were determined by methods described in Chapter 4, section 4.2.1.2.3. Approximation of K_m values of PAL in absence of L-aspartic acid (or presence of PAT activity) was by the method of Spears et al. (1971).

5.2.1.1. Plant material:

Discs of Solanum tuberosum L. var. Maris Piper were illuminated for 16 hours under white, fluorescent light (3,300 to 4,400 lux).

Hypocotyls excised from 7-day-old Phaseolus vulgaris seedlings were longitudinally bisected with a sterile razor blade.

Abbreviations: AAT, L-aspartate amino-tranferase;PAL, L-phenylalanine ammonia-lyase; PAT, L-phenylalanine amino-transferase; TAL, L-tyrosine ammonia-lyase; TLC, thin-layer chromatography.

Sets of half hypocotyls were placed, cut side uppermost, on moist filter paper in Petri dishes (10 segments.dish^{-1}). Sterile distilled water was applied to the cut surfaces and segments illuminated for several hours under white, fluorescent light (3,300 to 4,400lux).

Hypocotyl-derived suspension cultures and callus cultures were grown as described in chapter 3, section 3.2.1.

5.2.1.4. Identification of t-cinnamate, L-phenylpyruvate, L-phenylacetate and L-phenylalanine:

Upon termination of the enzyme assays for PAL and PAT, the reaction mixture was evaporated to dryness *in vacuo* at 18 to 25°C. The residue was dissolved in 0.1ml 90%(v/v) EtOH and applied to a Polygram Cel1 300 DEAE cellulose sheet using disposable 20µl pre-calibrated pipettes. Standard samples of 0.1% (w/v) in 90% EtOH each of t-cinnamate, L- phenylpyruvate, L- phenylacetate and L-phenylalanine were spotted in parallel. The chromatogram was developed in 50mM Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0 . The chromatogram was cut in 0.5cm bands and eluted in scintillation cocktail [0.7% (w/v) butyl-PBD, 8% (w/v) naphthalene, 60% (v/v) toluene and 40% (v/v) 2-methoxy-ethanol]. The samples were counted at 4°C in an LKB-1210 liquid scintillation counter. Position of standards was located at 366nm. Phenylpyruvate was also identified at 366nm after spraying the chromatogram with 0.05% (w/v) chromotropic acid.

5.2.2. MATERIALS:

The liquid scintillation counter was from LKB-Produkter AB, Bromma, Sweden; TLC sheets from Macherey-Nagel & Co., Doren, Germany and pre-calibrated pipettes from Modulohm 1/S, Vasekaer, Denmark.

Chemicals used were of the analar grade.

L-phenylalanine, L-phenylpyruvate, L-phenylacetate, t-cinnamate and L-aspartic acid were from Sigma Chemical Co.Ltd., Poole, Dorset.

5.3. RESULTS:

5.3.1. PAL activities are usually measured by a stopped or continuous spectrophotometric assay, as follows:

The enzyme (usually a crude or partially purified homogenate in sodium borate buffer) is incubated in presence of substrate L-phenylalanine. The end product (t-cinnamic acid) formed, is quantified by absorbance measurements at 265nm (in case of partially purified enzyme preparations) or 290nm (in case of crude enzyme preparations). Absorbance measurements are read against buffer (usually sodium borate) in presence or absence of the substrate L-phenylalanine, or its analogue, D-phenylalanine.

This spectrophotometric assay was non-linear with respect to time and protein concentration in crude and partially purified enzyme preparations of green and non-green tissue such as potato, bean hypocotyls and leaf tissue, and bean hypocotyl-derived suspension and callus tissue. The spectrophotometric assay was linear only at very low protein concentrations of $<100 \times 10^{-6}$g protein, in crude and partially purified enzyme preparations of green and non-green tissue such as potato, bean hypocotyls and leaf tissue, and bean hypocotyl-derived suspension and callus tissue, the activity ranging from 0.005 to 0.12nmoles min^{-1}. However, the assay became non-linear with respect to time and protein concentrations $> 100 \times 10^{-6}$g protein, in the same tissue homogenates.

5.3.2. Further, the assay above was higher than that would explain the induction of the phaseollin production in infected tissues. It was thought therefore, that we were measuring more than one enzyme or more than one end product. It was possible that other compounds absorb at 265 to 290nm in both crude and partially purified homogenates.

Among compounds that absorb strongly in the UV region, 265 to 290nm, (pure or in crude or partially purified homogenates) were t-cinnamic acid, (the end product of PAL activity), and p-coumaric acid (the end product of TAL activity). However, L-phenylalanine (the substrate for PAL), D-phenylalanine, its structural analogue, and phenylacetic acid (formed from L-phenylpyruvic acid), do not show an absorbance in the region 265 to 290nm. These results were obtained if absorption measurements were read against distilled water or sodium borate buffer

Fig. 45 CONTD.

The values obtained for apparent PAL activity were determined by sum of values obtained for PAL ant PAT activities shown in the figure. PAL and PAT activities were estimated by procedures described in 'methods'. Each spot represents the mean of 15 different experiments. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

'PAL and PAT activities are expressed as pmoles end product. min^{-1} (• — • — •) and specific activities as pmoles end product. $min^{-1}.mg^{-1}$ protein (x — x — x).

Fig. 46. CONTD.

The values obtained for apparent PAL activity were determined by sum of values obtained for PAL ant PAT activities shown in the figure. PAL and PAT activities were estimated by procedures described in 'methods'. Each spot represents the mean of 15 different experiments. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

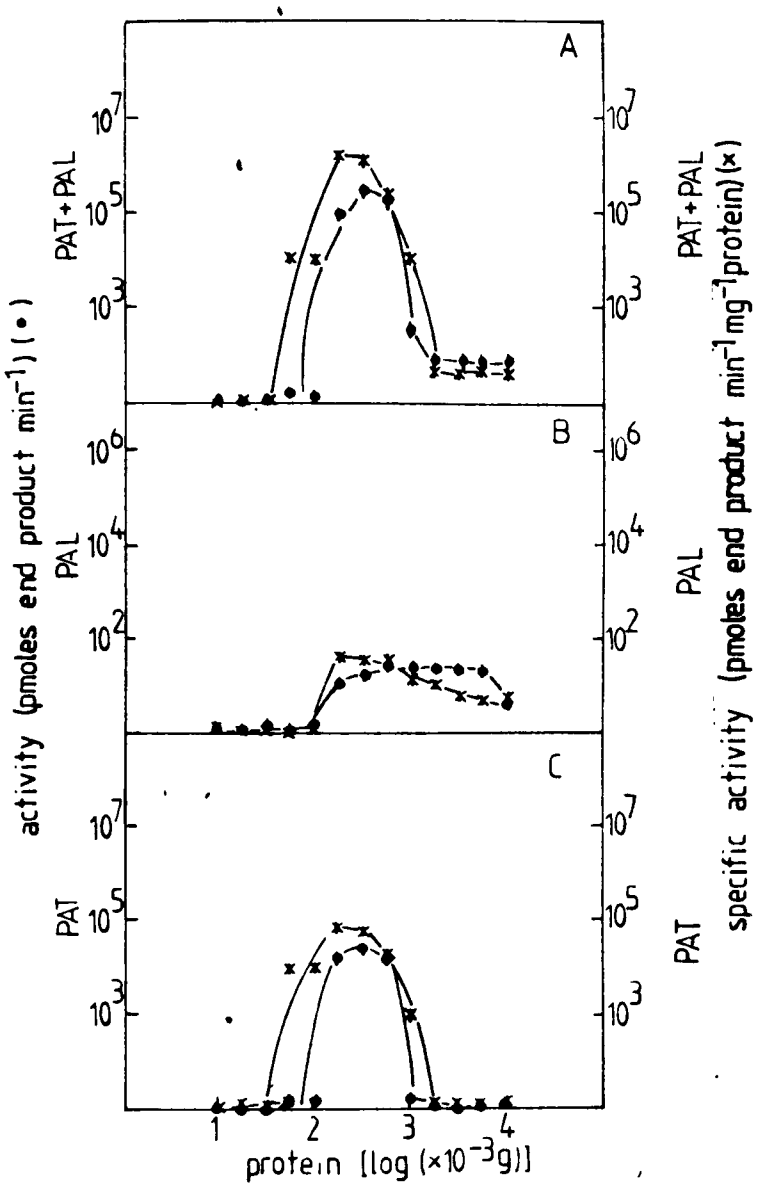


Fig. 45 Estimation of A, PAL + PAT (apparent PAL activity); B, PAL activity and C, PAT activity in crude and partially purified homogenates by the isotopic assay : non-linearity of PAL activity with protein concentration.

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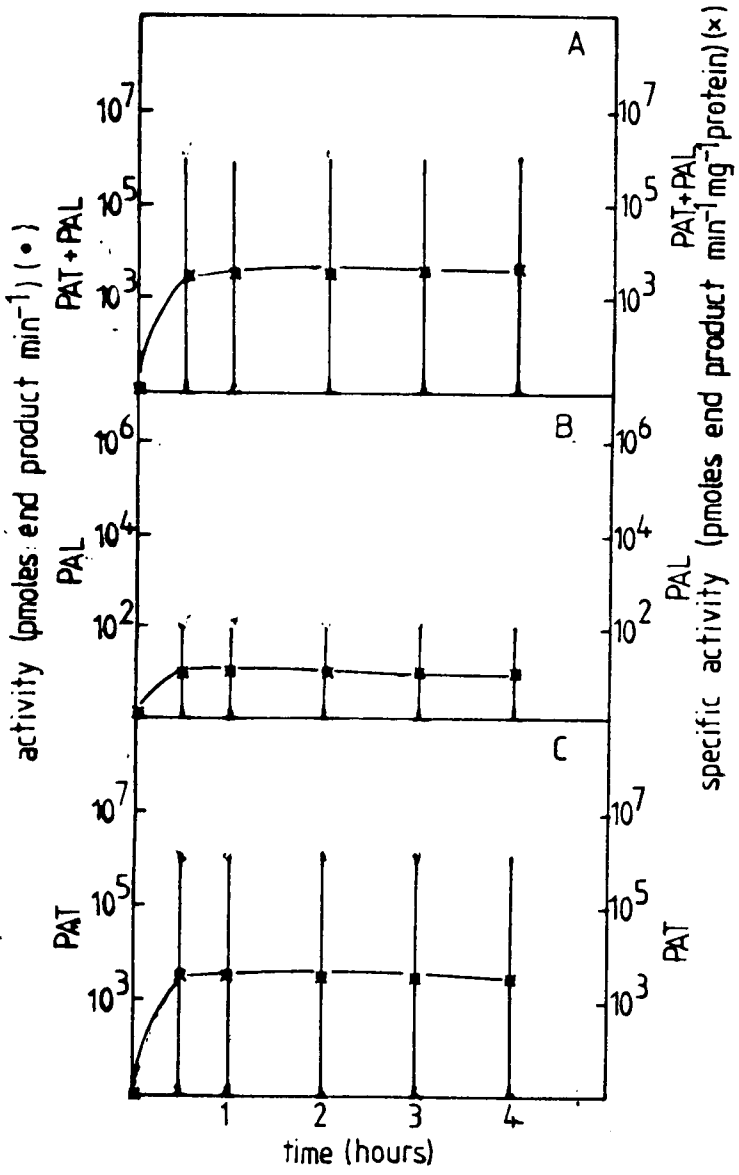

Fig. 46 Estimation of A, PAL+ PAT (apparent PAL activity); B, PAL activity and C, PAT activity in crude and partially purified homogenates by the isotopic assay : non-linearity of PAL activity with time.

(continued on facing page)

in presence or absence of D-phenylalanine (Fig. 47 A, B and C).

5.3.3. Another method for assaying PAL activities had to be chosen. The other method used is an isotopic assay, described as follows:

The enzyme (crude or partially purified homogenates) is incubated in presence of substrate, L- phenylalanine-U-^{14}C. At the end of the incubation period, the substrate is separated from the end product by differential solubility in organic solvents (e.g. toluene) and the end product t-cinnamic acid quantified by measurement of ^{14}C-label incorporated.

This isotopic assay for PAL was non-linear with time and protein concentrations in green and non-green tissues (bean hypocotyl and leaf tissue, bean hypocotyl-derived suspension and callus tissue). In green tissues, the assay was linear only for low amounts of enzyme having an activity of 0.005 to 0.12nmoles min^{-1} (Fig. 45, Fig.46).

5.3.4. It was possible, that the non-linearity of PAL activities could have been due to the incorporation of L-phenylalanine-U^{14}C in to ^{14}C-compounds other than t-cinnamate, enzymatically or non-enzymatically.

Among these compounds were t-cinnamic acid (the end product of PAL activity), L-phenylpyruvic acid (the end product of PAT activity), phenylacetic acid and hydroxyphenylacetic acid (formed non-enzymatically from L-phenylpyruvic acid) (Fig. 48).

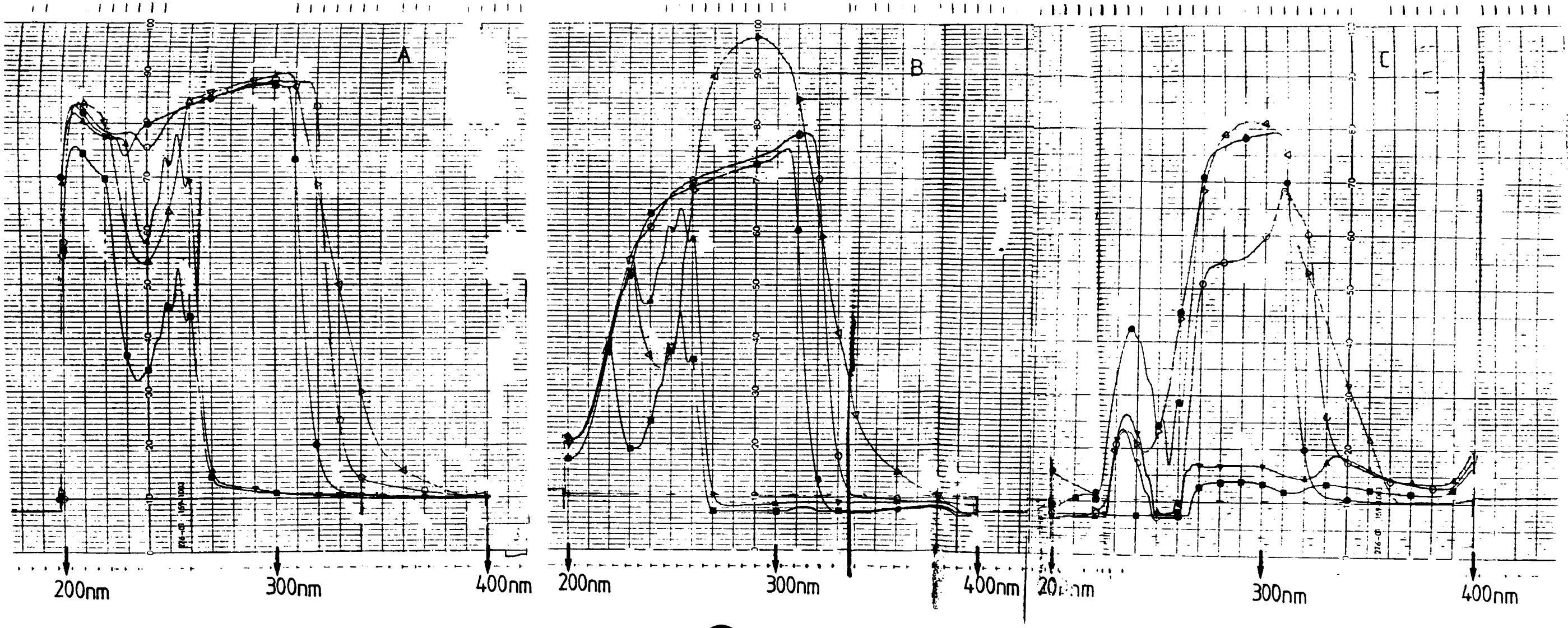

Fig. 47 Absorption of 10^{-3}M t-cinnamic acid (●—●—●), 10^{-2}M L-phenylpyruvic acid (○——○——○), 10^{-2}M L-phenylalanine (▶—▲—▲), 10^{-2}M L-phenylacetic acid (■—■—■) and 10^{-2}M p-coumaric acid (▽—▽—▽) versus A. distilled water, B. sodium borate buffer, and C. sodium borate buffer with D-phenylalanine, in the UV region (400 to 200nm). Absorbance scale: 0.000 to 3.000.

Fig. 48 CONTD.

For further details see `results'.

Fig. 49 CONTD.

For further details see `methods'.

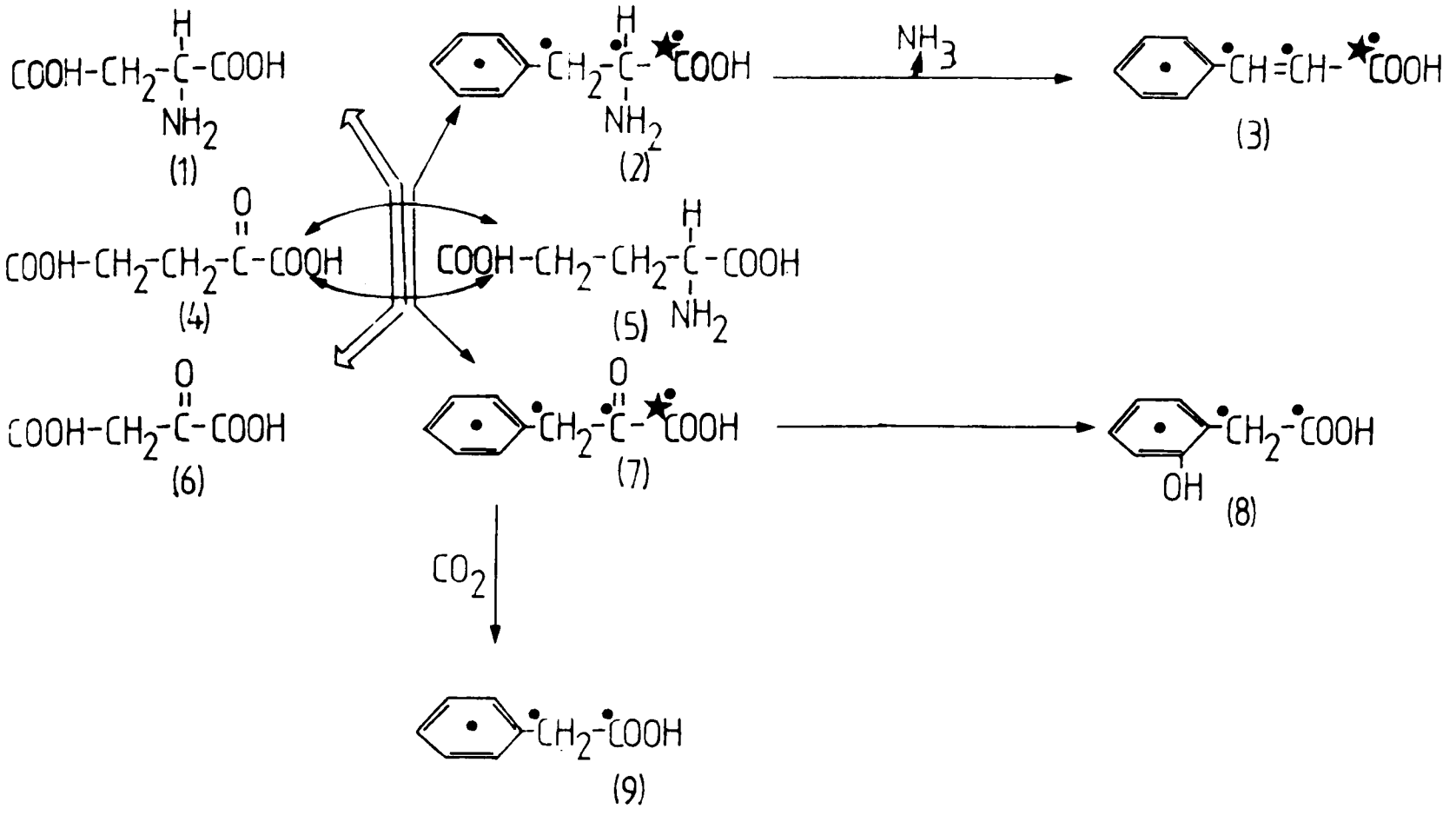

Fig. 48 Routes of L-U-^{14}C-phenylalanine (●), L-1-^{14}C-phenylalanine (★) during the isotopic assay for PAL activity and interaction of AAT (⟹) with PAT and PAL (⟶), where, (1) L-aspartic acid, (2) L-phenylalanine, (3) t-cinnamic acid (4) α-ketoglutaric acid, (5) L-glutamic acid, (6) oxaloacetic acid, (7) L-phenylpyruvic acid, (8) L-hydroxyphenylacetic acid and (9) phenylacetic acid. (continued on facing page)

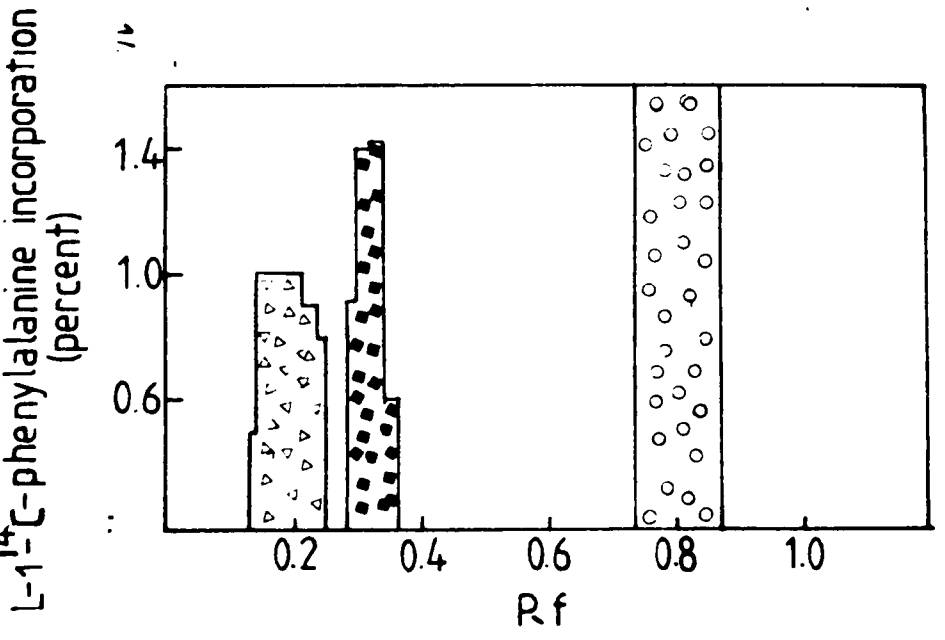

Fig. 49 Estimation of PAL activity in crude and partially purified homogenates : separation of L-^{14}C-phenylpyruvic acid (■ ■ ■) from 1-^{14}C-t-cinnamic acid (Δ Δ Δ Δ Δ) and L-phenylalanine (O O O O O) by thin-layer chromatography.

(continued on facing page)

From the results described above, we may conclude that the spectrophotometric and the L-U-^{14}C-phenylalanine isotopic methods of assaying PAL activity could not distinguish between phenylpyruvic acid, p-coumaric acid and t-cinnamic acid, and hence could not distinguish between PAT, TAL and PAL activities respectively.

5.3.5. It was therefore, likely that the use of L-1^{14}C-phenylalanine as a substrate for the isotopic assay of PAL would ensure the incorporation of ^{14}C-label in to t-cinnamic acid (the end product of PAL activity) and L-phenylpyruvic acid (the end product of PAT) only, as shown in Fig.48.

5.3.6. Hence, the L-1-^{14}C-phenylalanine isotopic assay for PAL could not distinguish between phenylpyruvic acid and t-cinnamic acid and hence between PAT and PAL activities respectively. However, the separate estimations of phenylpyruvic acid and t-cinnamic acid would enable the determination of PAT and PAL activities.

Phenylpyruvic acid and t-cinnamic acid could be separated by conventional methods such as (1) differential solubility, where t-cinnamic acid (unlike L-phenylpyruvic acid) is soluble in toluene and (2) thin-layer chromatography, where separate R_f values are obtained for t-cinnamic acid and L-phenylpyruvic acid. R_f values obtained for t-cinnamic acid, L-phenylpyruvic acid, L-phenylacetic acid and L-phenylalanine are 0.24, 0.34, 0.58 and 0.80 respectively (Fig. 49).

We could now estimate PAT and PAL activities.

5.3.7. However, PAL assayed by the above spectrophotometric and isotopic assays, had very low values of activity 0.005 to 0.12nmoles.min^{-1}, negligible values on the log scale, even at increased concentrations of L-phenylalanine (Fig. 50). These values could result from a loss of measurable t-cinnamic acid, or low levels of t-cinnamic acid production.

It was further found that low activities of PAL do not arise from loss of measurable t-cinnamic acid by its binding to protein in crude and partially purified homogenates (Table 4).

Table 4. Estimation of PAL activity in crude and partially purified homogenates: estimation of t-cinnamic acid bound to protein*:

Protein x 10^{-6} g	^{14}C-t-cinnamic acid recoverable K cpm	^{14}C-t-cinnamic acid bound to protein K cpm	percent
0.0	694.6	0	0
1.0	716.4	0	0
1.5	701.0	0	0
5.0	723.0	0	0
10.0	750.0	0	0
20.0	700.0	0	0

* Differing amounts of crude and partially purified homogenate protein were incubated for 0.5 hours with 10^{-4} M ^{14}C-t-cinnamic acid (specific activity 48.0 x 10^{3} Ci M^{-1}). The reaction was stopped with 20 x 10^{-6} ml 12M HCl, centrifuged in a microfuge and ^{14}C-t-cinnamic acid determined in the supernatant by procedures described for the PAL assay in `Methods´.

Fig. 50. CONTD

The values obtained for apparent PAL activity were determined by sum of values obtained for PAL ant PAT activities shown in the figure. PAL and PAT activities were estimated by procedures described in `methods'. Each spot represents the mean of 3 different experiments. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

Fig. 51. CONTD

Figures were approximated by the method of Spears et al. (1971). For further details see `methods'.

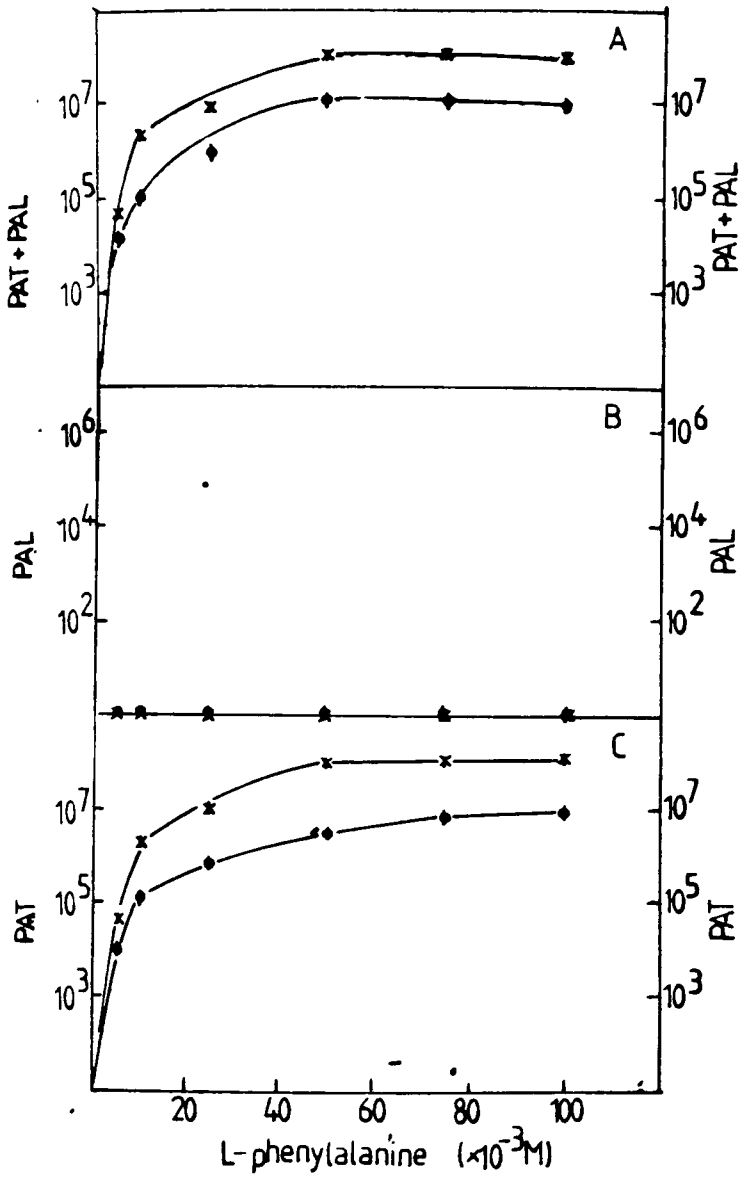

Fig. 50 Estimation of PAL + PAT activity (apparent PAL activity), B, PAL activity; and C, PAT activity in crude and partially purified homogenates: effect of different concentrations of L-phenylalanine in absence of L-aspartic acid.

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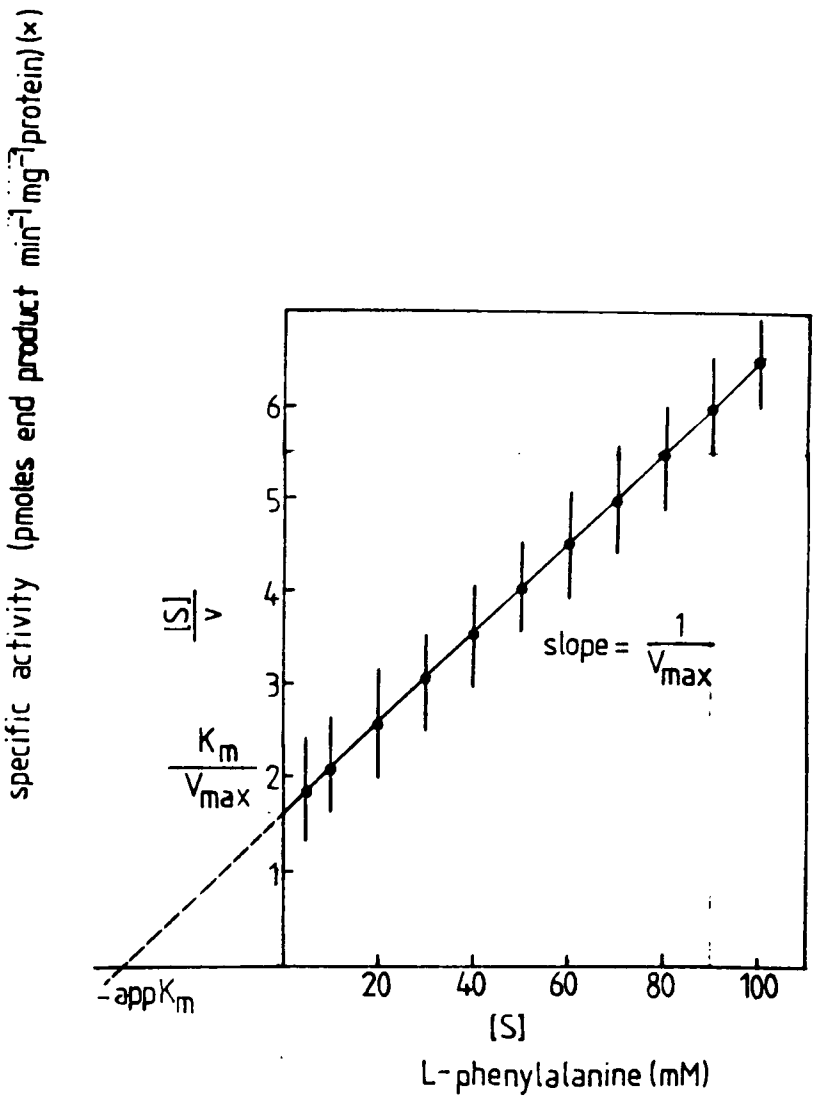

Fig. 24 Estimation of PAL activity in crude and partially purified homogenates: estimation of apparent K_m and V_{max} of PAL in absence of L-aspartic acid.

(continued on facing page)

It was possible that PAL had a low affinity for the substrate L-phenylalanine, responsible for the low values of activity obtained in the above assay. PAL was found to have an apparent K_m of 20×10^{-3}M (after approximation, by method of Spears et al., 1971), indicating an apparently low affinity for the substrate, L-phenylalanine in crude and partially purified homogenates. The apparent V_{max} of the enzyme under these conditions was much higher (12.5nmoles.min^{-1}) than the optimal functioning rate (0.005 to 0.12nmoles. min^{-1}) (Fig.51).

5.3.8. It was therefore possible that the apparently low affinity of PAL for the substrate L-phenylalanine was limited by the presence of another enzyme competing for the same substrate L-phenylalanine.

Relatively high concentrations of phenylpyruvic acid (the end product of PAT), as opposed to t-cinnamic acid concentrations (the end product of PAL) were estimated in the spectrophotometric and isotopic assays described above. These results suggested the occurrence of either an amino acid oxidase and/or an amino acid transferase in crude and partially purified homogenates. The experiment designed in Table 5 provided positive evidence in favor of the presence of an amino acid transferase, PAT.

The isotopic and spectrophotometric assays for PAL activity described above were not inhibited by D-phenylalanine.

Table 5. The presence of amino acid transferase activity in crude and partially purified homogenates:

Treatment*	activity**	recoverable activity percent
1. -step 1	0.185	13.82
2. + L-aspartic acid + α-ketoglutaric acid	1.339	100.00
3. + L-aspartic acid	0.000	0.00
4. + L-aspartic acid + α-ketoglutaric acid + H_2O_2	0.047	3.51
5. + L-aspartic acid + α-ketoglutaric acid + H_2O_2 + catalase***	1.401	100.00
6. + α-ketoglutaric acid + L-glutamic acid ****	0.019	1.36
7. + L-glutamic acid****	0.022	1.57

* step 1. The reaction mixture containing enzyme + 5×10^{-6} M L-ascorbic acid + 200×10^{-6} moles α-ketoglutaric acid + 20×10^{-6} moles pyridoxal phosphate in a final volume of 0.76ml, was incubated at 30°C for 0.5 hours.

step 2. To the reaction mixture was added 5×10^{-6} moles L-phenylalanine + 0.09×10^{-6} moles L-1-^{14}C-phenylalanine + 40×10^{-9} moles borate buffer (pH 8.8) and the volume made to 1.0ml.

The remaining steps are described in `methods'. Variation in estimation of activity = ±0.01

** $\times 10^{-6}$ M t-cinnamic acid.min^{-1}. 10^{-3} g protein.

*** 18,000 units

**** 60×10^{-3} M

Further, the occurrence of PAT in crude and partially purified homogenates was confirmed by use of a different assay from the spectrophotometric and isotopic assay described above - namely, the α-ketoglutarate-dehydrogenase-linked PAT assay in green and non-green tissues (Fig. 53).

5.3.9. The apparent K_m of PAT in crude and partially purified homogenates was found to be 0.03×10^{-3}M, indicating a much higher affinity for L-phenylalanine than that described for PAL above. This explained the low levels of PAL activity, non-linear with time and protein concentration obtained above. The maximal velocity of PAT (1.0nmoles min^{-1}) was higher than the optimal velocity of PAL (0.005 to 0.12nmoles min^{-1}) in the above conditions (Fig. 54).

The above results indicated the presence of 2 enzymes in crude and partially purified homogenates, PAT and PAL, with affinities for the same substrate L-phenylalanine.

5.3.10. It was apparent that PAT activity would have to be inhibited to facilitate estimation of PAL activity.

The following properties of PAT made the inhibition of PAT possible:

5.3.10.1. Concentrations of L-aspartic acid equal to or greater than L-phenylalanine are optimal for inhibition of PAT activity (Forest & Wightman, 1972).

Fig. 52 was removed during the revision of this thesis when it was realised that the conditions used for this preliminary experiment were not the standard ones and were not optimal for PAT. The values reported in Fig. 52 are therefore inappropriate. In place of Fig. 52 reference is made to Forest & Wightman (1972) who measured the aspartate inhibition of PAT under optimal conditions.

Fig. 53 contd.

For further details see 'Methods'.

Fig. 54. contd.

Values used for this calculation were from Fig. 50. Other values were fitted to these by drawing a regression line according to methods described by Campbell (1974).

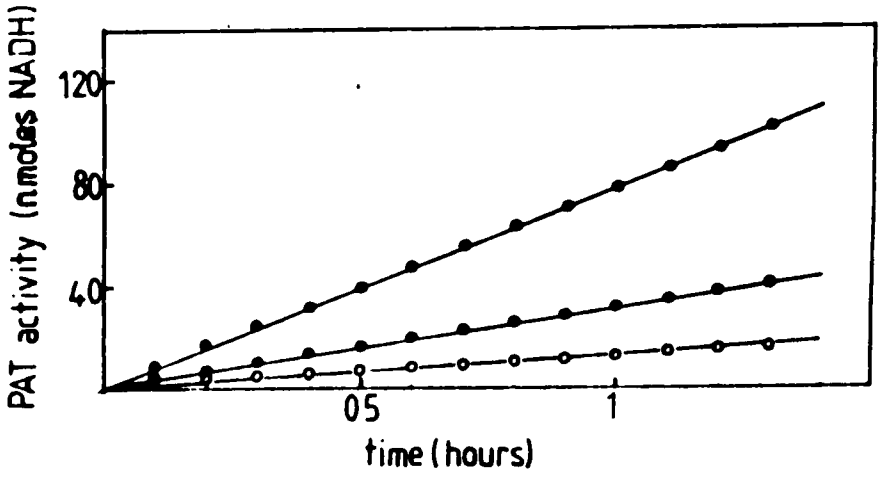

Fig. 53 Estimation of PAT activity in crude and partially purified homogenates: the α-ketoglutaric acid-dehydrogenase-linked PAT assay, where, 1 x protein (○); 2 x protein (◐) and (●) 4 x protein.

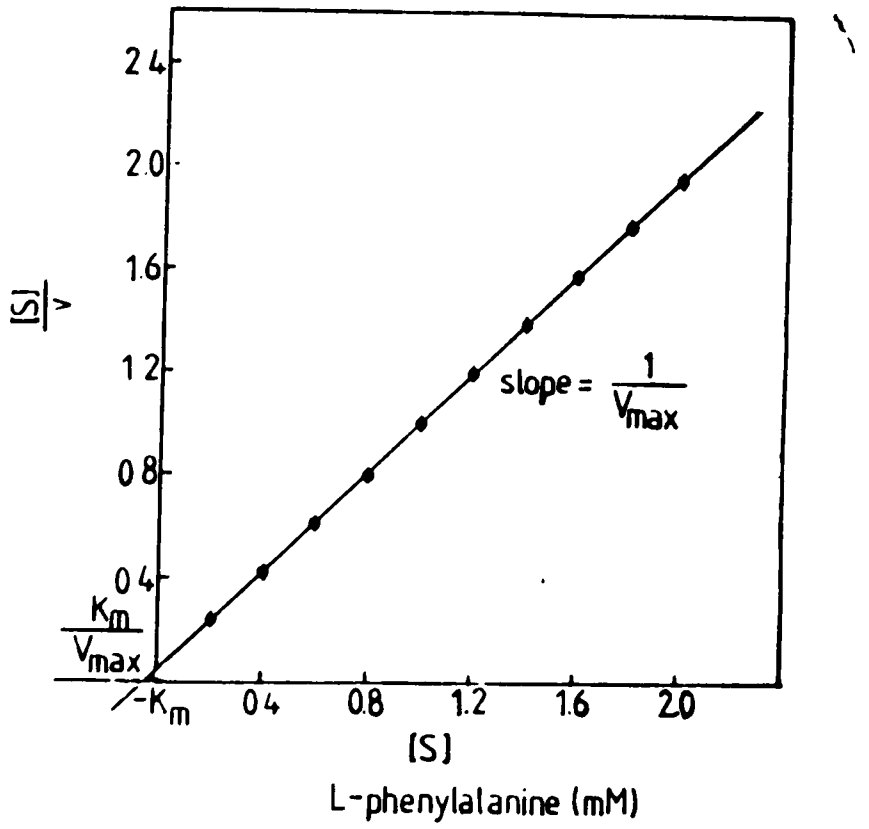

Fig. 53 Estimation of PAT activity in crude and partially purified homogenates : estimation of apparent K_m and V_{max} of PAT in absence of L-aspartic acid.

5.3.10.2. The activity of PAT increases with increasing concentrations of exogenous and endogenous ∝-ketoglutaric acid, in presence of L-aspartic acid (Fig. 55).

5.3.10.3. The activity of PAT is not influenced by concentrations of L-glutamic acid in presence of L-aspartic acid (Fig. 56).

5.3.10.4. The activity of PAT is inhibited by t-cinnamic acid (the end product of PAL activity) at relatively high concentrations, higher than that found in the cell (Fig. 57).

5.3.10.5. The pH optimum of PAT is 8.8 to 9.2 (similar to PAL).

5.3.10.6. The temperature optimum of PAT is 30°C (similar to PAL).

The following properties of PAL make the use of inhibition of PAT by L-aspartic acid possible:

5.3.10.1. PAL activity is not affected by increasing concentrations of L-aspartic acid (Fig. 52, Fig. 55, Fig. 56).

5.3.10.2. PAL activity is not affected by increasing concentrations of ∝-ketoglutaric acid in presence of L-aspartic acid (Fig. 55).

5.3.10.3. PAL activity is not affected by increasing concentrations of L-glutamic acid in presence of L-aspartic acid (Fig. 55).

Fig 55. CONTD.

The values obtained for apparent PAL activity were determined by sum of values obtained for PAL ant PAT activities shown in the figure. PAL and PAT activities were estimated by procedures described in 'methods'. Each spot represents the mean of 3 ± different experiments. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

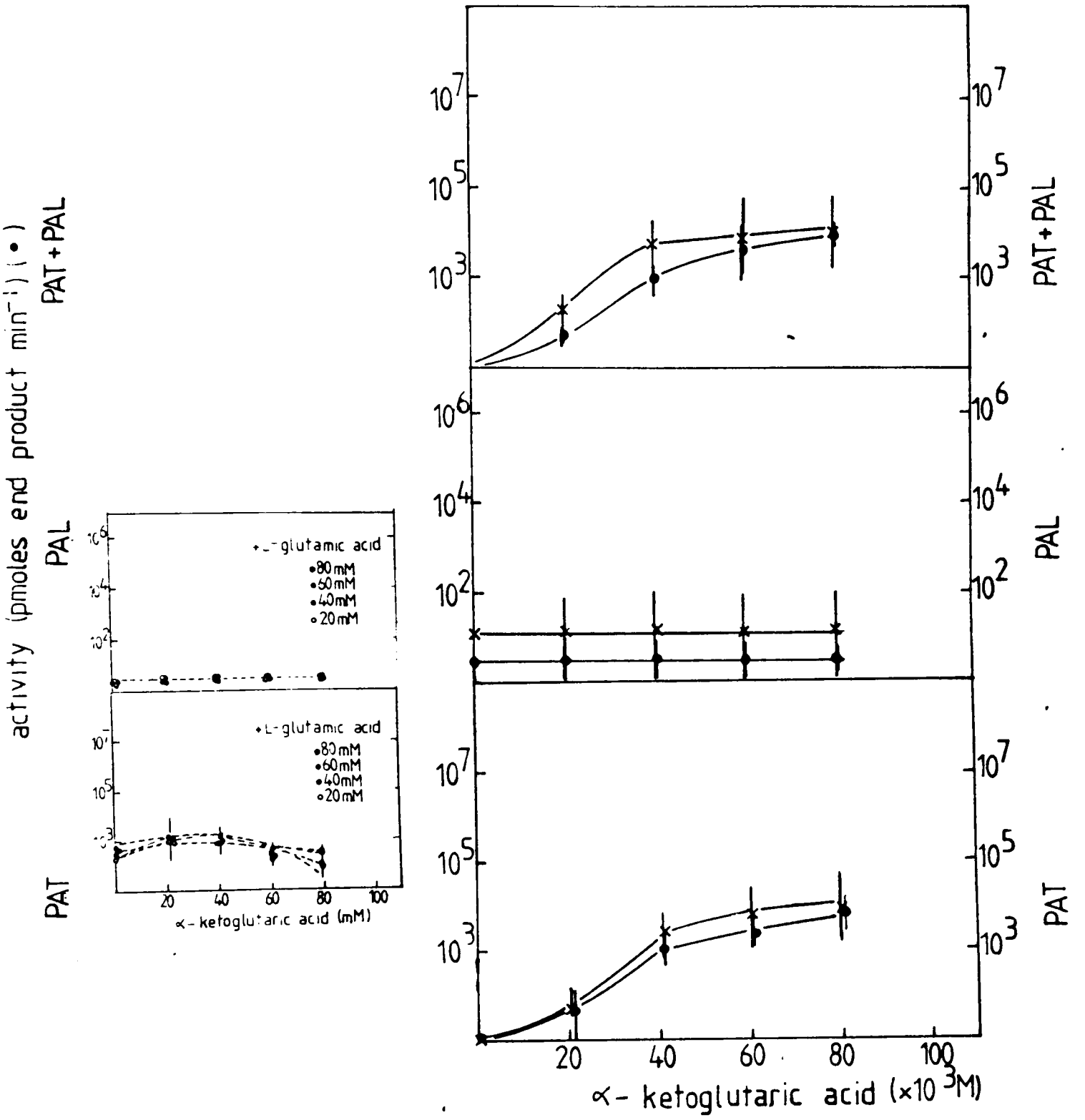

Fig. **49**. Estimation of A, PAL activity and B, PAT activity in crude and partially purified homogenates: effect of different concentrations of α-ketoglutaric acid and L-glutamic acid (insert) in presence of L-aspartic acid.

(continued on facing page)

Fig. 56 CONTD.

The values obtained for apparent PAL activity were determined by sum of values obtained for PAL ant PAT activities shown in the figure. PAL and PAT activities were estimated by procedures described in 'methods'. Each spot represents the mean of 3 different experiments. The bars indicate the maximum and minimum values obtained. The absence of a bar indicates that the variation is contained within each spot.

Fig 57. CONTD.

These values are obtained from an extension of those obtained at concentrations of L-phenylalanine used in Fig. 59. Other values were fitted to these by drawing a regression line according to methods described by Campbell (1974).

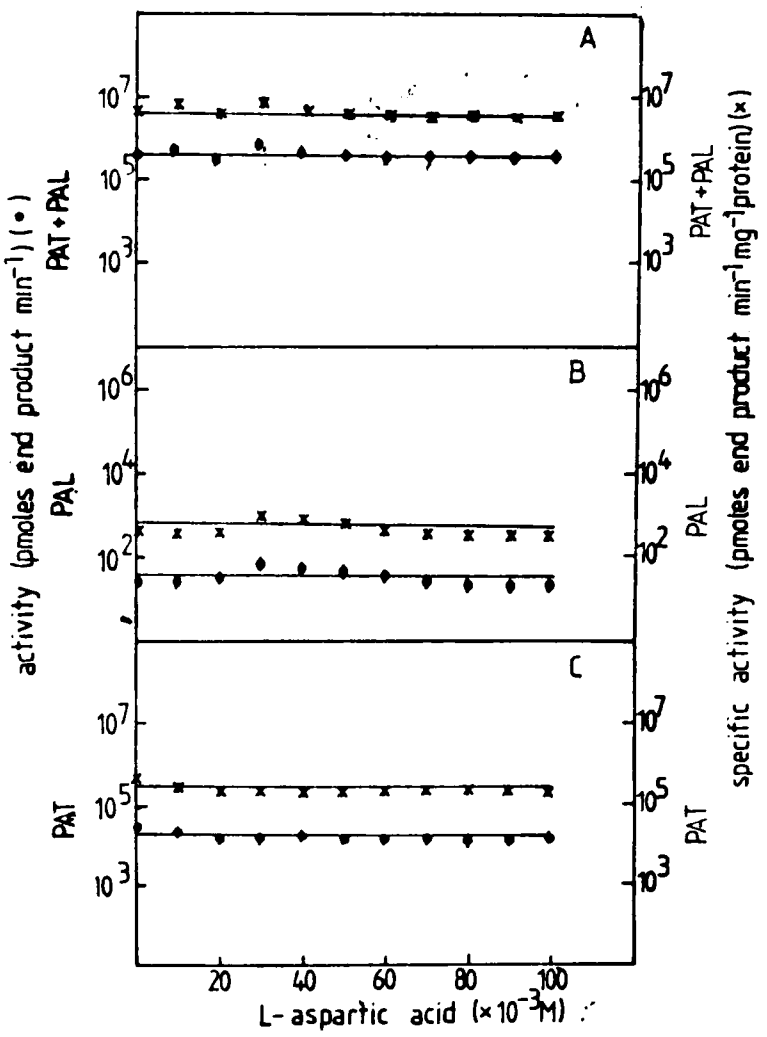

Fig. 56.

Estimation of A, PAL + PAT activity (apparent PAL activity); B, PAL activity; and C, PAT activity in crude and partially purified homogenates: effect of different concentrations of L-aspartatic acid when PAL is functioning optimally. (continued on facing page)

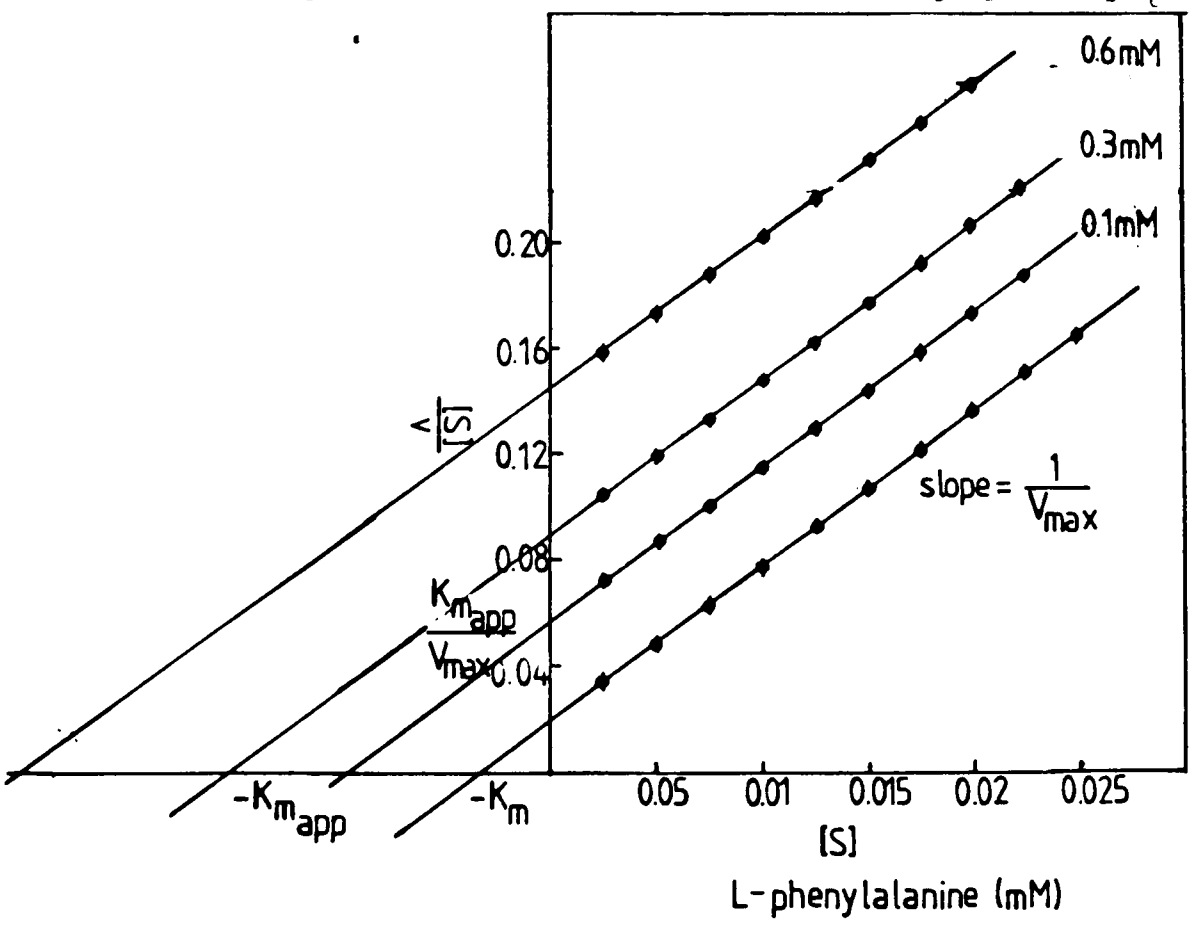

Fig. 57.

Estimation of PAT activity in crude and partially purified homogenates: inhibition by t-cinnamic acid. (continued on facing page)

Fig. 58 CONTD.

These values are obtained from an extension of those obtained at concentrations of L-phenylalanine used in Fig. 59. Other values were fitted to these by drawing a regression line according to methods described by Campbell (1974).

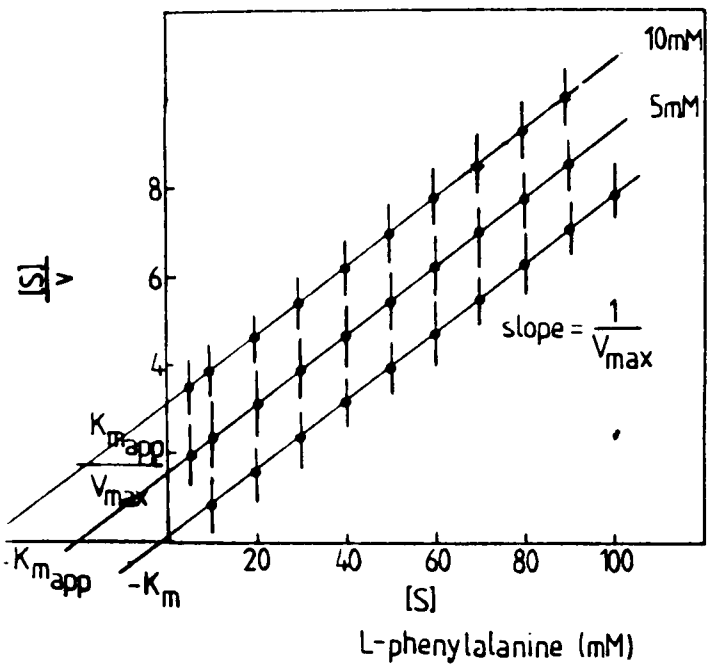

Fig. 57. Estimation of PAL activity in crude and partially purified homogenates: inhibition by L-phenylpyruvic acid.

(continued on facing page)

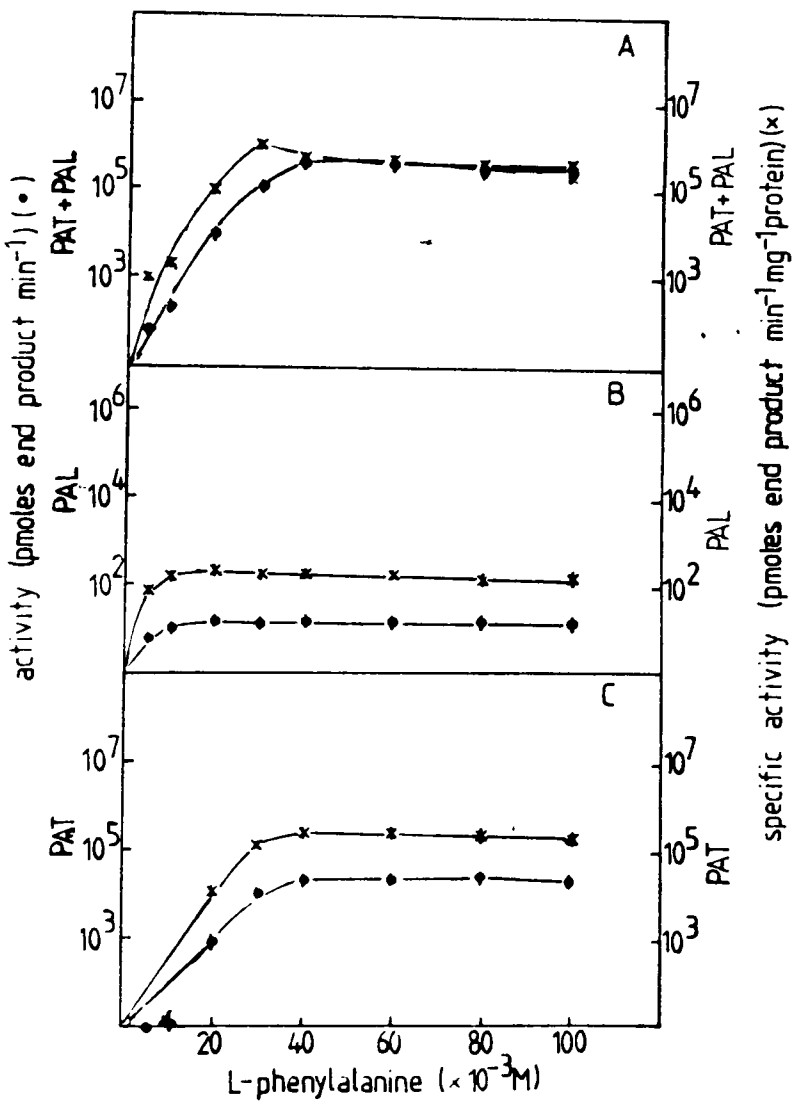

Fig. 57. Estimation of A, PAL + PAT activity (apparent PAL activity); B, PAL activity; and C, PAT activity in crude and partially purified homogenates: effect of different concentrations of L-phenylalanine in presence of L-aspartic acid. (for further details see Fig 50).

5.3.10.4. PAL is inhibited by L-phenylpyruvic acid (the end product of PAT activity), at relatively high concentrations, higher than that found in the cell (Fig. 58), as found by Hanson & Havir, (1981).

5.3.10.5. PAL activity has the same pH optimum as PAT, 8.8 to 9.2.

5.3.10.6. PAL activity has the same temperature optimum as PAT, 30°C.

5.3.11. The addition of L-aspartic acid to the asay and optimisation of the assay to the procedure described in `methods' resulted in a linear assay for PAL with time and protein concentration in crude and partially purified homogenates:

The L-1-^{14}C-phenylalanine isotopic assay for PAL gave identifiable values for t-cinnamic acid production on the log scale with varying concentrations of L-phenylalanine (Fig. 59).

This assay was linear with time for periods of up to 2 hours. At later time points, there occurred an increase in L-phenylpyruvic acid production for the following reasons: (1) release of L-aspartic acid which is probably related to the loss of L-aspartate in side reactions (2) non-enzymatic formation of L-phenylpyruvate in presence of L-aspartic acid and equimolar concentrations of pyridoxal phosphate and ∝-ketoglutaric acid; or (3) bacterial contamination (Fig. 60 A and B).

The isotopic assay for PAL was linear with protein concentrations having activities of 0.12nmoles to 0.8nmoles min^{-1} (Fig. 60C).

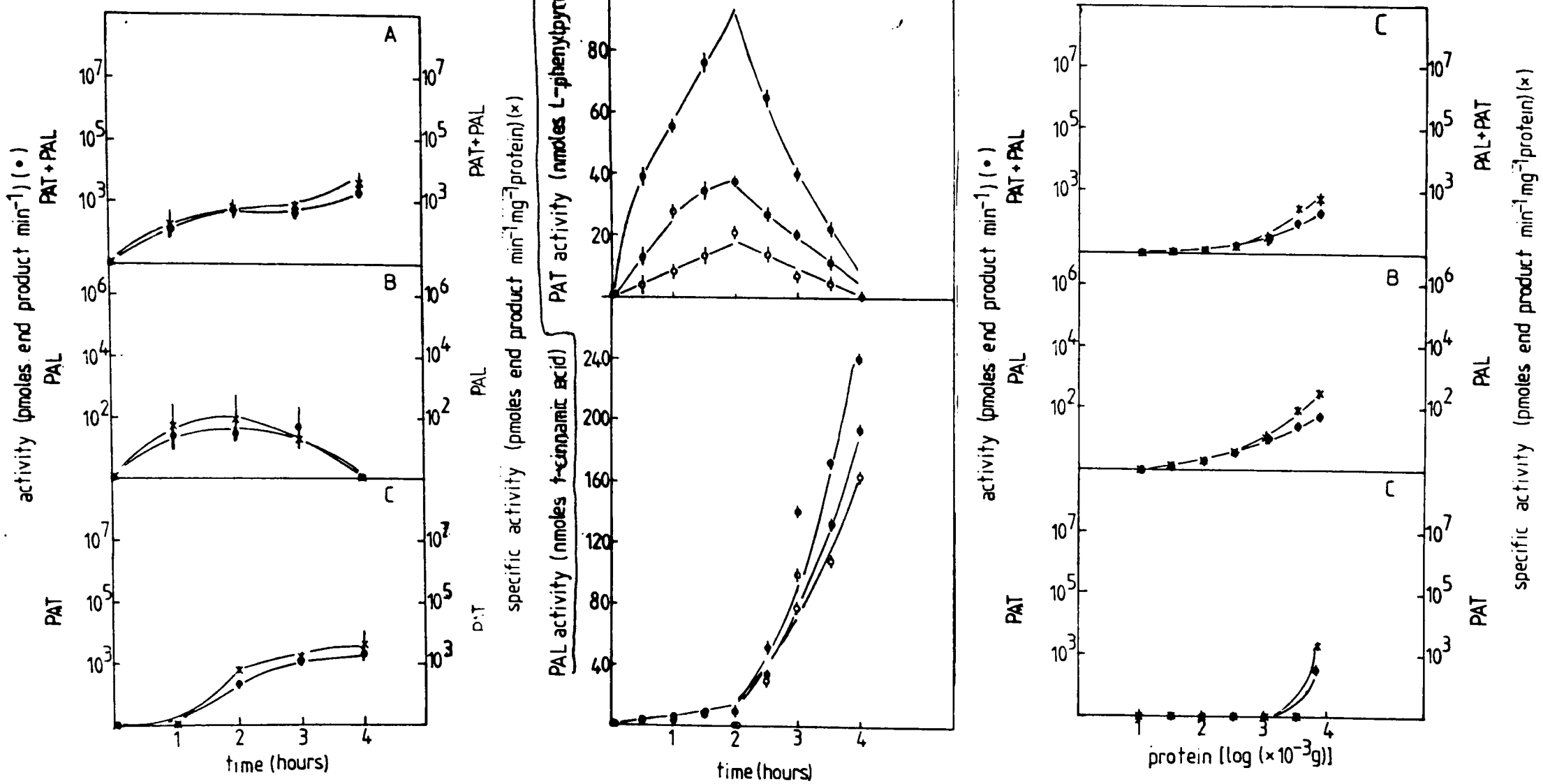

Fig. 60. Estimation of A, PAT + PAL activity (apparent PAL activity); B, PAL activity and C, PAT activity: linearity of PAL activity with 1, time (log scale); 2, time (linear scale) and 3, protein, where (○), 1 x protein; (◐), 3 x protein; (●), 5 x protein.

5.3.12. The relatively high increase in affinity of PAL for the substrate L-phenylalanine (as compared to PAT) was responsibile for the linearity in the determination of PAL activities by the L-1-^{14}C-phenylalanine isotopic assay described above.

The K_m of PAL in presence of L-aspartic acid was 1.25 x 10^{-3}M, indicating an increase in affinity of PAL for the substrate L-phenylalanine. The maximal activity of PAL attainable under these conditions was found to be reduced to 0.8nmoles min^{-1}, values nearer the optimal velocity (0.12nmoles to 0.8nmoles min^{-1}) (Fig. 61).

The K_m of PAT in presence of L-aspartic acid was 0.05 x 10^{-3}M, indicating a decrease in affinity for the substrate L-phenylalanine. The maximal attainable velocity was 2.5nmoles min^{-1}, higher than optimal velocities under these conditions (Fig. 62).

Further, PAL activity was inhibited by D-phenylalanine to 56% if added in equimolar concentrations with L-phenylalanine under these conditions.

5.4. DISCUSSION:

In order to determine the catalytic activity of PAL the concentrations of t-cinnamic acid produced have been measured by various methods, the most common method being spectrophotometric (Hanson & Havir, 1981). The enzyme (crude or purified homogenates) is incubated in presence of L-phenylalanine and sodium borate buffer at 30 to 40°C for 12 to 22 hours and concentrations of end product (t-cinnamic acid) measured by absorption at 268 to 290nm. (Koukol & Conn, 1961; Lamb et al. (1971); Hanson, 1981).

Fig. 61 CONTD

Values used for this calculation were from Fig. 59. Other values were fitted to these by drawing a regression line according to methods described Campbell (1974).

Fig. 62 CONTD.

Values used for this calculation were from Fig. 59 . Other values were fitted to these by drawing a regression line according to methods described Campbell (1974).

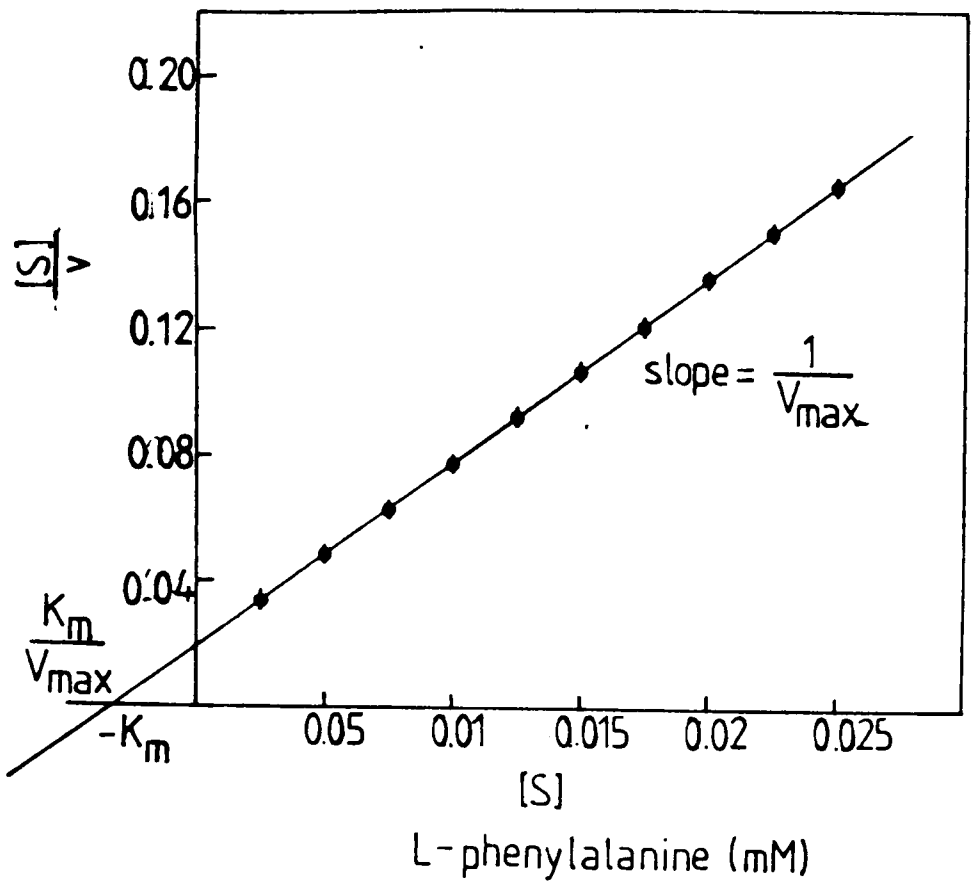

Fig. 61. Estimation of PAL activity in crude and partially purified homogenates: estimation of K_m and V_{max} of PAL in presence of L-aspartic acid. (continued on facing page)

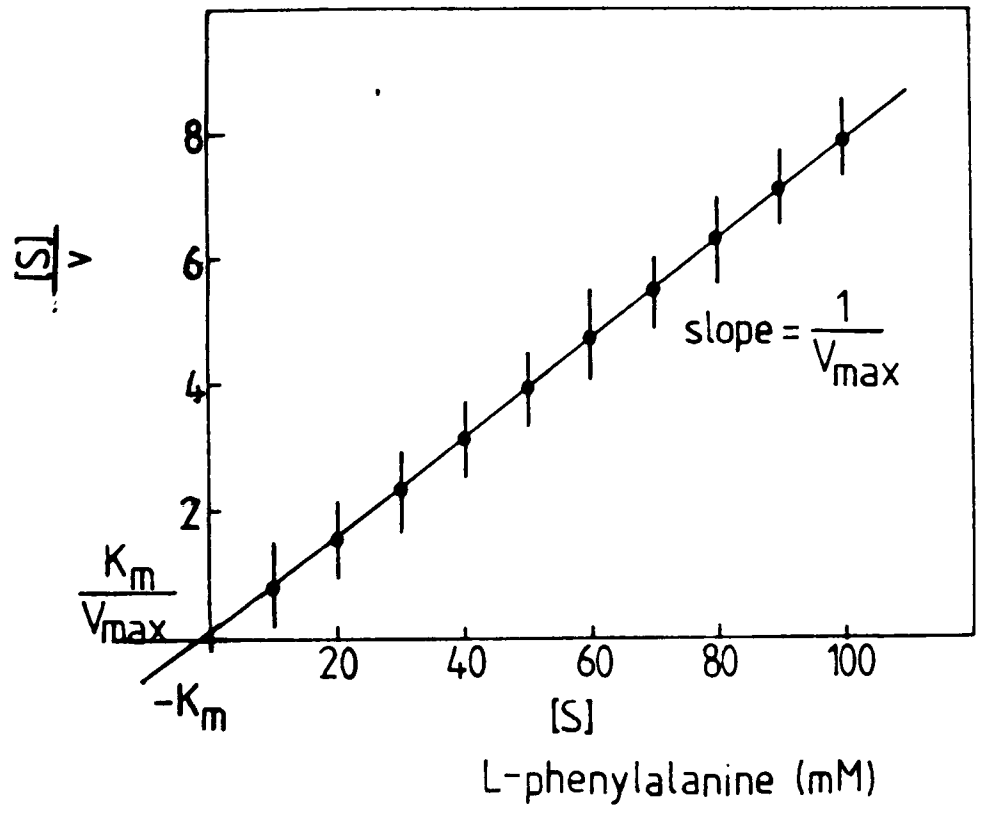

Fig. 62. Estimation of PAT activity in crude and partially purified homogenates: estimation of K_m and V_{max} of PAT in presence of L-aspartic acid. (continued on facing page)

The assay has been optimised for maximal activity by presence of (i) light for endogenous amino acid metabolism, (ii) L-phenylalanine to near saturation concentrations and (iii) D-phenylalanine to substitute L-phenylalanine in control samples (Zucker, 1965, 1968; Camm & Towers, 1969; Lamb et al., 1971; Klein-Eude et al., 1974; Nari et al., 1974; Faye, 1975; Fourcroy, 1980).

The use of this assay with optimal conditions described above yielded PAL activities that were nonlinear with time and protein concentration. The non-linearity in time and high protein concentration of the above assay, has also been found by several other workers (

Wilkinson, 1978).

To account for this non-linearity, the following reasons are described in the literature: (i) microbial contamination (Stafford & Lewis, 1977), (ii) absorption of several aromatic compounds between 200 and 400nm, for example, p-coumaric acid, the end-product of TAL activity (estimated by the absorption of p-coumaric acid at 290-320nm), (Camm & Towers, 1969 ; see Hanson & Havir, 1981) and L-phenylpyruvic acid, the end-product of PAT activity (estimated by the absorption of L-phenylpyruvate at 290nm)(Erez, 1973;

Stafford & Lewis, 1979)

and the end product of amino acid oxidase activity (Towers & Rao, 1972), (iii) phenylacetate, the end product of decarboxylase activity formed from phenylpyruvic acid , (

Stafford & Lewis, 1977,1979), (iv) unreliable estimations of protein

in green homogenates
(Eze & Dumbroff, 1982), and (v) the formation of keto-enol-borate complexes with phenylpyruvic acid absorbing at 298nm (Erez, 1973).

Experiments designed to test these possibilities indicated the presence of PAT activity in Phaseolus vulgaris. The confirmation of high PAT activities was carried out by estimation of L-phenylpyruvic acid, a more commonly used method of assaying PAT activity (Feliss & Martinez, 1970) and not L-phenylalanine (Forest & Wightman, 1972).

These results are further supported by the presence of endogenous substrates to PAT (such as keto acids) together with the widespread occurrence of PAT especially in tissues of Phaseolus vulgaris, the similar pH optimum of PAT and PAL, and the ability of PAT to use D-phenylalanine as substrate (Abraham et al., 1973; Ogawa & Fukuda, 1973; Forest & Wightman, 1972; Wightman & Forest, 1978; Stafford & Lewis, 1979).

Several methods have been used to assay PAL, with possible removal of PAT and prevention of the appearance of L-phenylpyruvic acid in extracts prior to assaying PAL. Among the methods are gel-filtration, ion-exchange chromatography, acetone and ammonium sulphate precipitation, and the addition of BSA (Koukol & Conn, 1961; Zucker, 1965; Creasy, 1976; Berlin & Widholm, 1977; Stafford & Lewis, 1979). The inhibition of PAT by L-aspartic acid enabled the measurement of PAL ativities in crude and partially purified homogenates of bean leaves.

Attempts have been made to devise a more rapid method for assaying PAL activities. The method described in the literature has been the isotopic assay for PAL using uniformly labelled L-phenylalanine as substrate and measuring the incorporation of label into t-cinnamate (Koukol & Conn, 1961; Legrand et al., 1976; Wilkinson, 1978; Jones & Northcote, 1984). However, this method like the spectrophotometric yielded results non-linear with time and protein concentration because other aromatic compounds also incorporate labelled L-phenylalanine into their structure as reported by other workers. These compounds have been found to be L-phenylpyruvic acid (30 to 50% incorporation of label) and L-phenylacetic acid (5 to 50% incorporation of label) (Stafford & Lewis, 1977; Jones & Northcote, 1984).

The presence of higher affinities of PAT for L-phenylalanine obtained in the present study, the presence of differing concentrations of PAT and PAL competing for the same substrate L-phenylalanine, as suggested by the successful use of the Woolf plot in the present study, may explain several features unique to PAL in Phaseolus vulgaris and several other systems described in the literature:

(i) the variation in rates of PAL ativities obtained, ranging from a very rapid rate as assessed by continuous methods of measurements of t-cinnamic acid formed to a very slow rate as assessed by a stopped time measure of t-cinnamic acid formed (Erez, 1973; Gilbert & Jack, 1981; Mayrisch et al., 1981);

(ii) the presence of decreasing levels of PAL activity with increasing

concentrations of L-phenylalanine, or negative cooperativity and the conversion of negative to positive cooperativity on gel filtration and hydroxypatite chromatography (Havir & Hanson, 1968 a, b; Nari et al, 1974; Hanson, 1981; Hanson & Havir, 1981);

(iii) the presence of differing Kms of PAL with differing degrees of purity of enzyme preparations, ranging from a high (>0.3mM) to a low (<0.3mM Km) (Iredale & Smith, 1974, Hanson & Havir, 1981; Jones, 1984; Bolwell et al., 1986 in press) and further, corrected estimates of affinities for L-phenylalanine (1.2mM) resembling results obtained for some preparations of PAL (1.7mM) (Koukol & Conn, 1961);

and (iv) the presence of different isoenzymes of PAL, differing in affinities for L-phenylalanine and with different degrees of inhibition by phenolics (Havir & Hanson, 1968b; Boudet et al., 1971; Faye, 1975).

CHAPTER 6

INDUCTION OF HYPERSENSITIVE NECROSIS AND PHYTOALEXIN SYNTHESIS IN THE PHASEOLUS VULGARIS L. - COLLETOTRICHUM LINDEMUTHIANUM L. INTERACTION : PURIFICATION OF PHENYLALANINE AMMONIA-LYASE

6.1. INTRODUCTION:

This chapter describes the purification of PAL from Phaseolus vulgaris, in order to further understand its mechanism of regulation. It describes the attempts that were made to separate PAL from PAT, the attempts that were made to purify PAL by affinity chromatography, the attempts that were made to stabilise pure PAL preparations and the attempts that were made to map the polypeptides of PAL in the active and inactive state.

6.2 MATERIALS AND METHODS:

6.2.1. Growth of plant material:

Seeds of Phaseolus vulgaris L. var. Prince were sown in autoclaved graded horticultural perlite and maintained under greenhouse

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; MSH, 2-mercaptoethanol; PMSF, p-methylsulphonyl fluoride; PVP, polyvinylpyrrolidone; SDS, lauryl sulphate (sodium salt); T, percentage total monomer i.e. grammes acrylamide plus bisacrylamide.

conditions for 1 week. Seedlings were transferred to pots containing Levington soilless potting compost. Plants were grown in a growth cabinet at 23 to 25°C, 65 to 75% relative humidity, under continuous light for a period of 34 to 720 hours. Leaves were harvested in liquid nitrogen (-198.5°C) and stored at -70°C.

6.2.1.2. Partial purification of PAL:

Leaf material was powdered in a mortar and pestle prefrozen at -70°C. The powder was ground in liquid N_2 (-198.5°C). 1/10(w/v) insoluble PVP was added in chilled extraction buffer [0.1M $Na_2B_4O_7$-HCl buffer, pH 8.8, 5mM L-ascorbic acid and 1mM PMSF] at a concentration of 4ml.gFW^{-1} leaf material. The extract was thawed to 4°C and filtered through 3 layers of cheesecloth followed by centrifugation at 20,000 x g for 10 minutes at 4°C in a Sorvall RC5C centrifuge. The supernatant was subjected to precipitation with $(NH_4)_2SO_4$ in presence of of 0.04% (w/v) Na_2EDTA. A protein concentration of 0.5mg.ml^{-1} was essential for optimal protein precipitation. The precipitate formed from 30 to 50%$(NH_4)_2SO_4$ saturation was suspended in a minimal amount of 0.1M $Na_2B_4O_7$-HCl buffer, pH 8.8 at 4°C and rapidly desalted by centrifugation at 625 to 1875 x g through a 5 x 1cm Sephadex G-25 column in a swinging-bucket centrifuge. The extract was then subjected to molecular gel exclusion on a Sephacryl S-200 column, 3.75 x 30cm in dimension at a flow rate of 1ml.min^{-1}. Fractions were assayed for PAL activity as follows: A 1.0ml reaction mixture containing enzyme, 10mM L-phenylalanine in extraction buffer [0.1M $Na_2B_4O_7$-HCl buffer, pH 8.8] and 5mM L-ascorbic acid] was incubated at 25 to 28°C for 30 to 120

minutes. Absorption was read at 290nm in a Perkin-Elmer 551S UV/VIS spectrophotometer against an identical mixture save that D-phenylalanine was substituted for L-phenylalanine. Fractions rich in PAL activity were pooled together, made to 0.02M sodium borate, pH 8.8 by dilution with distilled water at 18 to 25^{o}C and the pH adjusted to 6.0 with dilute acetic acid. Protein was adjusted to approximately 0.1mg.ml^{-1} for efficient binding.

6.2.1.3. Purification of PAL by affinity chromatography:

6.2.1.3.1. Preparation of adsorbents: Preparation of succinyl-aminoethyl-L-phenylalanine/pyridoxamine phosphate-Sepharose 4B was by CNBr-activation of Sepharose 4B by the method of Cuatracasas (1970), and addition of a succinyl-aminoethyl side arm by the method of Thompson (1974). Sepharose 4B-200 was washed in several volumes of 0.1M NaCl and distilled water in a glass-sintered funnel over gentle vacuum. `X´ ml distilled water was added to `X´ml settled gel with a few chunks of ice. 250mg.ml^{-1} CNBr was added to the slurry and the temperature maintained near 20^{o}C by addition of ice. The pH was immediately titrated to a constant pH of 11.0 with 4N NaOH. A large quantity of ice was added and the gel filtered rapidly, followed by a wash with 20 volumes of 0.1M $NaHCO_3$ at 4^{o}C. 0.3M ethylene diamine in 0.2M $NaHCO_3$, pH 9.8 was immediately added till an increase in volume of 15% over `X´. The whole mixture was rotated on an end to end shaker at 18 to 25^{o}C for 24 hours. The gel was washed extensively with distilled water and suspended in an equal volume (of distilled water containing 2moles succinic anhydride.ml^{-1} slurry at 4^{o}C. The pH was

immediately titrated to 6.0 with 5N NaOH and the gel rotated for 5 to 12 hours on an end to end shaker at 18 to 25°C. The gel was washed extensively with distilled water at 18 to 25°C and mixed rapidly with 2 x `X´ml distilled water containing 0.5mg L-phenylalanine.ml^{-1} packed gel or 0.01mg pyridoxamine phosphate.ml^{-1} packed gel. The pH was adjusted to pH 4.7. 50mg water soluble 1- ethyl-3-(3-dimethylamino propyl) carbodiimide. ml^{-1} packed gel was rapidly added in 0.15ml distilled water.ml^{-1} packed gel. The slurry was held at 18 to 25°C, pH 4.7 for 5 to 12 hours. The gel was washed with 8 litres 0.1M NaCl over a 6 hour period on a glass-sintered funnel under gentle vacuum, followed by extensive washes with 1% (w/v) BSA and distilled water. The adsorbent was stored with 0.02% merthiolate at 4°C. Coupling efficiencies were estimated by addition of L-phenylalanine-1-^{14}C at a specific activity of 57 $mCi.mmole^{-1}$ [2.11 $GBq.mmol^{-1}$] during the coupling procedure.

6.2.1.3.2. Affinity chromatography of partially purified PAL:

15ml packed gel volume of affinity-adsorbent was washed with 0.1M $Na_2B_4O_7$-HCl, pH 8.8, and equilibrated 12 hours in 0.02M $Na_2B_4O_7$-HOAc buffer, pH 6.0. Partially purified PAL was added to the affinity-adsorbent and the mixture left to incubate 12 hours to ensure binding of enzyme to L-phenylalanine. In initial studies binding was carried out on a column (80 x 2.2cm) to facilitate differential elution of PAL and PAT. Batch adsorption and elution were used in later studies. The eluate was removed by filtration using Whatman No. 1 filter paper. The affinity-adsorbent was washed with 0.02M

$Na_2B_4O_7$-HOAc buffer, pH 6.0. PAL was eluted with 0.1M $Na_2B_4O_7$-NH_4OH buffer pH 9.3. Elution was performed for 6 to 12 hours at 4°C.

6.2.1.3.3. Purification of affinity chromatographed extract:

The eluate obtained by affinity chromatography was extensively dialysed versus 0.02M $Na_2B_4O_7$-HOAc buffer, pH 6.0 at 4°C. The pH was adjusted to 6.0 at 15 to 20°C using dilute acetic acid. 15 ml packed gel volume affinity-adsorbent was equilibrated 12 hours with 0.02M $Na_2B_4O_7$-HOAc buffer, pH 6.0. Enzyme extract was added to the affinity-adsorbent, and the mixture left to incubate 6 to 12 hours to ensure binding of enzyme to L-phenylalanine. The eluate was removed by filtration using Whatman No. 1 filter paper. The affinity-adsorbent was washed with 0.02M $Na_2B_4O_7$-HOAc buffer, pH 6.0 to remove all unbound protein. PAL was eluted with elution buffer (0.02M $Na_2B_4O_7$-HOAc buffer, pH 6.0 and 5 mM L-phenylalanine). Elution was performed for 6 hours at 20 to 22°C. PAL was concentrated under N_2 in an ultrafiltration cell (model 8050), using a membrane with an exclusion limit of MW 10,000.

6.2.1.4. Preparation of ligand-free PAL:

PAL isolated by the above procedure (in the ligand-bound form) was subjected to extensive dialysis at 4°C versus 0.1M $Na_2B_4O_7$-HCl, pH 8.8 to remove ligand. Removal of ligand was assessed by addition of L-phenylalanine-1-^{14}C at specific activity of 57mCi.mmol^{-1} [2.11 GBq.mmol^{-1}]. Aliquots of sample were added to scintillation cocktail

[0.7%(w/v) butyl-PBD, 8% (w/v) naphthalene, 60% (v/v) toluene and 40% (v/v) 2-methoxy-ethanol] and counted at $4^{o}C$ in an LKB-1210 liquid scintillation counter.

6.2.1.5. Analysis of purity of PAL:

PAL purified by the above procedure was subjected to native gel electrophoresis in the anodic and cathodic direction.

6.2.1.5.1. The anodic gel:

The anodic gel was prepared according to the procedures of Davis, (1964). The resolving gel was 5,7.5 or 10%T. The stacking gel was 2.5%T. The sample was prepared by adding 1:1(v/v) glycerol to 1 to 2 $mg.ml^{-1}$ protein in 0.1M $Na_2B_4O_7$-HCl buffer, pH 8.8 or 0.02M $Na_2B_4O_7$-HOAc buffer, pH 6.0 with 0.1% (v/v) 1% (w/v) bromophenol blue. Electrophoresis was carried out once before loading the sample. The electrophoresis buffer contained 4.95mM Tris-base and 38.36mM glycine, pH 8.3. Electrophoresis was conducted at $18^{o}C$ under a constant current setting of 7mA for 12 hours or 35mA for 4.5 hours.

6.2.1.5.2. The cathodic gel:

The cathodic gel was prepared by the method of Reisfield *et al.* (1962). The resolving gel was 7.5%T. The stacking gel was 5%T. The sample was prepared by adding 1:1(v/v) glycerol to 1 to 2 $mg.ml^{-1}$ protein in 0.1M $Na_2B_4O_7$-HCl buffer, pH 8.8 or 0.02M $Na_2B_4O_7$-acetic acid buffer,

pH 6.0 and 5.0µl 5%(w/v) basic fuchsin solution. Electrophoresis was performed once prior to loading the sample. The electrophoresis buffer contained 350mM ∝-alanine and 133mM HOAc, pH 4.5. Electrophoresis was conducted at 18°C at constant current setting of 7mA for 12 hours or 35mA for 4.5 hours in the cathodic direction.

A preparation of PAL purified from Rhodotorula glutinis was run alongside PAL prepared by the above procedure in the above systems for comparison.

6.2.1.5.3. Determination of PAT activity:

PAL preparations were stained for PAT activity according to the method of Valeriote et al. (1969) using L-phenylalanine as substrate. The gel was incubated at 30°C in the dark for 2 to 12 hours in the following reaction mixture: 1ml 20mM pyridoxyl-5´-phosphate, 2ml 0.5M ∝-ketoglutarate, 10ml p-iodonitrotetrazolium violet, 2.5ml 0.4mg.ml^{-1} phenazine methosulphate and 35ml 20mM L-phenylalanine in 0.02M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.6 made to 100ml.

6.2.1.6. Analysis of denatured PAL:

Samples were denatured in the presence of SDS, guanidine-hydrochloride and urea and subjected to SDS-polyacrylamide gel electrophoresis.

6.2.1.6.1. Denaturation in SDS:

Denaturation in SDS was by dissolving the protein in sample buffer [0.1875M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, +/- 15% (w/v) MSH, and 0.1% (v/v) 1% (w/v) bromophenol blue, Laemmli, 1970]. Samples were heated in a boiling water bath for 2 minutes and cooled to room temperature prior to loading on the gel.

6.2.1.6.2. Denaturation in guanidine-hydrochloride:

Denaturation in guanidine-hydrochloride was by adding 0.1M Tris-HCl ($100^{o}C$) to protein and immediately transferring the sample to a boiling water bath. 15µl MSH was added to the tube and capped. The samples were alkylated with 0.25 to 1.0ml iodoacetate solution [260mg iodoacetate.ml^{-1} 1M NaOH]. The pH was titrated to between pH 8 and pH 9 with 2M NaOH, and then to pH 10.5 with iodoacetate solution. Samples were incubated at 18 to $25^{o}C$ for 10 minutes. 30 to 50µl MSH was added to each sample and the samples titrated to pH 7.0. Samples were dialysed against urea buffer [0.1M Tris-HCl, pH 8.0 and 9M urea], followed by extensive dialysis against SDS-buffer [0.01M sodium phosphate buffer, pH 7.0 and 0.1% (w/v) SDS], and extensive dialysis against sample buffer [0.1875M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (v/v) glycerol, +/- 15% (w/v) MSH] to remove SDS and urea. Samples were then loaded on the gel.

6.2.1.6.3. Denaturation in urea:

Denaturation in urea was by addition of sample buffer [4M urea, 1%(w/v) SDS +/- 1% (v/v) MSH] at a concentration of 0.5$ml.mg^{-1}$

protein. Samples were denatured at 18 to 25°C. To ensure completion of this carbamylation reaction,samples were treated for a minimum period of 1 hour and immediately loaded on the gel to avoid ionisation of urea. Cyanate ions were removed from urea when necessary, by passage of urea through a AG50 1-X8 (D)(20 to 50 mesh) mixed bed-ion exchange resin. 30ml of 7M urea was deionized on a 40 x 2.5cm column at a flow rate of $10ml.min^{-1}$ at 18 to 25°C (Salmon et al. 1978). Analar water was used to prepare all solutions used above and in the electrophoretic procedure, to prevent ionisation of urea.

6.2.1.6.4. SDS-polyacrylacrylamide gel electrophoresis:

Proteins were analysed by one-dimensional separation on the discontinuous SDS-PAGE system described by Laemmli (1970). The resolving gel was 10%T. The stacking gel was 4% T. The above protein samples and the molecular weight marker protein mixture [α-lactalbumin (MW 14,200), soybean trypsin inhibitor (MW 20,100), trypsinogen (MW 24,000), bovine erythrocyte carbonic anhydrase (MW 29,000), rabbit muscle glyceraldehyde-3-phosphate (MW 36,000), egg albumin (MW 45,000) and bovine serum albumin (MW 66,000)] were denatured according to methods described above. Electrophoresis was carried out in electrophoresis buffer [0.03M Tris-base, 193M glycine and 0.1% (w/v) SDS, pH 8.3] at a constant current of 35mA for 4.5 hours in the anodic direction at 18°C. For samples denatured in presence of urea, 4M urea was added to the electrophoresis buffer. The gels were subsequently stained with silver stain using a horizontal shaker, according to the technique of Morrissey (1981) modified as follows: Gels were fixed in

fixative [50% (v/v) MeOH and 10% (v/v) glacial acetic acid] for 30 minutes followed by a wash in solution [50% (v/v) MeOH and 7% (v/v) glacial acetic acid] for 30 minutes. The gels were then fixed for 45 minutes in 10% glutaraldehyde and washed extensively for 2 to 12 hours in distilled water. Gels were immersed in 0.5mg.ml^{-1} DTT for 30 minutes, 0.1%(w/v) $AgNO_3$ for 45 minutes and rapidly rinsed with distilled water. Gels were developed in developing solution [3% (w/v) sodium carbonate and 0.5µl.ml^{-1} 37% formaldehyde] until the desired levels of staining intensity was attained. Staining was stopped by addition of 23mM citric acid. ml^{-1} and incubation for 10 minutes. Gels were washed several times in distilled water over a 30 minute period and molecular weights determined.

In later studies, gels were stained by the Merrill silver stain procedure (M.T.McManus, personal communication). Gels were fixed in fixative [40%(w/v) MeOH and 10%(v/v) glacial acetic acid] for 30 minutes. The gels were then fixed for (15 min. x 2 times) in dilute fixative solution [10% (v/v) EtOH and 5% (v/v) glacial acetic acid].Gels were oxidised in 0.0034M potassium dichromate and 0.0032N HNO_3, and washed extensively over 30 minutes in distilled water. Gels were immersed in 0.2% (w/v) $AgNO_3$ for 30 minutes and rapidly rinsed in distilled water. Gels were developed in developing solution [3%(w/v) sodium carbonate and 0.5µl.ml^{-1} 37% formaldehyde] until the desired level of staining intensity was attained. Staining was stopped by immersing the gel in 5% (v/v) glacial acetic acid.

6.2.1.6.4. Determination of molecular weight of PAL in the native and

denatured state:

Molecular weight of native PAL was determined in the native state by (a) polyacrylamide gel electrophoresis using the following proteins as standards: bovine milk α-lactalbumin (M.W. 14,200), bovine erythrocyte carbonic anhydrase (M.W. 29,000), chicken egg albumin (M.W. 45,000), bovine serum albumin (monomer) (M.W. 66,000), and dimer (M.W. 132,000) and jack bean urease dimer (M.W. 240,000) and tetramer (M.W. 480,000); and (b) gel filtration using the following proteins as standards: bovine serum albumin monomer (M.W. 66,000) and dimer (M.W.132,000) and jackbean urease dimer (M.W. 240,000) and tetramer (M.W.480,000); (c) sedimentation analysis, using an ultracentrifuge. The molecular weight of denatured PAL was determined by polyacrylamide gel electrophoresis using the following proteins as standards: standard BDH protein cross-linked with diethylpyrocarbonate, to give a monomer (M.W. 14,000), dimer (M.W. 28,600), trimer (M.W. 42,900), tetramer (M.W. 57,200), pentamer (M.W. 71,500) and hexamer (M.W. 85,800).

6.2.2. MATERIALS:

Seeds of Phaseolus vulgaris L. var. Prince were from Clause (UK) Ltd., Charvil, Reading. The centrifuge was from DuPont Co., Wilmington, Delaware; swinging-bucket centrifuge from MSE Scientific Instruments, London; end to end shaker from Taab Labs. Equipment Ltd., Berkshire and spectrophotometer from Perkin-Elmer & Co., GMBH/Uberlingen, Bundesrepublik Deutschland. The ultrafiltration cell unit was from

Amicon Corporation, Danvers, Massachusetts; gel electrophoresis equipment (apparatus GE-2/4) from Pharmacia Fine Chemicals Ltd., Uppsala, Sweden and horizontal shaker from Northern Media Supply Ltd., Humberside. Graded horticultural perlite was from Silvaperl Products Ltd., Harrogate, North Yorkshire; dialysis tubing from Medicell International Ltd., London and Levington soilless potting compost from Fisons Horticultural Division, Bramford, Ipswich, Suffolk. Chemicals used were of the analar grade. AG 50 1-X8 (D) (20-50 mesh) mixed bed-ion exchange resin was from Biorad, Richmond, California; Sepharose 4B, CNBr-Sepharose-4B-L-phenylalanine [10μmoles L-phenylalanine.ml^{-1} packed gel], Sephacryl S-200 and Sephadex G-25 from Pharmacia Fine Chemicals Ltd, Uppsala, Sweden; butyl-PBD from Fisons Scientific Apparatus, Loughborough, Leicestershire; radioactive chemicals from Amersham International, Amersham, Buckinghamshire; DTT, $AgNO_3$, molecular weight markers, pyridoxyl-5´-phosphate, α-ketoglutarate, p-iodonitrotetrazolium violet, iodoacetate, phenazine methosulphate, glycine, pyridoxamine phosphate, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide, riboflavin, ethylene diamine, Tris-base, Tris-HCl, merthiolate, BSA, CNBr, L-phenylalanine, D-phenylalanine, Rhodotorula glutinis-PAL, PMSF, L-ascorbic acid and insoluble PVP from Sigma Chemical Co.Ltd., Poole, Dorset. Other chemicals were from BDH Chemicals Ltd., Poole, Dorset. Dialysis tubing was boiled in 0.01M $NaHCO_3$ and 1mM Na_2EDTA for 15 minutes prior to usage.

6.3. RESULTS

6.3.1. Crude and partially purified homogenates contain PAT and PAL activites (see chapter 5, section 5.3). The following attempts were made to optimise PAL activites and protein (as opposed to PAT activity and protein) during various stages in the purification of PAL:

6.3.1.1. Optimisation of PAL activities in crude homogenates:

A method had to be found for obtaining large quantities of plant material with relatively high concentrations of PAL activity as opposed to PAT activity. Plants infected with fungal pathogen showed transient increases in PAL activities only in plants of younger age (see chapter 2, section 2.3). The material obtainable for PAL extraction was therefore low.

Amongst the methods tried, was the abiotic elicitor, continuous light.

PAL activities increase simultaneously with PAT activities on continuous exposure of plants to light. During later stages of the induction process, PAL activities equilibrate while PAT activities decrease to levels found in controls (Fig. 63). The equilibration of PAL activities is due to the production of inactive PAL protein (see chapter 7, section 7.3). Leaves induced by such treatment senesced and resembled leaves exhibiting hypersensitive necrosis (Fig. 9)

Attempts were made to purify PAL during various stages of induction by

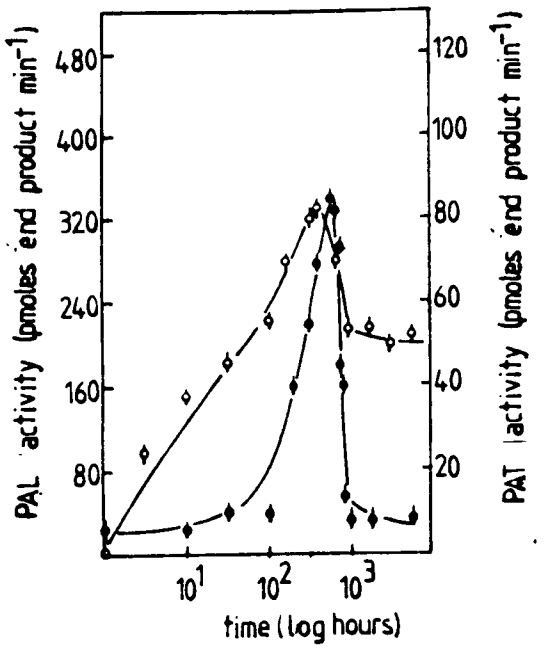
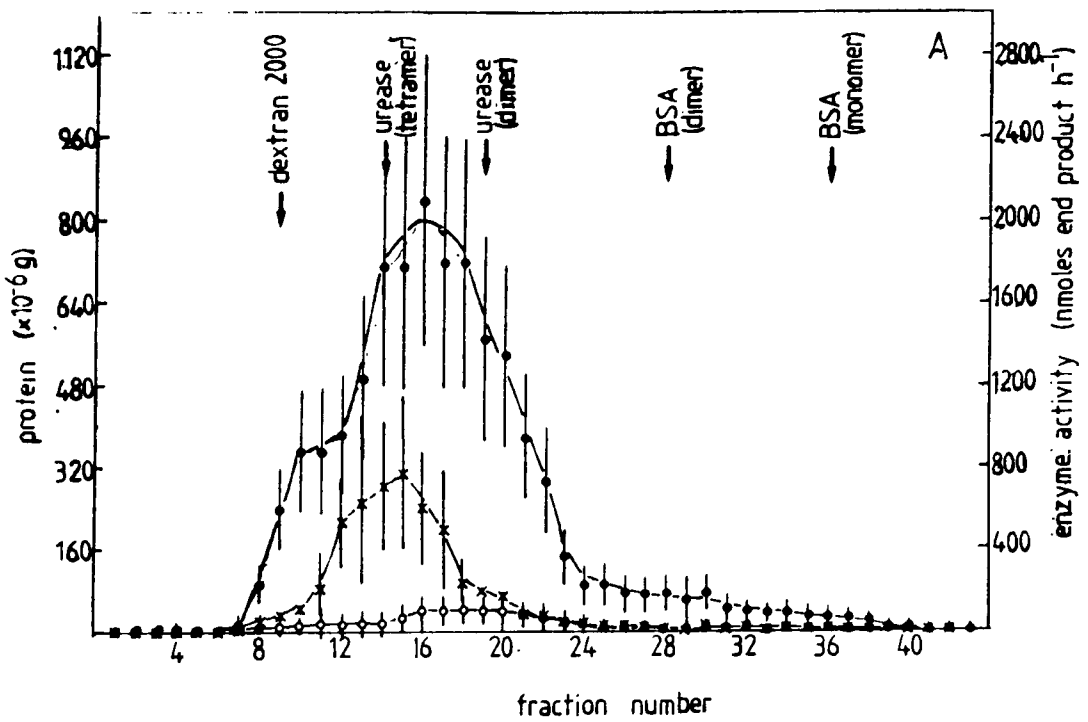


Fig. 61 Purification of PAL by affinity chromatography: optimisation of PAL activities (o—o—o), as opposed to PAT activities (●—●—●) in crude homogenates. (for further details see Fig. 37 & methods).



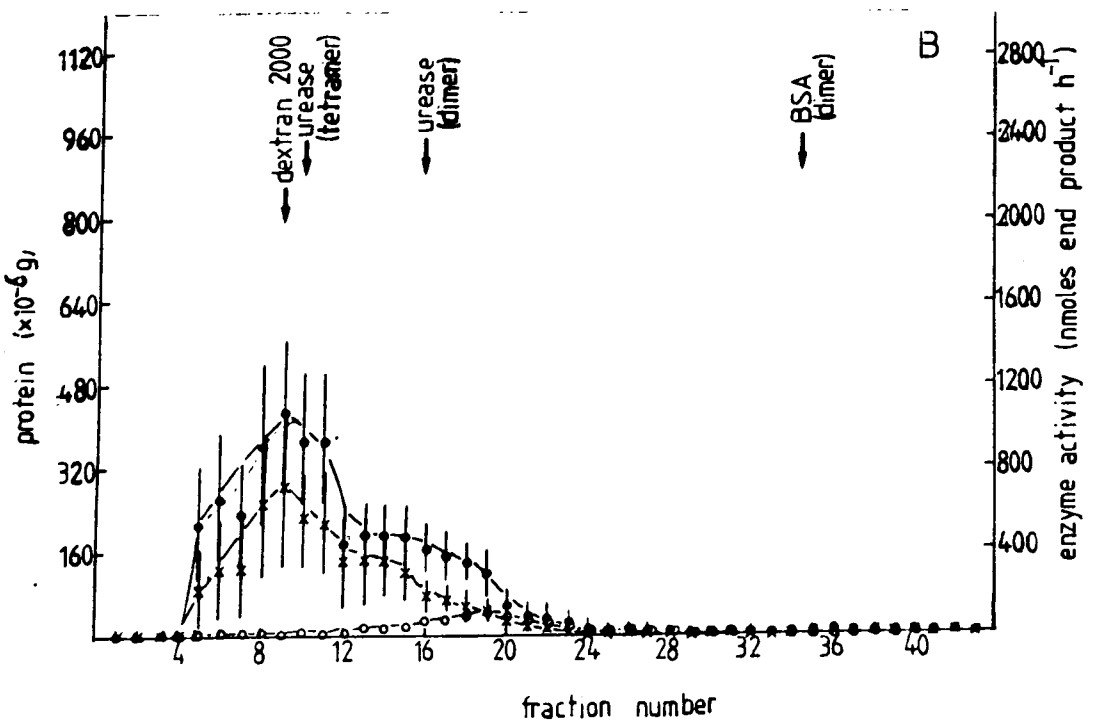


Fig. 62 Purification of PAL by affinity chromatography: optimisation of PAL activities (●—●—●), as opposed to PAT activities (o—o—o) in partially purified homogenate protein (×—×—×—). A. gel filtration on a Sephacryl S-300 column; B. gel filtration on a Sephacryl S-200 column. Each fraction = 1.5ml. (for further details see Fig. 38 & methods).

continuous exposure to light. Purification of PAL during the initial stages of induction by continuous light resulted in a higher fold purification of PAL accompanied by a higher fold purification of PAT. Purification of PAL during later stages of induction by continuous light resulted in a lower fold purification of PAL in absence of a higher fold purification of PAT (Table 6).

6.3.1.2. Optimisation of PAL activites in partially purified homogenates:

Attempts were made to separate PAT from PAL by use of gel filtration techniques.

S-300 fractions have PAT as contaminants. S-200 fractions have no PAT as contaminants. This was because S-300 gel filtration columns separate PAL from PAT during inclusion in the gel matrix, while S-200 gel filtration columns separate PAL from PAT by exclusion of PAL from included PAT in the gel matrix (Fig. 64 A and B, Table 7).

Inactive PAL protein, when in abundance (as in longer periods in continuous light), was found to elute prior to and with traces of PAT protein. The inactive form of PAL protein was identifiable by immunoprecipitation (see chapter 7, section 7.3).

6.3.1.3. Optimisation of PAL activities by affinity chromatography:

6.3.1.3.A. Attempts were made to separate PAT from PAL by differential

Table 6: Purification of PAL by affinity chromatography: effect of continuous light on contamination of PAL by PAT.

fraction	activity*		recovery %		protein x 10^{-3} g	specific activity		fold purification	
	PAT	PAL	PAT	PAL		PAT	PAL	PAT	PAL
Experiment I***									
crude extract	331	72	-	-	87.5	3.8	0.8	-	-
pH 9.3 eluate	164	67	50	93	4.0	41	16.8	10.8	20.4
Experiment II****									
crude extract	47	54	-	-	87.5	0.5	0.6	-	-
pH 9.3 eluate	-	19	-	35	4.0	-	4.8	-	7.7

* nmoles.min^{-1}, variation in activity = ±5.0
** nmoles.min^{-1}.10^{-3} g protein
***I. 295 hours in continuous light
****II. 600 hours in continuous light

Table 7: Purification of PAL by affinity chromatography: separation of PAL from PAT by gel filtration:

fraction	activity*		recovery %		protein x 10^{-3} g	specific activity**		fold purification	
	PAT	PAL	PAT	PAL		PAT	PAL	PAT	PAL
Crude extract	25	60	-	-	87.5	0.3	0.7	-	-
S-300 gel filtration	1.5	60	6	100	25.0	0.1	2.4	0.2	3.5
S-200 gel filtration	-	42	-	70	25.0	-	1.7	-	2.5

* nmoles.min^{-1}, variation in activity = ±5.0
** nmoles.min^{-1}.10^{-3} g protein

hydrophobicity in presence of L-phenylalanine:

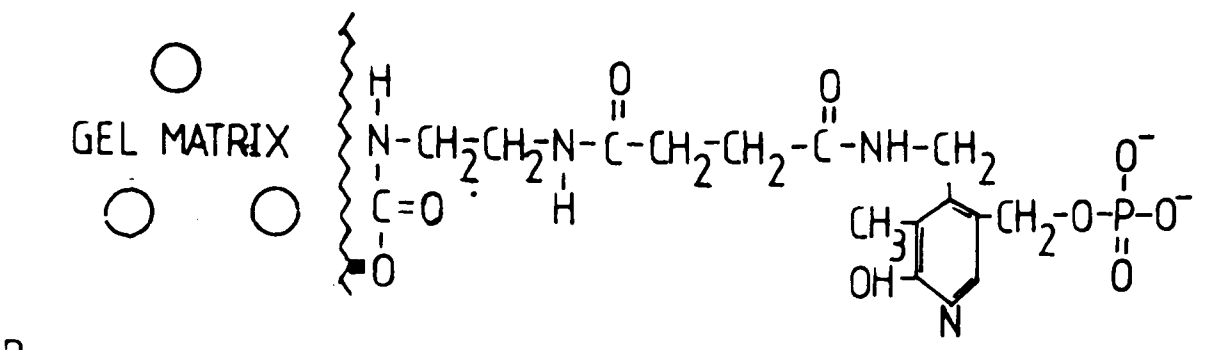
PAL (as opposed to PAT) had a greater affinity for a hydrophobic environment, in presence of L-phenylalanine. Partially purified homogenates containing PAT and PAL activity were loaded on to a hydrophobic succinyl-amino-ethyl- L-phenylalanine Sepharose 4B column (Fig. 65B). This resulted in a greater amount of recoverable affinity-purified PAL, as compared to PAL (Table 8).

Table 8. Purification of PAL by affinity chromatography: separation of PAT from PAL by hydrophobic affinity chromatography.

fraction	activity*		recovery %		protein $\times 10^{-3}$ g	specific activity**		fold purification	
	PAT	PAL	PAT	PAL		PAT	PAL	PAT	PAL
Crude extract	25	60	-	-	87.5	0.3	0.7	-	
Experiment I*** pH 9.3 eluate	25	29	100	48	25.0	1.0	1.2	4	2
Experiment II*** pH 9.3 eluate	0.5	41	2	68	25.0	0.02	1.6	0.1	2.5

* nmoles.min^{-1}, variation in activity = $\pm$5.0
** nmoles.min^{-1}.10^{-3} g protein
*** Experiment I, L-phenylalanine Sepharose 4B
Experiment II, succinyl-amino-ethyl L-phenylalanine Sepharose 4B

A



B

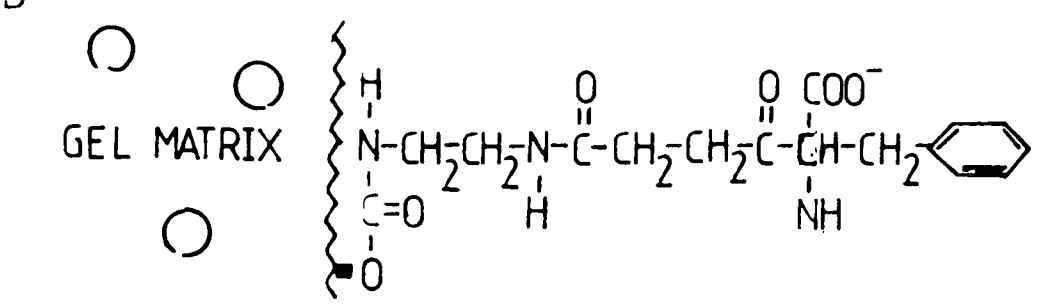


Fig. 62 Purification of PAL by affinity chromatography: structure of affinity adsorbents used : A. succinyl-amino-ethyl-pyridoxamine phosphate Sepharose 4B; B. succinyl-amino-ethyl-L-phenylalanine Separose 4B.

6.3.3.B. Attempts were made to separate PAT from PAL by differential ligand affinity in presence of a hydrophobic environment. PAT (unlike PAL) has a higher affinity for pyridoxamine phosphate in presence of a hydrophobic environment.

Partially purified homogenates containing PAT and PAL activity were loaded on to a hydrophobic succinyl-amino-ethyl-pyridoxamine phosphate Sepharose 4B column (Fig. 65A) to bind PAT protein. The eluate was then loaded on a L- succinyl-amino-ethyl L-phenylalanine Sepharose 4B column, to bind PAL protein.

However, PAT failed to bind to the pyridoxamine phosphate column. This may have been due to unsatisfactory binding conditions, such as prior incubation with co-factors or substrates with or without sub-optimal concentrations of ∝-ketoglutaric acid, L-glutamic, pyridoxyl phosphate and L-aspartic acid. Since the various possibilities for attaining optimal binding conditions were enormous, further attempts were abandoned.

6.3.1.4. Optimisation of PAL activites in affinity eluates:

Attempts were made to elute PAT with an alternative substrate (L-aspartic acid), the affinity of which may be increased by increasing concentrations of ∝-ketoglutaric acid:

Partially purified homogenates containing PAT and PAL activities were

Table 9. Purification of PAL by affinity chromatography:

Fraction (ml)	activity*		recovery %		protein x 10^{-3} g	specific activity**		fold purification	
	PAT	PAL	PAT	PAL		PAT	PAL	PAT	PAL
Crude extract (175.0)	25	60	-	-	87.5	0.3	0.7	-	-
$(NH_4)_2SO_4$ precipitation 30 to 50% (3.5)	2	45	8	75	26.5	0.1	1.7	-	2.5
S-200 gel filtration (5.0)	-	42	-	70	25.0	-	1.7	-	2.5
20mM solution (50.0)	-	134	-	223	25.0	-	5.4	-	8
pH 6.0 (50.0)	-	206	-	343	25.0	-	8.2	-	12
pH 9.3 eluate (50.0)	-	22	-	37	25.0	-	0.9	-	1.3
L-phenylalanine eluate (50.0)	-	43	-	72	2.5	-	17.2	-	25

* nmoles.min^{-1}, variation in activity = ± 5.0
** nmoles min^{-1} 10^{-3} g protein

loaded on a succinyl-amino-ethyl-L-phenylalanine Sepharose 4B column. The column was then incubated for 12 hours and washed with 1 x 10^{-3}M L-aspartic acid. This resulted in elution of only 0.53% of the bound PAT activity at room temperature (not at 4^{o}C). The column was then incubated for 12 hours and washed with 1 x 10^{-3}M L-phenylalanine. This resulted in elution of only 0.23% PAL activity, most of the PAL having been proteolytically degraded. This was probably because the whole elution procedure expanded into 2 days of washing and elution, longer times of incubation with ligands being obligatory.

6.3.2. The best results obtained from the above experiments were combined as described in `Methods´ to give a profile of PAL activities, free of PAT activity and protein (Fig. 66, Table 9). The low fold purification of PAL was probably indicative of the presence of inactive protein formed after decrease of PAT during longer periods of exposure to continuous light. Inactive PAL protein was identifiable by immunoprecipitation (Chapter 7).

6.3.3. Analysis of purity of PAL:

PAL obtained by the above procedures was free of PAT activity and protein:

6.3.3.1. PAT activity was not excluded with PAL activity from the Sephacryl S-200 column (Fig. 64B).

6.3.3.2. Spectrophotometric assays of PAL fractions excluded from the

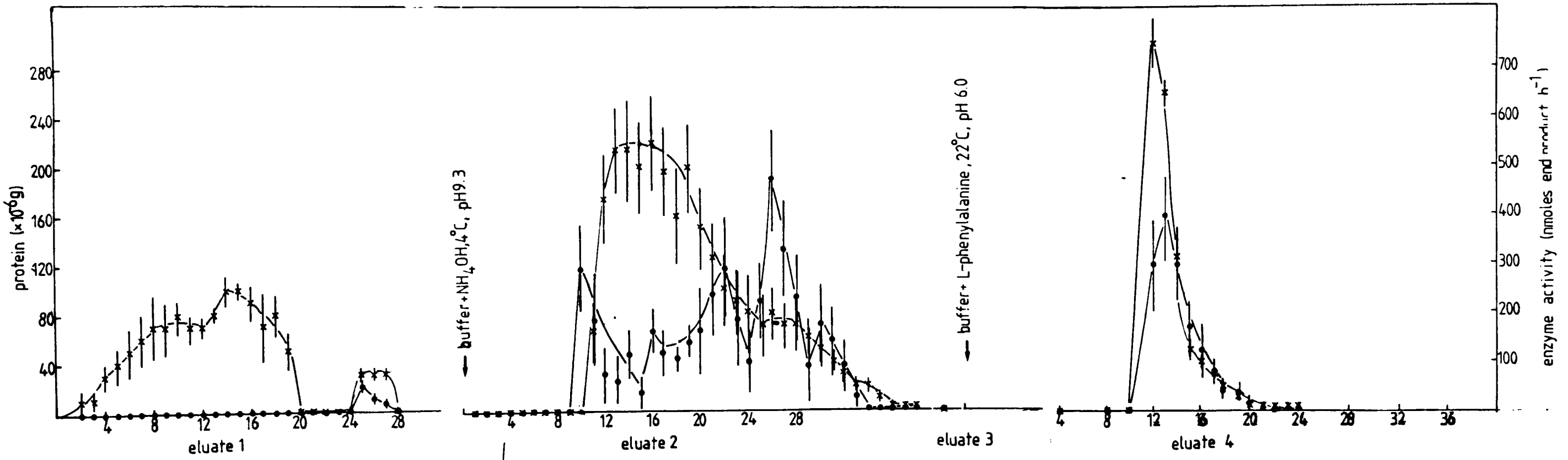


Fig. 66 Purification of PAL by affinity chromatography: profile of PAL activities (●—●—●); with protein (×—×—×) during purification procedures described in `methods´.

(for further details see Fig. 38)

Sephacryl S-200 column exhibit a linear increase in incorporation of L- phenylalanine into t-cinnamic acid with time and protein concentration, unlike crude extracts of bean leaves. This is indicative of the non-interference of PAT, which if present, would compete for incorporation of L-phenylalanine into phenylpyruvic acid.

6.3.3.3. The stained protein band representing PAT activity on native anodic gels is lost after separation of PAT from PAL by S-200 gel filtration chromatography (Fig. 67). Subsequent affinity purification resulted in a single homogenous protein band (Fig. 68; Fig. 69).

6.3.3.4. PAL isolated from Rhodotorula glutinis also shows a protein band of near electrophoretic mobility to that of affinity purified PAL (Fig. 69). Other bands in the preparation are possibly degraded PAL as the preparations were 1 week old (see below).

6.3.3.5. No cationic proteins (including PAT) were found in PAL (unlike crude homogenates) after purification by affinity chromatography and in PAL from Rhodotorula glutinis L. (Fig. 70).

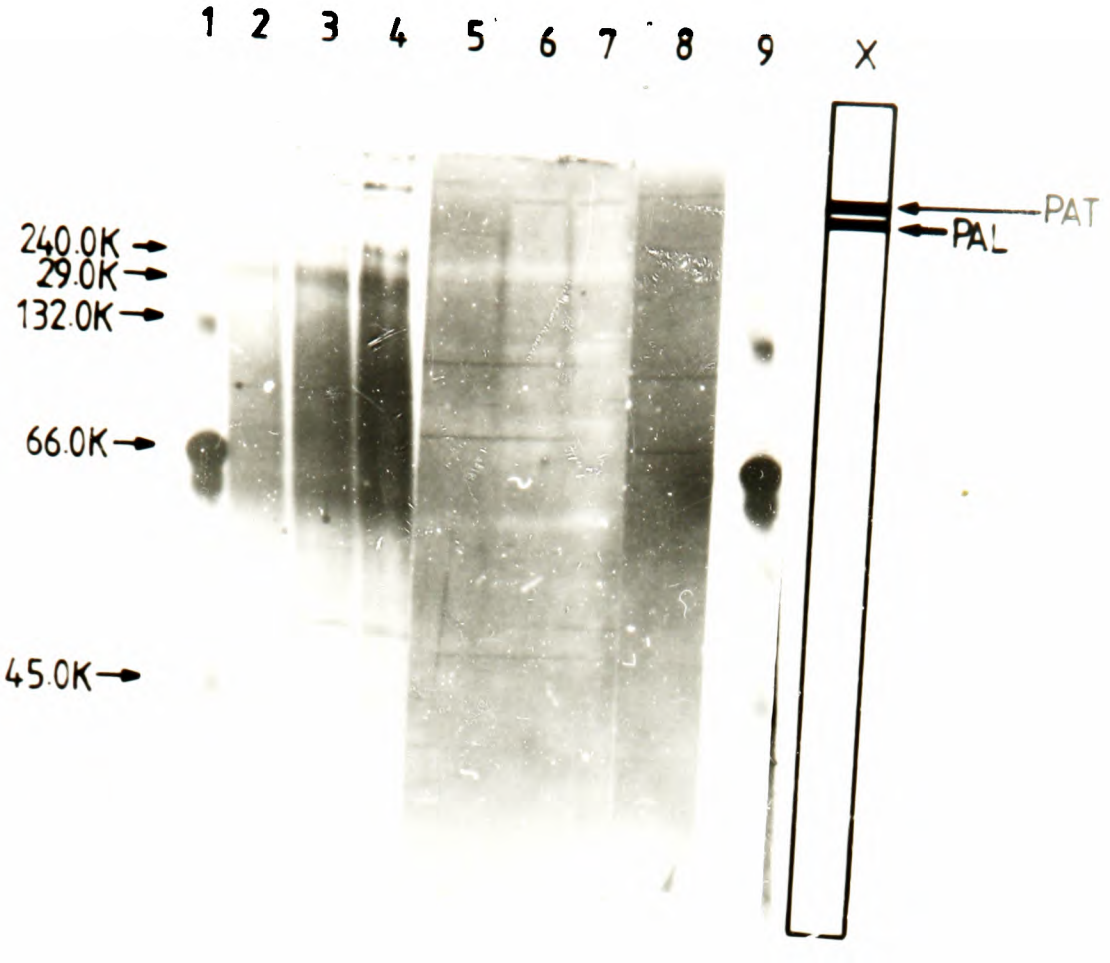
6.3.3.6. A single molecular weight was obtained by native gel electrophoresis (Fig. 71A and B) and gel filtration (Fig. 72) of PAL purified by affinity chromatography. The molecular weight of PAL was found to be approximately 330kD. The estimation of the molecular weight of PAL by sedimentation coefficients were unsuccessful because lower concentrations of $<1 mg.ml^{-1}$ PAL protein than $>1 mg.ml^{-1}$ required for the use of the method were obtainable by affinity chromatography.

Fig. 47 Purification of PAL by affinity chromatography: analysis of pure PAL, free of PAT protein. anodic-PAGE : 10%T. Tracks: X, facsimile of native PAL and PAT protein; 1, MWM; 2 to 4: PAL + PAT protein (before Sephacryl S-200 gel filtration): 2, x 1 protein; 3, x 2 protein; 4, x 3 protein; 5 to 7, PAL + PAT protein stained for PAT activity (before Sephacryl S-200 gel filtration): 5, x 1 protein; 6, x 2 protein; 7, x 3 protein; 8, PAL protein (after Sephacryl S-200 gel filtration) x 1 protein; 9, MWM.

Fig. 68. Purification of PAL by affinity chromatography: analysis of pure PAL, free of PAT protein. anodic-PAGE : 10%T. Tracks: X, facsimile of native PAL and PAT protein; 1, MWM; 2, PAL + PAT protein (before Sephacryl S-200 gel filtration); 3, PAL + PAT protein (after Sephacryl S-200 gel filtration) x 1 protein; 4, PAL + PAT protein (after S-200 gel filtration) x 2 protein; 5, MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 66 Purification of PAL by affinity chromatgraphy, analysis of purity of PAL, free of PAT protein. anodic-PAGE = 5%T. Time after isolation = 12 hours (except Rhodotorula glutinis L, = 168 hours) Tracks: Y, facsimile of native PAL protein; 1, MWM; 2, PAL + PAT protein (after Sephacryl S-200 gel filtration); 3 to 5: PAL protein (after affinity chromatography): 3, 1 x protein; 4, 2 x protein; 5, 3 x protein; 6 to 8: PAL protein (after affinity chromatography, and ligand removal): 6, 1 x protein; 7, 2 x protein; 8, 3 x protein 9, PAL from Rhodotorula glutinis L.; 10, MWM.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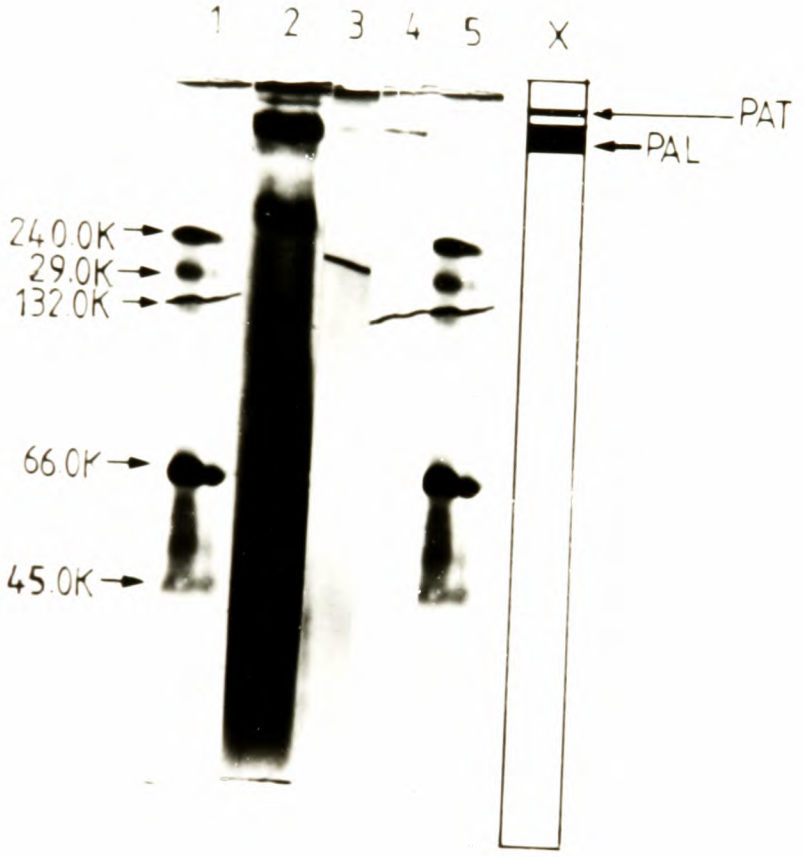
240.0K
29.0K
132.0K
66.0K
45.0K
PAT
PAL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29.0K
132.0K
66.0K
45.0K
PAT
PAL

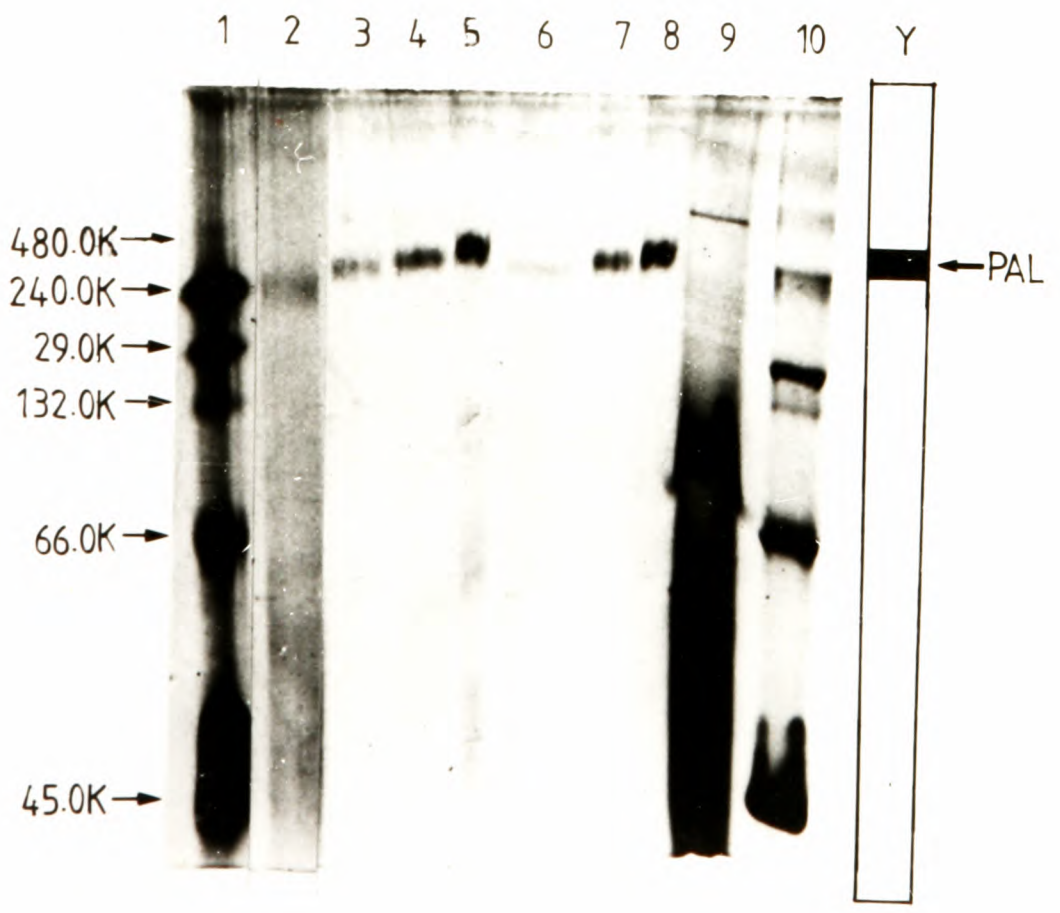

1 2 3 4 5 6 7 8 9 10 Y
480.0K
240.0K
29.0K
132.0K
66.0K
45.0K
PAL

Fig. 20 Purification of PAL by affinity chromatography: analysis of purity of PAL protein, free of PAT protein. cationic-PAGE : 15%T. Tracks: 1, MWM; 2, PAL + PAT protein (crude extract); 3, PAL + PAT protein [$(NH_4)_2SO_4$ precipitate]; 4, PAL + PAT protein (pH 9.3 eluate) 5, PAL + PAT protein (after Sephacryl S-200 gel filtration); 6, PAL protein from Rhodotorula glutinis L.; 7 to 9: PAL protein (after affinity chromatography): 7, 1 x protein; 8, 2 x protein; 9, 3 x protein; 10 to 12: PAL protein (after affinity chromatography and removal of ligand): 10, 1 x protein; 11, 2 x protein; 12, 3 x protein; 13, MWM.

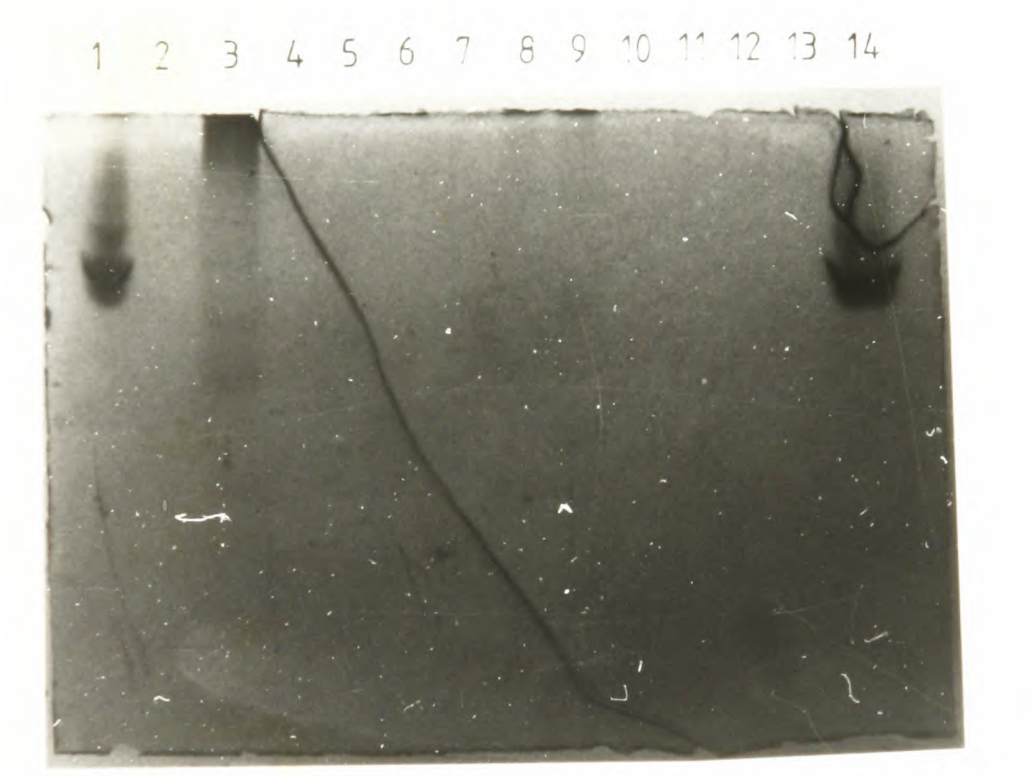
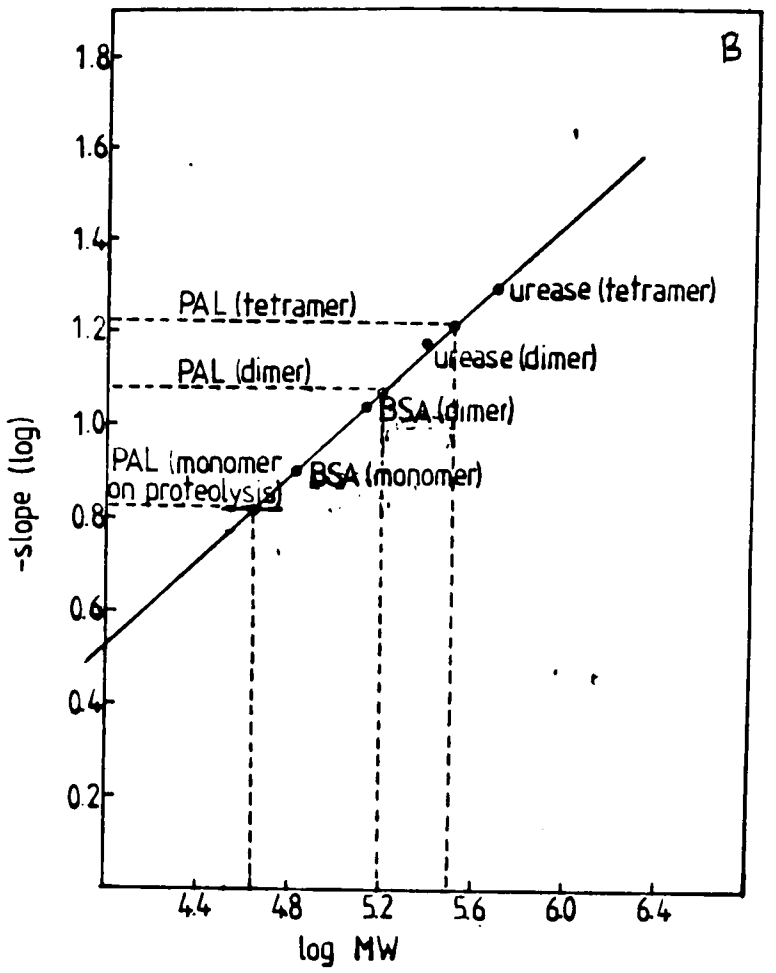
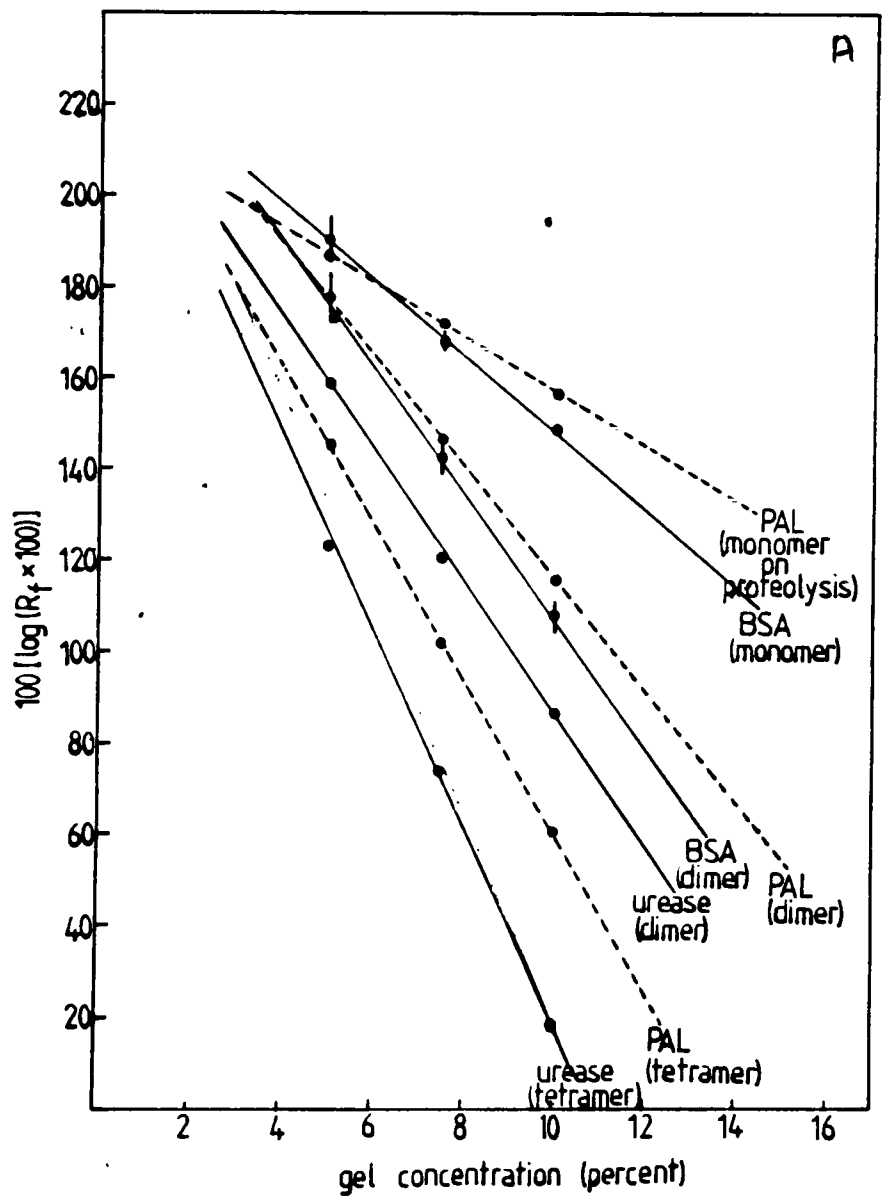

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 71 CONTD.

The relative mobility (R_f) of PAL was determined by dividing its migration distance from the top of the separating gel to the centre of the protein band by the migration distance of the bromophenol tracking dye from the top of the separating gel. In Fig. 71a, 100 [log (R_f x 100)] values (ordinate) were plotted against the gel concentration as percent (abscissa) on standard graph paper for PAL and the relative marker proteins. In Fig. 71b, the negative slopes from these graphs (ordinate) were plotted against the known molecular weights of the standards (abscissa) on 2 cycle log-log paper. The molecular weight of the PAL protein was determined from the graph in Fig. 71b. Each spot represents the mean of 3 determinations at 3 to 6 different protein concentrations and at 3 different gel concentrations of 5, 7.5 and 10%T. For further details see 'methods'. The bars indicate the maximum and minimum values in a gel. The absence of a bar indicates that the variation is contained within each spot.





71

Fig. Purification of PAL by affinity chromatography: analysis of purity of PAL, free of PAT protein: analysis of molecular weight by gel electrophoresis.

(continued on facing page)

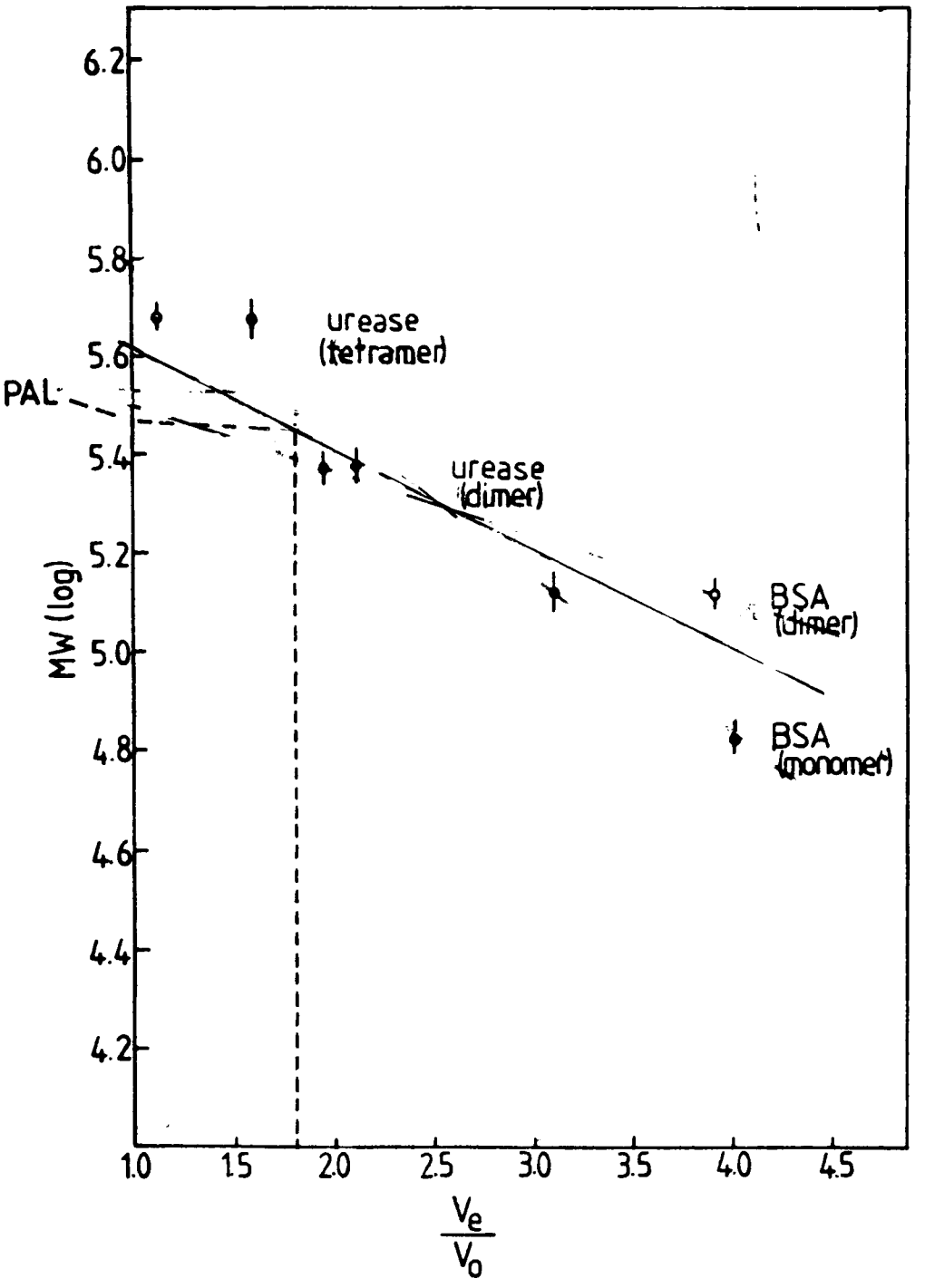


Fig. 72. Purification of PAL by affinity chromatography: analysis of purity of PAL, free of PAT protein: analysis of molecular weight by gel filtration.

For further details see 'methods'. Each spot represent the mean of 3 determinations at 3 to 6 different protein concentrations. The bars indicate the maximum and minimum values in a determination. The absence of a bar indicates that the variation is contained within each spot.

6.3.3.7. PAL purified by affinity chromatography does not contain any charge isomers. The separation of PAL protein before and after purification by affinity chromatography, on the basis of size and charge, by native gel electrophoresis, gave only two bands (in case of crude homogenates, before gel filtration on S-200, one PAT and one PAL) and one band of protein (in case of PAL purified after gel filtration and affinity chromatography) moving in the anodic direction (Fig. 68; Fig. 69). Since this system could sucessfully separate charge isomers of carbonic anhydrase 29kD and 132kD (Fig. 68; Fig. 69), it is unlikely that PAL before and after purification by affinity chromatography exists as charge isomers.

6.3.3.8. Affinity-purified PAL showed no polypeptides in the region of the molecular weight of PAT (Fig. 80).

6.3.4. Stability of PAL after purification by affinity chromatography:

6.3.4.1. Stability of ligand-bound PAL:

The ligand-bound form of PAL isolated by the above procedure maintained its activity and native molecular weight (stability) for a short period of 12 hours (Fig. 69). Inactivation and degradation of PAL started between 12 and 19 hours after isolation with the production of a acidic polypeptide (Fig. 73). Between 24 and 42 hours after isolation, the production of a dimeric form of PAL (molecular weight 180kD, Fig. 71A and B), and traces of the monomeric form were obtained (molecular weight 95kD, Fig. 71A and B; Fig. 74).

Crude and partially purified preparations of PAL also exhibit the same pattern of degradation on storage at $4^{o}C$ for 2 to 3 days (Fig. 73; Fig. 74, tracks 2 and 3).

Further, PAL isolated from Rhodotorula glutinis also converted to the dimeric state on storage at $4^{o}C$ for a week. The smaller acidic polypeptide was also visible (Fig. 69, track 9).

The associated loss of the small acidic polypeptide with basic/neutral dimer formation of PAL, indicated the possible structural function of the small polypeptide, being to hold the dimeric subunits of PAL together while in the ligand-bound form.

6.3.4.2. Stabilisation of the ligand-bound form of PAL:

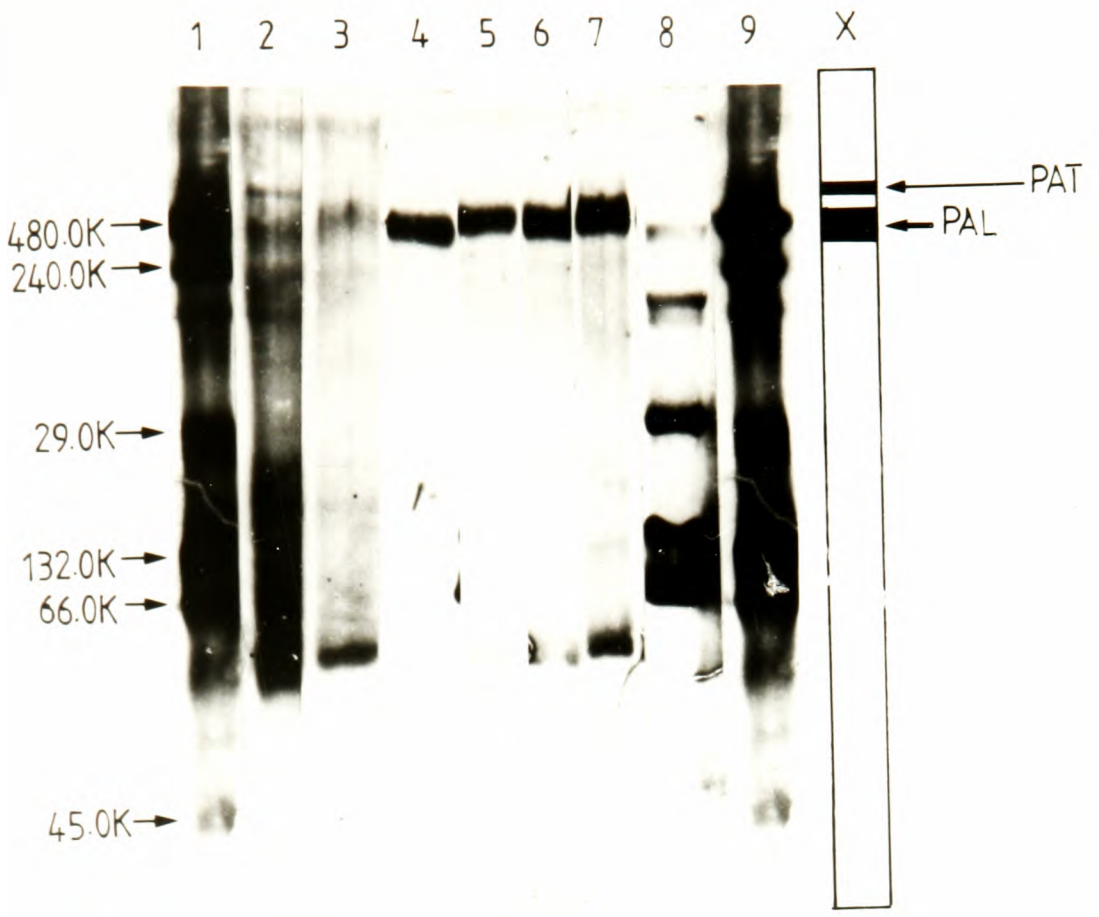
Attempts were made to prevent proteolysis during purification. Other workers have used PMSF to prevent proteolytic degradation of PAL (Gilbert & Jack, 1981). However, the addition of PMSF (1mM) inhibited elution of PAL by L-phenylalanine by as much as 100%. This was presumably because the phenyl group on PMSF competed with the phenyl group on L-phenylalanine for the same binding site on PAL attached to the L-phenylalanine affinity gel. Such competitive interaction may explain the low (<5.0%) recovery obtained in initial attempts at purification of PAL, when PMSF was added to the extraction buffer. Further, competitive inhibition was also found with other substrate analogues added during affinity elution. For example, D-phenylalanine and t-cinnamic acid resulted in only 3.4% and 0% recovery respectively

Fig. 73. Purification of PAL by affinity chromatography: stability of affinity-purified PAL, free of PAT protein. anodic-PAGE : 5%T. Time after isolation : 19 hours. Tracks: X, facsimile of native PAT and PAL protein; 1, MWM; 2, PAL + PAT protein (crude extract); 3, PAL + PAT protein (after Sephacryl S-200 gel filtration); 4 to 7: PAL protein (after affinity purification): 4, 1 x protein; 5, 2 x protein; 6, 3 x protein; 7, 4 x protein, 8, BSA + PAL (after affinity purification); 9, MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 73. Purification of PAL by affinity chromatography: stability of affinity purified PAL, free of PAT protein. anodic-PAGE = 10%T; Time after isolation : 42 hours. Tracks: X, facsimile of native PAL and PAT protein; 1, MWM; 2, PAL + PAT protein (crude extract); 3, PAL + PAT protein (after Sephacryl S-200 gel filtration); 4 to 7: PAL protein (after affinity purification): 4, 1 x protein; 5, 2 x protein; 6, 3 x protein; 7, 4 x protein; 8, BSA + PAL protein (after affinity purification); 9, MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.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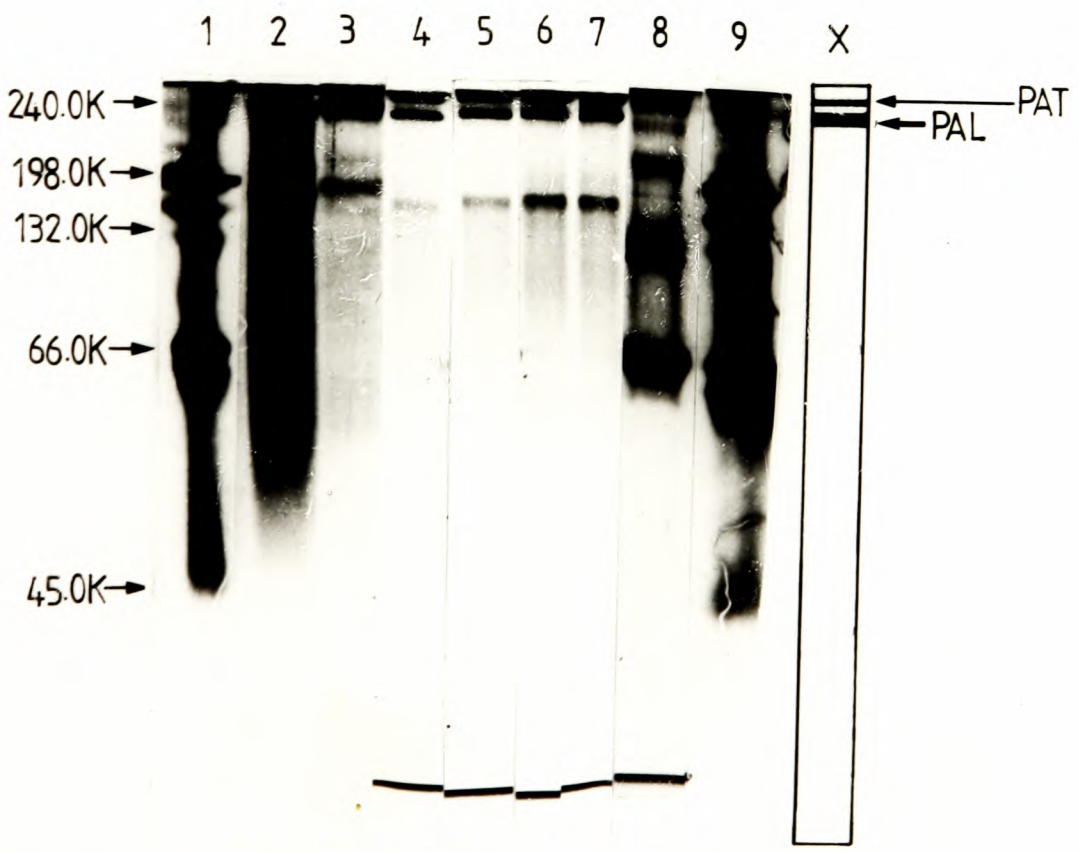
480.0K
240.0K
29.0K
132.0K
66.0K
45.0K
PAT
PAL


1 2 3 4 5 6 7 8 9 X
240.0K
198.0K
132.0K
66.0K
45.0K
PAT
PAL

of PAL activity from the L-phenylalanine affinity gel.

6.3.4.3. Stability of ligand-free PAL:

The removal of the ligand from ligand-bound PAL resulted in inactivation of PAL activity. However, the ligand-free form of PAL had the same native molecular weight as the ligand-bound form of PAL, 24 hours after isolation (Fig. 69). Hence, the inactivation of ligand-free PAL (unlike ligand-bound PAL) was not because of proteolytic degradation into a dimer, 24 hours after isolation.

The removal of ligand from ligand-bound PAL resulted in the formation of a turbid solution indicative of a possible change in structural conformation of PAL, related to the association of the acidic polypeptide with the dimeric subunits of protein.

6.3.4.4. Stabilisation of ligand-free PAL:

Attempts were made to restore activities of ligand-bound PAL during and after removal of ligand. BSA was found to stabilise PAL if added prior to removal of the ligand. However, this complicated the determination of purity of PAL preparations since the BSA pentamer and PAL had the same molecular weight of 330kD (Fig. 73, track 8; Fig. 74, track 8).

6.3.5. Analysis of denatured PAL:

Attempts were made to study the subunit size of PAL by denaturation of native PAL at high and low protein concentrations.

6.3.5.1. Denaturation of PAL at high protein concentrations:

6.3.5.1.1. Denaturation in SDS: Denaturation of affinity-purified PAL in SDS, at high protein concentrations, of >1mg ml^{-1} resulted in a subunit molecular weight of 83kD (Fig. 75; Fig. 76). This denaturation pattern was obtained with (i) high concentrations of affinity purified PAL protein (Fig. 75; Fig. 76); (ii) high concentrations of BSA added to low concentrations of affinity purified PAL protein (Fig. 77); this molecular weight being higher than the monomeric form of BSA, 65kD, Fig. 71A and B; Fig.72); and (iii) highly concentrated PAL protein by $(NH_4)_2SO_4$ (Fig. 78, tracks 6 and 7).

This dissociation pattern was stable to heating at 37°C for 3 hours or at 100°C for 20 minutes (Fig. 77).

6.3.5.1.2. Denaturation in guanidine-HCl:

Denaturation by guanidine-HCl at high protein concentrations resulted in a higher subunit molecular weight than that obtained in presence of SDS, i.e.95kD (similar to that obtained by proteolytic degradation above) (Fig. 78). This indicated a possible change in conformation of PAL on stabilisation of activity of PAL. Because of this reason additives which would serve as alternatives to BSA such as egg albumin, of differing molecular weight to native and denatured PAL

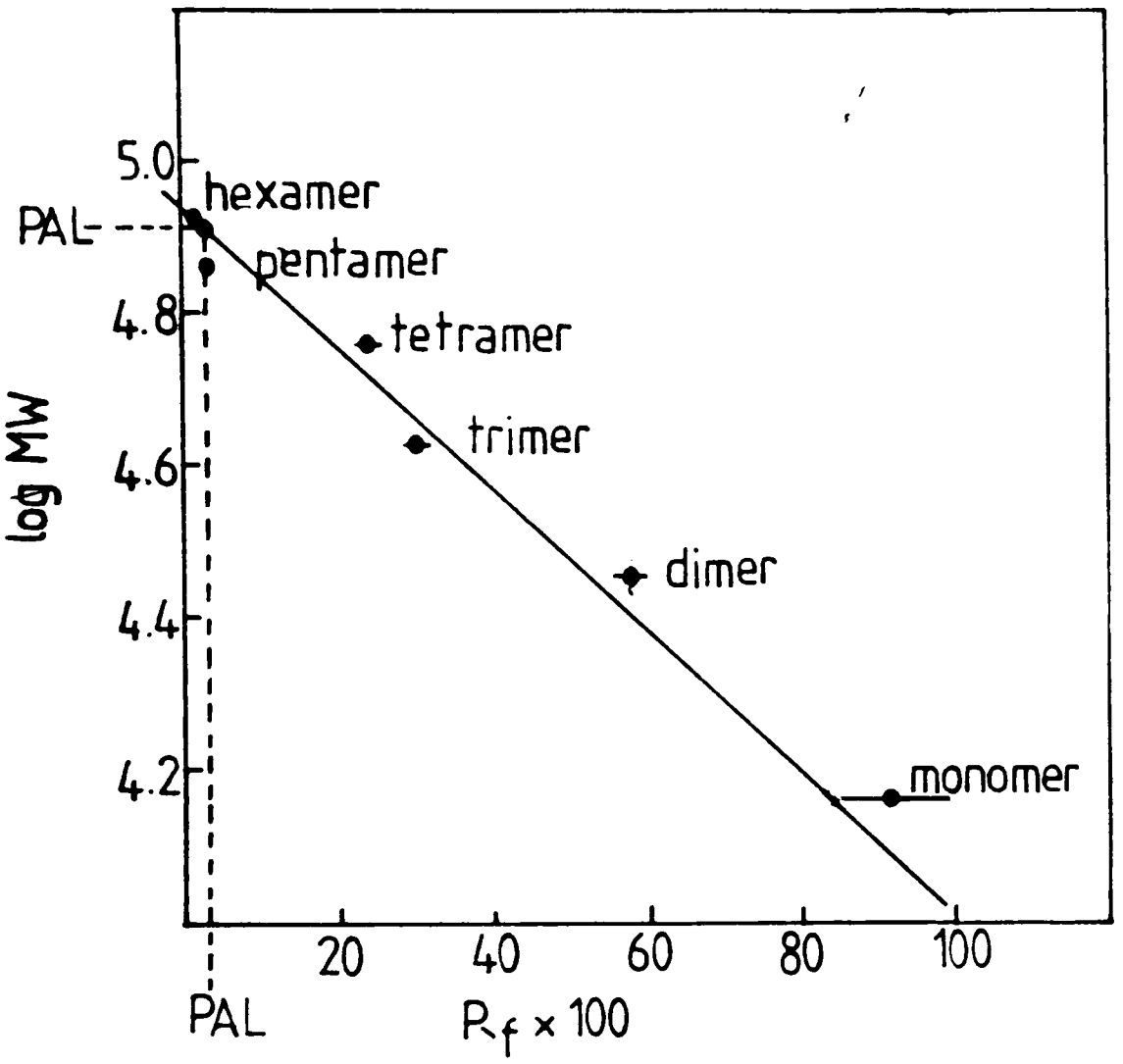


Fig. 72. Purification of PAL by affinity chromatography: determination of molecular weight of denatured PAL at high protein concentrations. Molecular weight markers were synthetic BDH polypeptides.

The molecular weight of denatured PAL was determined by SDS-PAGE as described in 'methods' using the following proteins as standard BDH protein cross-linked with diethylpyrocarbonate, to give a monomer (M.W. 14,000), dimer (M.W. 28,600), trimer (M.W. 42,000), tetramer (M.W. 57,200), pentamer (M.W. 71,500) and at hexamer (M.W. 85,800). Each spot represents the mean of 3 gels at different protein concentrations. The bars indicate the maximum and minimum values in a gel.

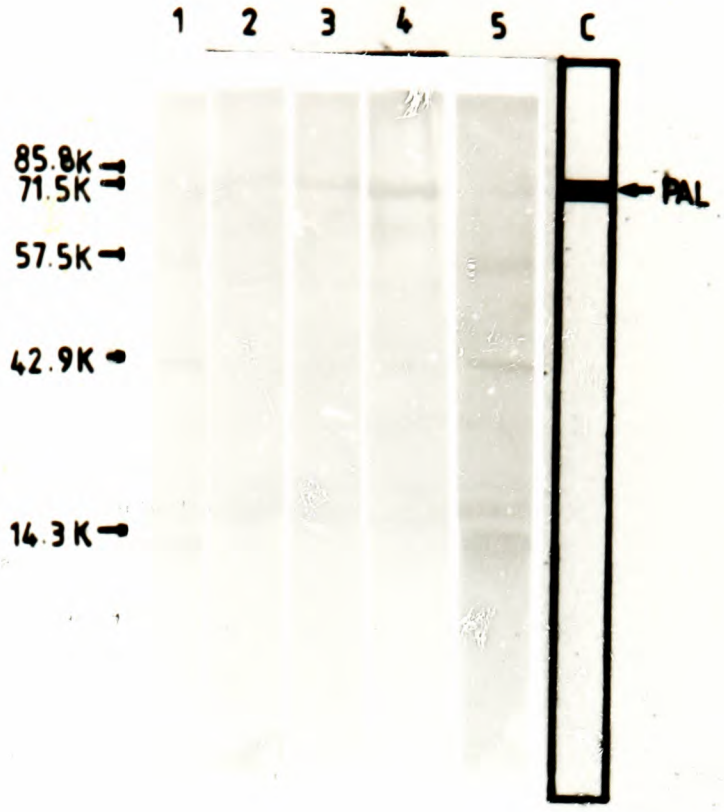
Fig. 73. Purification of PAL by affinity chromatography: analysis of denatured PAL at high protein concentrations. SDS-PAGE : 10%T. Tracks: C, facsimile of denatured PAL at high protein concentrations; 1, MWM; 2 to 4: PAL protein (after affinity chromatography): 2, 1 x protein; 3, 2 x protein; 4, 3 x protein; 5, MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 73. Purification of PAL by affinity chromatoraphy: analysis of denatured PAL at high protein concentrations. SDS-PAGE : 10%T. Tracks: C, facsimile of denatured PAL at high protein concentrations; 1, MWM; 2, BSA + PAL protein (after affinity chromatography) not heated; 3 to 5: BSA + PAL protein (after affinity chromatography), heated at 37°C for 3 hours: 3, 1 x protein; 4, 2 x protein; 5, 3 x protein; 6, BSA + PAL protein (after affinity chromatography) heated at 100°C for 2 minutes; 7, BSA + PAL protein (after affinity chromatography) heated at 100°C for 20 minutes: 8. MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 73. Purification of PAL by affinity chromatography: analysis of denatured PAL at high protein concentrations. SDS-PAGE = 10%T. Tracks: C, facsimile of denatured PAL protein in SDS at high protein concentrations; D; facsimile of denatured PAL protein in guanidine at high protein concentrations; E; facsimile of denatured PAT protein; 1, MWM; 2 to 5: BSA + PAL (after affinity purification): 2, SDS-denatured; 3, guanidine-HCl denatured 1 x protein; 4, guanidine-HCl denatured 2 x protein; 5, guanidine-HCl denatured 3 x protein; 6, PAL + PAT protein [$(NH_4)_2SO_4$ precipitation] SDS denatured 1 x protein; 7, PAL + PAT protein [$(NH_4)_2SO_4$ precipitation] SDS denatured 2 x protein; 8, MWM.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2
3
4
5
C
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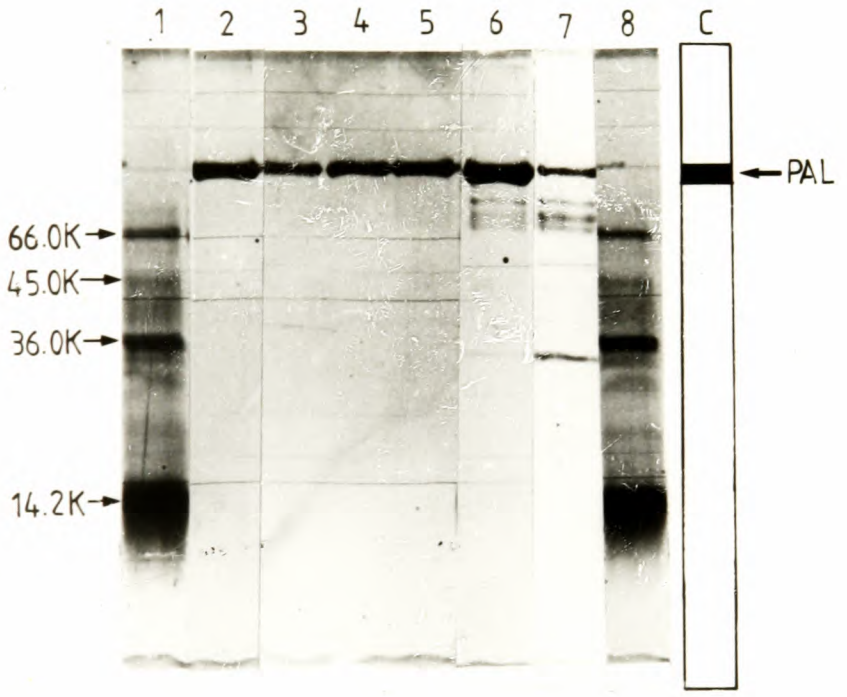
71.5K
57.5K
42.9K
14.3K
PAL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3
4
5
6
7
8
C
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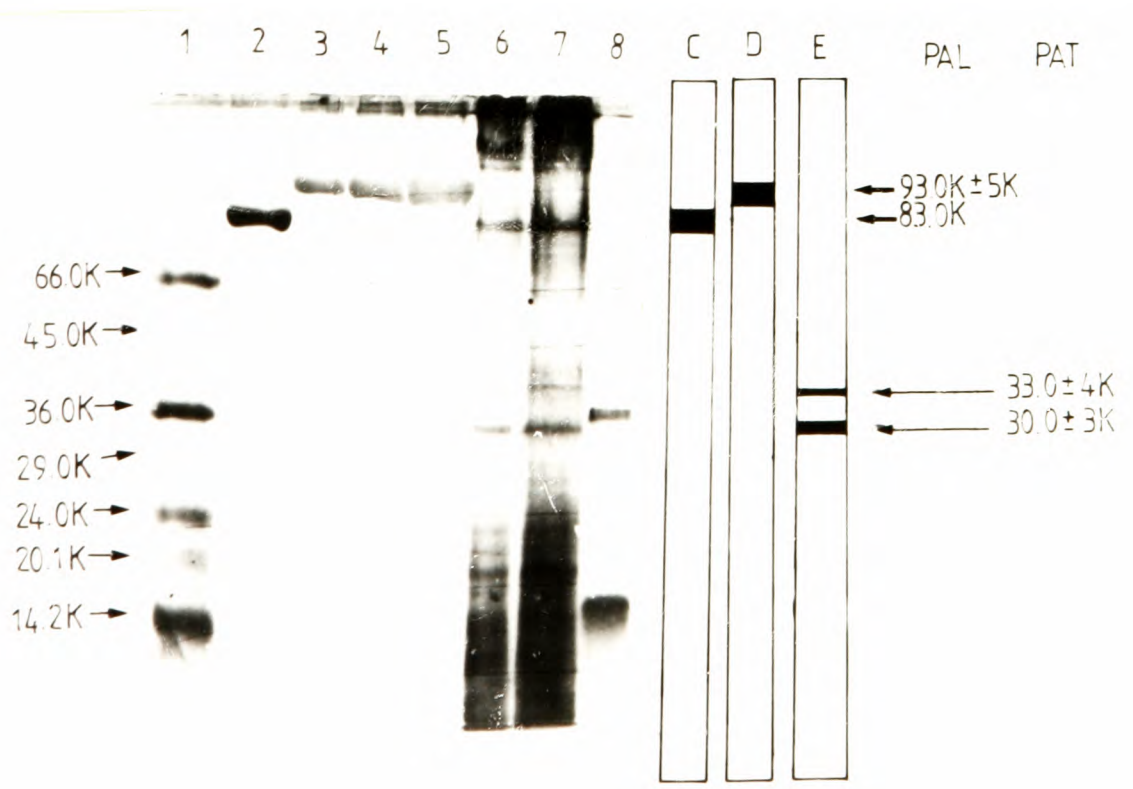
45.0K
36.0K
14.2K
PAL


1
2
3
4
5
6
7
8
C
D
E
PAL
PAT
66.0K
45.0K
36.0K
29.0K
24.0K
20.1K
14.2K
93.0K ± 5K
83.0K
33.0 ± 4K
30.0 ± 3K

were avoided.

6.3.5.2. Denaturation of PAL at low protein concentrations:

6.3.5.2.1. Denaturation in SDS:

Denaturation of PAL with SDS at low protein concentrations resulted in 3 major polypeptides smaller than 83kD (Fig. 79A; Fig.80).

6.3.5.2.2. Denaturation in guanidine-HCl:

Denaturation of affinity-purified PAL in presence of guanidine-hydrochloride gave 2 major polypeptides, the smallest polypeptide obtained above, with SDS, being absent (Fig. 79B). This is probably because guanidine-HCl is a weak denaturing agent.

6.3.5.2.3. Denaturation in urea:

Denaturation of affinity-purified PAL in urea gave 2 major polypeptides similar to that obtained in guanidine-HCl (Fig. 79C). This is probably because urea is a weak denaturing agent.

6.3.5.3. The degradation pattern that was obtained at low protein concentrations could have been due to non-enzymatic hydrolysis of peptide bonds or due to ionic interactions of peptides within the protein. The latter possibility was proven by ionisation of native PAL

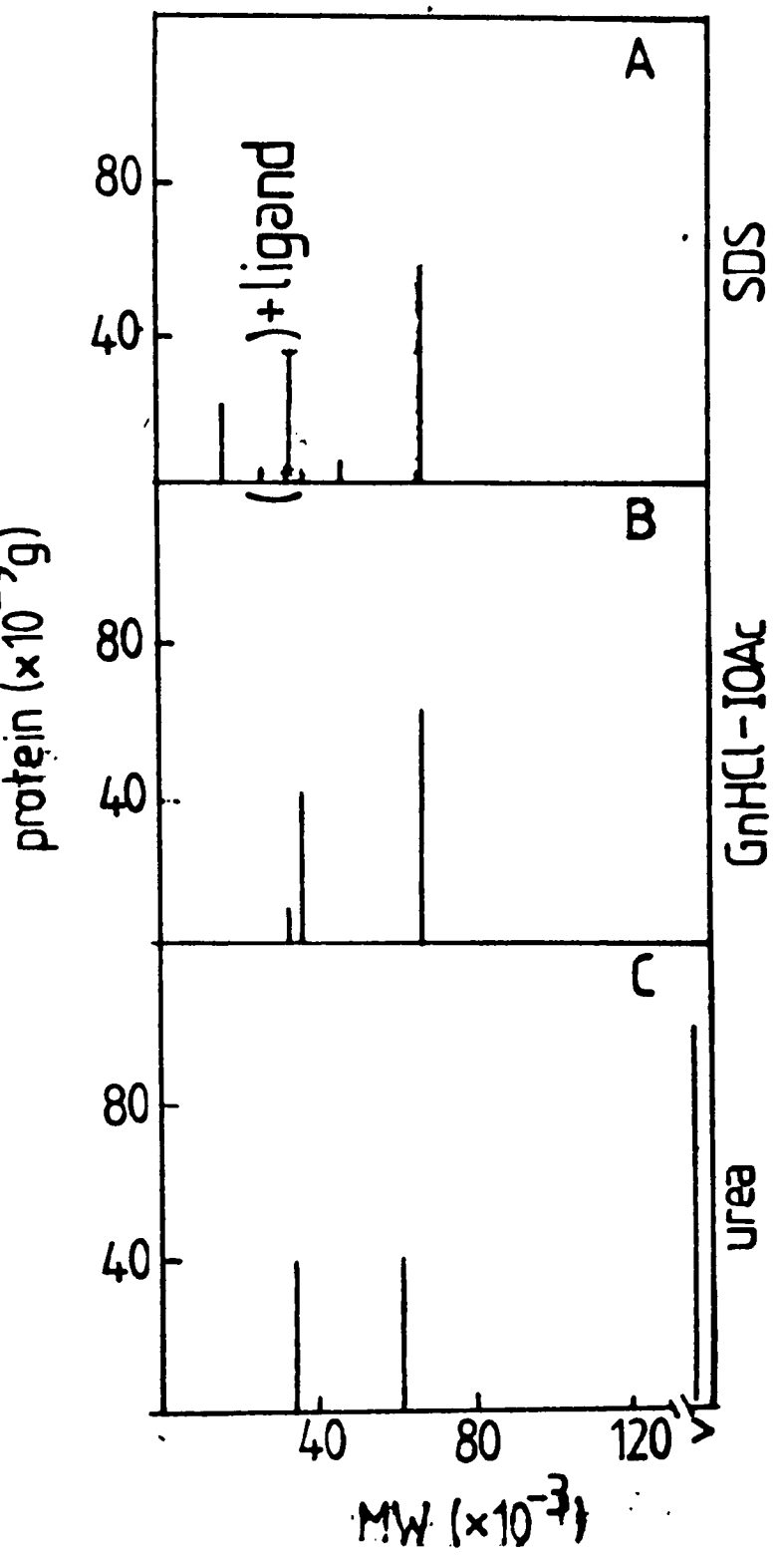


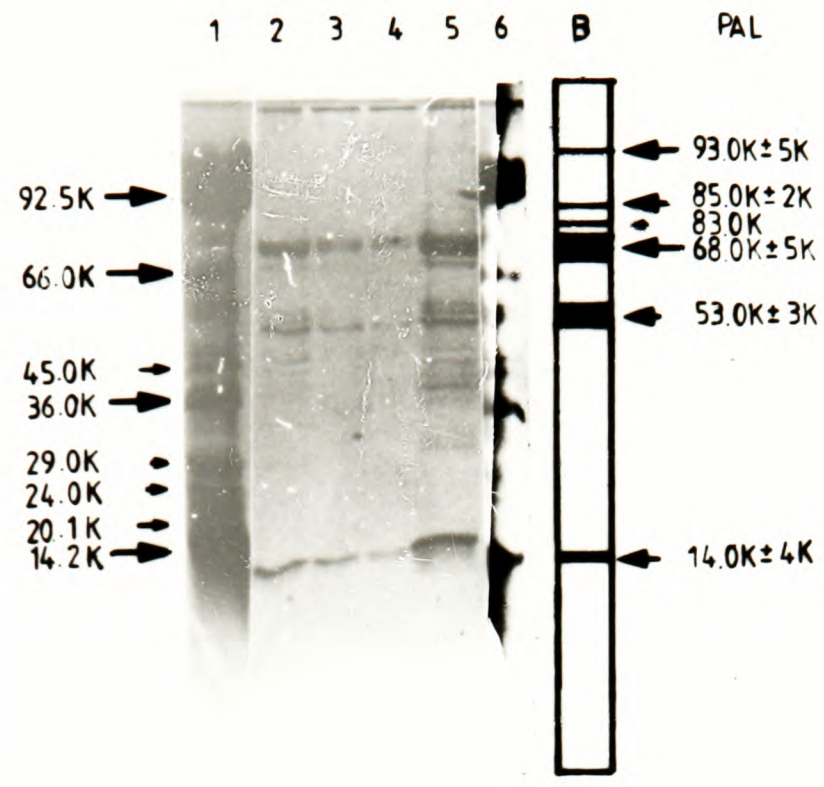
Fig. 79. Purification of PAL by affinity chromatography: analysis of denatured PAL at low protein concentrations in A, SDS; B, guanidine-HCl; C, urea.

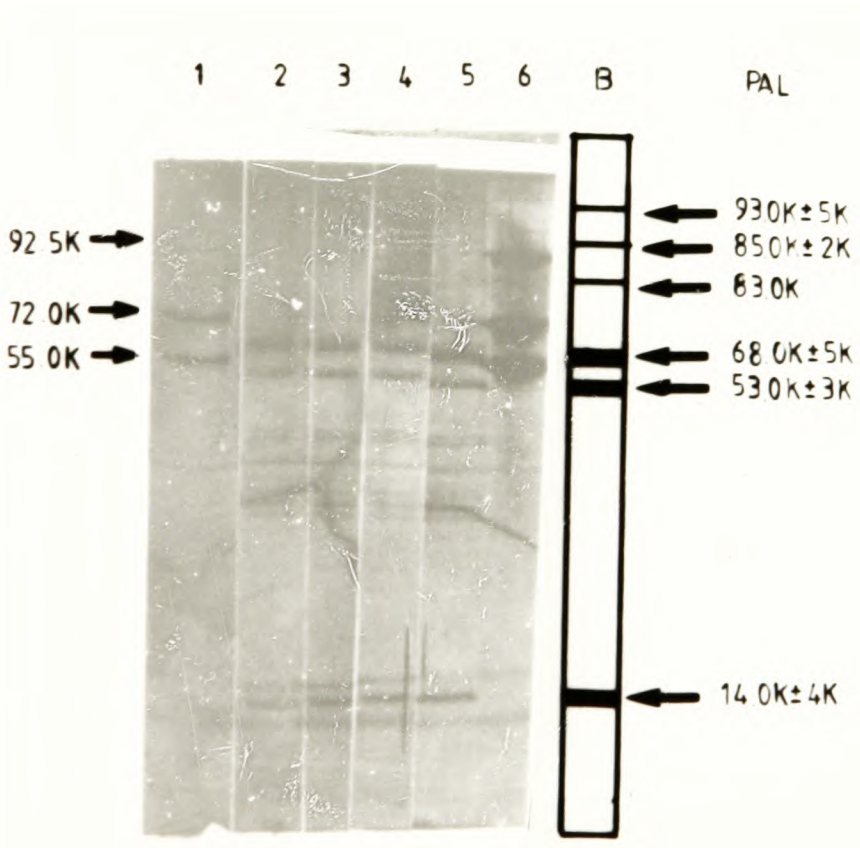
Fig. 60. Purification of PAL by affinity chromatography: analysis of denatured PAL at low protein concentrations. SDS-PAGE = 10%T. Tracks: B, facsimile of denatured affinity purified PAL protein; 1, MWM; 2 to 5: PAL protein (after affinity purification) SDS denaturation: 2, 1 x protein; 3, 2 x protein; 4, 3 x protein; 5, 4 x protein; 6, MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 81. Purification of PAL by affinity chromatgraphy: analysis of denatured PAL at low protein concentrations. SDS-PAGE : 10%T. Tracks: B, facsimile of denatured affinity purified PAL protein; 1, MWM; 2 to 5: PAL protein (after affinity purification) ionised urea denaturation: 2, 1 x protein; 3, 2 x protein; 4, 3 x protein; 5, 4 x protein; 6, MWM.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.


1
2
3
4
5
6
B
PAL
92.5K
66.0K
45.0K
36.0K
29.0K
24.0K
20.1K
14.2K
93.0K±5K
85.0K±2K
83.0K
68.0K±5K
53.0K±3K
14.0K±4K


1
2
3
4
5
6
B
PAL
92 5K
72 0K
55 0K
930K±5K
850K±2K
830K
68 0K±5K
530K±3K
14 0K±4K

during denaturation.

This reaction was performed by ionised urea, and was the result of carbamylation of polypeptides released from native PAL. In this reaction, cyanate ions act on the amino groups of PAL protein to form stable carbamlyated polypeptides with non-basic carbamyl amino groups (as opposed to basic amino groups on proteins).

$$PAL\text{-}NH_2 + NHCO \longrightarrow PAL\text{-}NH\text{-}CO\text{-}NH_2$$

The non-basic carbamyl amino groups make it possible to differentiate polypeptides of native PAL on the basis of charge and size. 3 major polypeptides similar to that obtained above for degradation of PAL at low protein concentrations were obtained by carbamylated denaturation of PAL at high protein concentrations using ionised urea (Fig. 81). The highly acidic nature of the smallest polypeptide (14kD) may explain the higher affinity of PAL for the carboxyl groups of L-phenylalanine on the L-phenyalanine affinity column during purification at low pHs of 6.0. The presence of 4 polypeptides at 55kD (2 of which are major) of near identical electrophoretic mobility and the presence of 2 bands of 46kD of near identical electrophoretic mobiity is indicative of the larger subunits contining polypeptides differing in a combination of basic/neutral charge and size.

The presence of a band running across the gel was often observed in preparations of PAL in SDS-PAGE. This property has been observed for other proteins and is due to a non-covalently associated phospholipid

or covalently attached fatty acid molecule to the PAL protein (Sarris & Palade, 1982a,b).

6.4. DISCUSSION:

Early steps in the methods used to purify PAL from Phaseolus vulgaris were common in some respects to those used to purify PAL from other sources. For example protein precipitation with ammonium sulphate and gel filtration. Later steps in the purification procedure used for Phaseolus vulgaris bean cultures and several other systems were not used. Examples include sucrose density centrifugation, ion exchange chromatography and chromatofocusing (Havir & Hanson, 1968a, 1973, ; Hodgins, 1971; Fritz et al., 1976; Jack, 1978; Gilbert & Jack, 1981; Havir, 1981; Bolwell et al., 1986). The reasons for not choosing these methods were not only their laborious and time-consuming nature but also the discrepancy in results obtained. For example preparations of PAL are thought to exist as aggregates, and exist in several isoenzymic forms, besides preparations obtained were non-homogeneous or contained more than one protein band (Hodgins, 1971; Havir & Hanson, 1973; Ussuf & Nair, 1980; Hanson & Havir, 1981; Havir, 1981; Bolwell et al., 1986). Further these methods use one and not all the unique properties of PAL.

Affinity chromatography was chosen as a later step in the purification of PAL from Phaseolus vulgaris leaves, because of the special properties of the enzyme which are involved in its binding to L-phenylalanine. These properties are a certain amount of

hydrophobicity and a certain number of basic amino groups on the surface and a characteristic pI (Blondel et al. 1973; Jack, 1978; Gilbert & Jack, 1981; Havir, 1981; Gupta & Creasy, 1984). Affinity chromatography has been attempted for purification of PAL from several sources e.g. yeast, potato and radish cotyledons (Blondel et al., 1973; Ussuf & Nair, 1980; Gilbert & Jack, 1981).

Similar methods of affinity chromatography were used for Phaseolus vulgaris as were used by the above workers and with similar results. PAL protein was bound to immobilised L-phenylalanine using its unique requirement for hydrophobic environmental conditions, required by PAL and not PAT (Jack, 1978).

PAL protein was eluted from immobilised L-phenylalanine using a combination of its properties. For example its elution at high ionic strength (>20mM) and high pH (>6.5) made use of its pI (

Blondel et al., 1973; Jack, 1978). Its elution at high temperature ($20^{o}C$), as opposed to low temperature ($4^{o}C$) made use of its unique requirement for a lipophilic environment, PAL being a membrane protein.

There have been several indications in the literature of PAT contaminants in purified PAL preparations. The following reasons increase the possibility of obtaining PAT in PAL preparations: (i) the presence of PAT protein as a common component of Phaseolus vulgaris systems (ii) the wide distribution of PAT protein

in the chloroplast, peroxisomes, and mitochondria, (iii)

the anionic nature of PAT protein , similar to PAL protein (not cationic, as animal mitochondrial PAT) and (iv) the soluble nature of PAT protein (Rehfeld & Tolbert, 1972; Wightman & Forest, 1978). The above procedure for purification of PAL from Phaseolus vulgaris resulted in preparations of PAL free of PAT protein. Like other workers, the removal of PAT contaminants from PAL protein could be carried out only prior to affinity chromatography (Jack, 1978).

It must be noted that the purification procedures described above yielded homogeneous preparations of PAL but with low increases in specific activity. Further, recoveries of PAL from preparations containing low amounts of PAL protein resulted in poor recoveries of PAL protein. Similar findings have been reported by other workers (Havir, 1981). The enzyme is therefore induced considerably prior to purification (Zucker, 1968). Similar methods (e.g. light) used by other workers prior to purification of PAL protein were used in the present study (Zucker, 1968). The reasons for these procedures have been found to be the considerably high amounts of inactive PAL enzyme that accumulate on induction and purification found for other systems such as potato and maize (Koukol & Conn 1961, Havir & Hanson, 1968[a,b], Havir & Hanson, 1973). This property seems to be shared by other major enzyme proteins with a slow turnover rate such as ribulose-bisphosphate carboxylase (Toman & Schmidt, 1985).

PAL preparations obtained by the above methods exhibited properties common to other isolated preparations[a] of PAL protein:

(i) PAL was not stable, a property shared by PAL isolated from other systems (eg. soybean, Havir, 1981).

(ii) PAL was inactivated on removal of ligand, a property found for other systems (e.g. Rhodotorula glutinis, Gilbert & Jack, 1981).

(iii) PAL formed a single peptide of 83kD, on degradation with SDS in preparations purified in considerably larger quantity as found in other systems (e.g. Rhodotorula and maize, Hodgins, 1971; Havir & Hanson,1973). This makes PAL like other enzymes that have an even number of subunits (Darnall & Klotz, 1972).

(iv) PAL formed several peptides on degradation with different denaturing agents, a property found for several other systems (e.g. wheat, Phaseolus vulgaris cultures, Rhodotorula, potato, soybean, Gilbert & Jack, 1981; see Hanson & Havir, 1981; Bolwell et al., 1986). While the reason here may be due to low protein concentrations, the reasons for the disparities in other systems may be due to (a)proteolysis during purification, (b) cleavage of a labile linkage (see Gilbert & Jack, 1981; see Hansen & Havir, 1981), (c) hydrolysis of the enzyme due to removal of

noncovalently associated phospholipids , covalently attached fatty acids (Sarris & Palade 1982 a , b) and (d) ineffectiveness of the denaturing agent for example guanidine HCl is a less effective denaturing agent for membrane proteins (Juliano, 1972).

(v) similar peptide tertiary structure but not primary structure for inactive and active enzymes as found in other systems (e.g. Rhodotorula, Gilbert & Jack, 1981).

and (vi) stabilisation by BSA as found for several enzymes (e.g. aminotransferases, Thompson, 1974).

CHAPTER 7

INDUCTION OF HYPERSENSITIVE NECROSIS AND PHYTOALEXIN SYNTHESIS IN THE PHASEOLUS VULGARIS L. - COLLETOTRICHUM LINDEMUTHIANUM L. INTERACTION : PREPARATION AND PURIFICATION OF POLYCLONAL ANTISERUM TO PHENYLALANINE AMMONIA-LYASE

7.1. INTRODUCTION:

This chapter describes the raising of a polyclonal antiserum to a homogeneous preparation of PAL protein. It describes the recognition of the antiserum for active PAL protein, the recognition of the antiserum for PAT protein, the attempts made to remove determinants which recognise PAT protein and the recognition of PAL polypeptides in the active and inactive state.

7.2. MATERIALS AND METHODS:

Abbreviations: BSA, bovine serum albumin; DEA, diethanolamine; ELISA, enzyme-linked immunoadsorbent assay; PAL, phenylalanine ammonia-lyase; PAT, phenylalanine amino-transferase;PBS, phosphate buffered saline; PVP, polyvinylpyrrolidone; SDS-PAGE, lauryl sulphate (sodium salt)-polyacrylamide gel electrophoresis.

7.2.1. METHODS:

7.2.1.1. Immunisation:

Ligand-bound PAL immunogen was suspended in PBS [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4], at a concentration of $1mg.ml^{-1}$ and mixed with an equal volume of Freund's complete adjuvant. Primary immunisation consisted of intradermal injections of 100µg ligand-bound PAL immunogen in several sites on the back of a New Zealand white laboratory rabbit. Injections were carried out by Mr James Davis at the Department of Biochemistry, University of Oxford, Oxford.

Secondary immunisation was carried out 10 days later. Ligand-bound PAL immunogen was suspended in phosphate buffered saline [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 and 8.1mM Na_2HPO_4] at a concentration of $1mg.ml^{-1}$ and mixed with an equal volume of Freund's incomplete adjuvant. Intradermal injections of 100µg ligand-bound PAL immunogen were carried out on several sites on the rabbit's back.

7.2.1.2. Bleeding:

The rabbits were bled from the ear vein. The efficient recovery of rabbits after bleeding allowed 15ml blood to be removed each time when required.

7.2.1.3. Isolation of serum from rabbit blood:

The blood was allowed to clot at 15 to 25°C and concentrated 12 hours at 4°C. The clot was detached from the walls of the container and clot-free liquid centrifuged for 30 minutes at 2500 x g at 4°C in a Sorvall RC5C centrifuge. The serum was assayed for antibody activity by the procedure given below. The serum was aliquoted and stored at -20°C or used for the isolation of IgG.

7.2.1.4. Isolation of IgG from rabbit serum:

Serum protein IgG was precipitated to 50% with $(NH_4)_2SO_4$ for 30 minutes at 25°C. The sample was centrifuged at 3,000 x g for 30 minutes at 4°C in a Sorvall RC5C centrifuge. The protein pellet was redissolved in phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl, pH 7.2] to the original volume of serum. The solution was dialysed extensively versus phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl, pH 7.2]. The IgG was aliquoted and stored at -20°C.

7.2.1.5. Enzyme-linked immunoadsorbent assay (ELISA):

7.2.1.5.1. ELISA for antibody:

Antigen was prepared at 1 to 10µg.ml^{-1} in coating buffer [1.6g l^{-1} Na_2CO_3, 2.9g l^{-1} $NaHCO_3$, pH 9.6]. 200µl antigen solution was pipetted into each well of the 96-well flat bottom microELISA plate and the plate incubated 8 to 12 hours at 4°C to coat the wells with antigen.

Unbound antigen solution was removed from the wells with 3 x 2 minute rinses at 0.250ml.well^{-1} PBS-Tween solution [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2PO_4 and 0.05% (v/v) Tween-20] at 18 to 25^{o}C. The remaining binding sites on the wells were blocked with PBS-BSA [0.14M NaCl, 2.7mMKCl, 1.5mM KH_2PO_4, 8.1mM Ha_2HPO_4 and 1% (w/v) BSA] at 18 to 25^{o}C for 1 hour at 0.250ml.well^{-1}. The plates were again washed 3 x 2 minutes with PBS-BSA-Tween [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 0.05% (v/v) Tween-20 and 1% (v/v) BSA at 0.250ml.well^{-1}. Wells coated with 0.2ml coating buffer [1.6g l^{-1} Na_2CO_3, 2.9g l^{-1} $NAHCO_3$, pH 9.6] were used as the controls.

7.2.1.5.2. Antibody coating:

Dilutions of antibody solution were prepared in distilled water and added in duplicate to each of the antigen coated wells. The plates were incubated at 4^{o}C for 8 to 12 hours and washed twice with PBS-Tween [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4 and 0.05% (v/v) Tween-20] at 0.250ml.well^{-1} for 3 minutes at 18 to 25^{o}C.

7.2.1.5.3. Enzyme conjugation and assay:

Horse-radish peroxidase conjugated to goat-anti-rabbit IgG or rabbit-anti-rat IgG were made up as a stock of 1 ml lyophilised conjugate reconstituted in 4ml 50% (v/v) glycerol. Aliquots of the stock were diluted 0.3%(v/v) in PBS-Tween ([0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 0.05% (v/v) Tween-20, 0.2% (w/v) ovalbumin and 2% (w/v) PVP] and 0.2ml pipetted into each washed well.

The plates were incubated for at least 4 hours at $4^{o}C$ before excess conjugate was removed with PBS-Tween [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4 and 0.05% (v/v) Tween-20]. Localisation of the bound enzyme was achieved by the addition of 0.2ml peroxidase substrate solution [0.05% (w/v) o-phenylenediamine, 0.03% (v/v) H_2O_2 in 0.02M CH_3COONa-NaOAc buffer, pH 5.0] to each well and the plates incubated in the dark for 30 minutes at 18 to $25^{o}C$. The reaction was stopped by addition of $0.05ml.well^{-1}$ 3M H_2SO_4. The absorption at 495nm was immediately read for each well in a microELISA MR 580 plate reader.

7.2.1.6. Purification of antibodies by immuno-affinity chromatography:

7.2.1.6.1. Preparation of immunoaffinity adsorbents:

Sepharose 4B-200 was washed in several volumes of 0.1M NaCl and distilled water in a glass-sintered funnel over gentle vacuum. `X´ ml distilled water was added to `X´ml settled gel with a few chunks of ice. $300mg.ml^{-1}$ CNBr was added to the slurry and the temperature maintained near $20^{o}C$ by addition of ice. The pH was immediately titrated to pH 11.0 with 4N NaOH. A large quantity of ice was added, and the gel filtered rapidly through a glass-sintered funnel, followed by a wash with 20 volumes of distilled water at $4^{o}C$. Protein [14.0mg PAL protein, 29.0mg PAT protein, 6.25 mg pig PAT protein] was prepared for binding by extensive dialysis versus 0.1M $Na_2B_4O_7$-HCl buffer, pH 8.3 to remove any $(NH_4)_2SO_4$. `X´ ml coupling buffer [0.1M $Na_2B_4O_7$-HCl buffer, pH 8.3 and 0.5M NaCl] containing 4mg $protein.ml^{-1}$ packed gel

was immediately added to the gel and rotated on an end to end shaker for 12 hours at 4°C. The gel was extensively washed in each of the following: blocking agent [0.1M $Na_2B_4O_7$ and 0.2M glycine , pH 8.0], coupling buffer [0.1M $Na_2B_4O_7$-HCl, pH 8.3], acetate buffer [0.1M CH_3COONa-NaOAc and 0.4M NaCl, pH 4.0], coupling buffer [0.1M $Na_2B_4O_7$-HCl, pH 8.3], acetate buffer [0.1M CH_3COONa-NaOAc and 0.4M NaCl, pH 4.0), coupling buffer [0.1M $Na_2B_4O_7$-HCl, pH 8.3], phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl, pH 7.2], DEA buffer [50mM DEA, 0.5mM Na_2EDTA, 100mM NaCl, pH 11.0], phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl,pH 7.2], glycine buffer [0.1M glycine, pH 2.5] and phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl, pH 7.2]. The gels were stored in phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 , 0.55M NaCl and 0.1%(w/v) sodium azide, pH 7.2], at 4°C.

7.2.1.2. Purification of antibodies by immunoadsorption:

Immunoadsorption was carried out on a 10 x 1cm glass column with sintered glass base, containing the desired immunoadsorbent equilibrated in phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl, pH 7.2]. A dilute solution of the antiserum was applied to the column, and the column run at a flow rate of $20ml.h^{-1}$. The absorbance was monitored at 280nm using a UVcord II connected to a Servoscribe 1s RE 546.20 potentiometric recorder. Columns were washed extensively with phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl, pH 7.2]. Adsorbed protein was eluted with 50mM DEA, pH 11.0, followed by 0.1M glycine,pH 2.5. Fractions containing protein

were pooled, immediately adjusted to pH 6.5 with Tris-base or Tris-HCl and stored at $4^{o}C$.

7.2.1.7. Purification of anti-rabbit IgG (from donkey):

Whole antibody ^{125}I-F(ab´) 2 fragment-anti-rabbit IgG (from donkey), at a specific activity of $20mCi.mg^{-1}$ [$740x\ 10^{3} KBq\ mg^{-1}$] was diluted with phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl , pH 7.2) and passed through a 10 x 1cm column containing CnBr-Sepharose-4B- chicken serum equilibrated in the same phosphate buffer [0.05M Na_2HPO_4, 0.01M KH_2PO_4 and 0.55M NaCl, pH 7.2] at a flow rate of $10ml.h^{-1}$. The eluate was collected and stored at $4^{o}C$.

7.2.1.8. Estimation of purity of antibody by immunoblotting (Western blotting):

The antigen was electophoresed in the native gel or SDS-PAGE gel by methods described in chapter 6. The tank of the `trans-blot´ cell was filled with transfer buffer [24.8mM Tris-base, 192mM glycine and 20% (v/v) MeOH (0.01% (w/v) SDS was added for denatured gels)]. The cassette was unfolded, and the following soaked in transfer buffer and placed on it: scouring pad, Whatman No. 1 filter paper, native or denatured gel, nitrocellulose sheet, 0.45nm, Whatman No. 1 filter paper, scouring pad. All air bubbles were expelled. The cassette was secured tightly and inserted in the tank containing transfer buffer, the nitrocellulose sheet facing the anode and the gel facing the cathode. Electrophoresis was carried out at a constant voltage of 30mV

for 12 hours at 4°C. The gel and nitrocellulose sheet were stained for protein with Coomassie blue as follows: The gel or blotted nitrocellulose sheet were incubated in 3 to 5 volumes of Coomassie blue stain solution [0.025% (w/v) Coomassie brilliant blue R, 45% (v/v) MeOH and 9% (v/v)glacial acetic acid, for 12 hours at 18 to 25°C. The stain solution was discarded, the gel rinsed in distilled water and placed in destain solution [7.5% (v/v) acetic acid and 5% (v/v) MeOH]. A horizontal shaker was used. The gel was destained until a clear background was obtained.

The nitrocellulose sheet containing the transferred protein was blocked with PBS-BSA [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4 and 1% (w/v) BSA] for 12 hours and washed several times in PBS [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4]. The sheet was blocked with PBS-BSA-chicken serum [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 1% (v/v) BSA and 1/100 dilution chicken serum] 2 to 12 hours and washed several times with PBS [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4]. The nitrocellulose sheet was then shaken for 4 to 16 hours with PBS-BSA-antiserum solution [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 1%(w/v) BSA and 1/100 dilution PAL antiserum, PAL IgG, pre-immune serum or non-immune serum] 4 to 12 hours and washed several times with PBS [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4]. The nitrocellulose sheet was incubated in PBS-BSA-anti-rabbit IgG [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 1% (w/v) BSA and 10^{-4}%(w/v))$^{-1125}$I-F(ab´) 2 fragment anti-rabbit IgG (from donkey) purified by affinity chromatography as described in section 7.2.1.7]. The nitrocellulose sheet was washed

extensively over 18 hours with PBS-Tween [0.14M NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 0.05%(w/v) Tween-20], blotted dry and exposed to a pre-flashed Fujifilm RX-P in a cassette containing an intensifying screen for 31 to 48 hours at -70°C. The X-ray film was developed in a 1:3 dilution of developer for 3 minutes, 1:3 dilution fixer for 6 minutes, washed in distilled water and air-dried.

7.2.2. MATERIALS:

The isolation, purification and characterisation of PAL was by methods described in Chapter 6, section 6.2. Native and denatured proteins were run on gels as described in Chapter 6, section 6.2. Chicken serum, CNBr-Sepharose-4B-chicken serum and ^{125}I-Fb(ab') 2 fragment-anti-rabbit IgG (from donkey) were a gift from Dr M.T. McManus, Department of Biochemistry, University of Oxford, Oxford. The centrifuge was from Du Pont Co., Wilmington, Delaware; electro-blot system from Biorad, Richmond, California; UVcord from LKB-Produkter AB, Bromma, Sweden; end to end shaker from Taab Labs. Equip. Ltd., Berkshire; horizontal shaker from Northern Media Supply Ltd., Humberside; microELISA plate reader and microELISA plates from Dynatech Labs. Ltd., Sussex and cassette from Genetic Research Instrumentation Ltd., Bishops Stortford, Hertfordshire. The X-ray film was from Fujimex Ltd., Swindon, Wiltshire; dialysis tubing from Medicell Int., Ltd., London; nitrocellulose from Schleicher & Schull, GmBH, Dassel, W. Germany and Whatman filter paper from Whatman Labsales Ltd., Maidstone, Kent. Freund's complete adjuvant and Freund's incomplete adjuvant were from Difco Labs, Detroit, Michigan;

Coomassie blue, Tris-base, Tris-HCl, pig PAT, CNBr, Sepharose 4B-200, PVP and BSA fraction V from Sigma Chemical Co.Ltd., Poole, Dorset; horse-radish peroxidase conjugated to goat-anti-rabbit IgG or rabbit-anti-rat IgG were obtained from Nordic Immunological Labs., Berkshire; radioactive chemicals were obtained from Amersham International, Amersham, Buckinghamshire.

Dialysis tubing was boiled in 0.01M $NaHCO_3$ and 1mM Na_2EDTA for 15 minutes prior to use.

7.3 RESULTS:

7.3.1. Recognition of native PAL by polyclonal antiserum using the ELISA:

Antisera (crude and partially purified IgG) raised to affinity-purified PAL recognised native PAL in affinity-purified eluates when diluted as much as 2×10^4 fold (Fig. 82).

7.3.2. Recognition of native PAL by polyclonal antiserum using Western blotting:

Antisera (crude and partially purified IgG) raised to affinity-purified PAL recognised native PAL in crude homogenates, partially purified homogenates and in affinity-purified eluates (Fig. 83; Fig. 84).

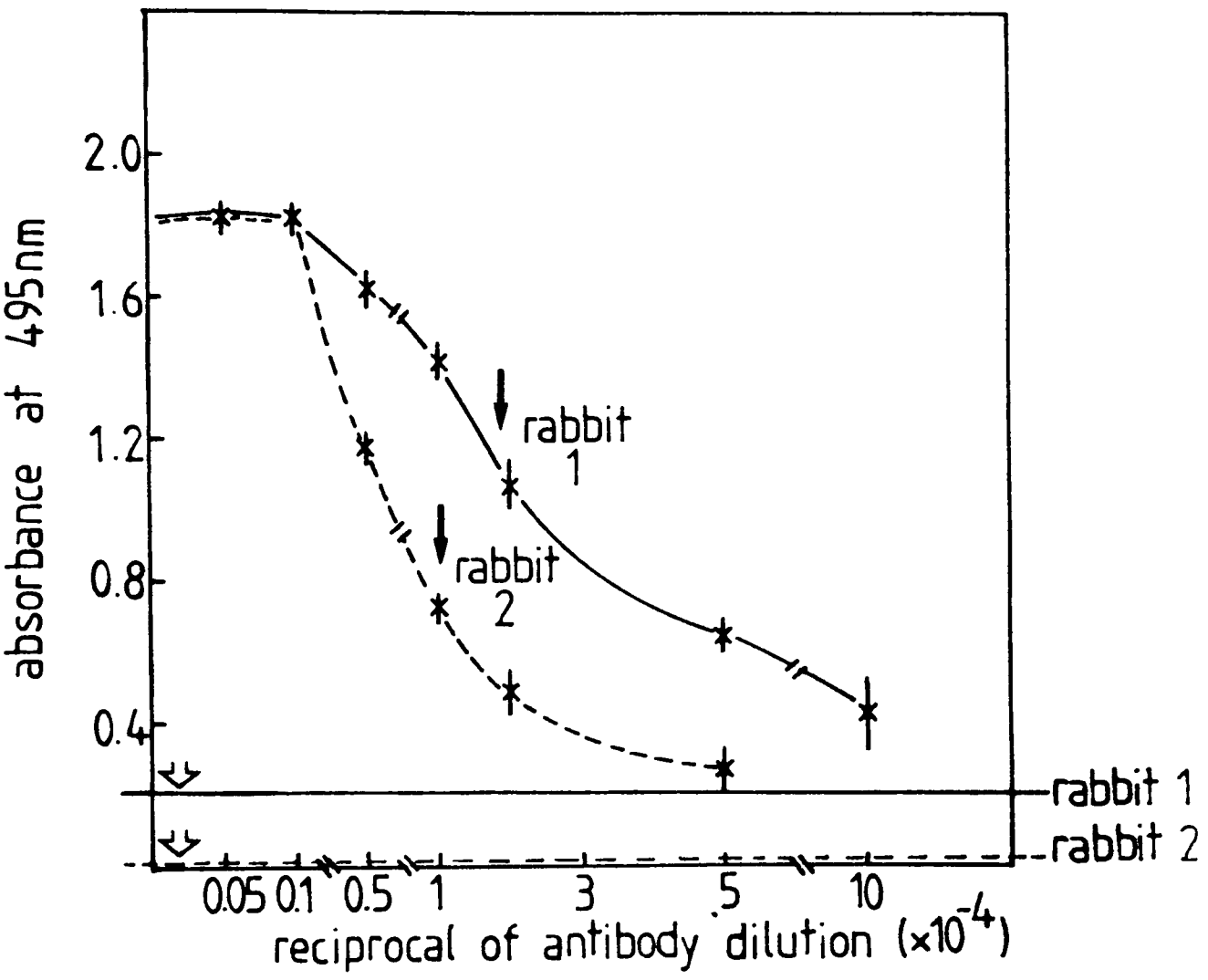


Fig. 62 Preparation of polyclonal antiserum to PAL: Comparison of ELISA of PAL antisera raised in 2 rabbits, (×——×) rabbit 1 and (×- - -×) rabbit 2. ELISA of pre-immune serum of the 2 rabbits, rabbit 1 (————)and rabbit 2 (- - - -). Arrows indicate titre values obtained (⇩) for pre-immune serum, and (⇩) for PAL antiserum.

7.3.3. Recognition of native PAT by polyclonal antiserum using Western blotting:

However, antisera (crude and partially purified IgG) raised to affinity-purified PAL recognised both native PAL and native PAT in crude homogenates and partially purified homogenates (Fig.83; Fig. 85, tracks 4,5 and 6; Fig. 86, tracks 1,2,3,4 and 5).

7.3.4. Recognition of native PAT (and not native PAL) by rabbit serum:

The serum of 2 out of 3 rabbits prior to immunisation, recognised native PAT and not native PAL in crude homogenates and partially purified homogenates (Fig. 85, tracks 1,2 and 3; Fig. 86, tracks 6, 7 and 8). However, 1 rabbit prior to immunisation showed no recognition of native PAT or native PAL in crude homogenates and partially purified homogenates (Fig. 86, tracks 9, 10 and 11).

7.3.5. Recognition of native PAL and native PAT by immuno-affinity purified polyclonal antiserum:

7.3.5.1. The polyclonal antiserum was purified on a plant-PAT-Sepharose 4B column, to remove all native PAT recognising determinants. However, this immuno-affinity - purified polyclonal antiserum still recognised PAT despite an increase in intensity of native PAL recognition (Fig. 85, tracks 7, 8 and 9).

7.3.5.2. The polyclonal antiserum was purified on an animal

(pig-heart)-PAT-Sepharose 4B column to remove all native PAT recognising determinants. This was followed by passage through a plant-PAT-Sepharose 4B column, followed by a rabbit non-immune-PAT-recognising-serum-Sepharose 4B column, to ensure removal of all PAT recognising determinants. However, this immuno-affinity purified polyclonal antiserum still recognised native PAT despite an increase in intensity of native PAL recognition (Fig. 85, tracks 13, 14, and 15).

7.3.6. Recognition of denatured PAL by polyclonal antiserum by Western blotting:

The polyclonal antiserum which recognised native PAL did not recognise PAL in the denatured state, under the conditions used for Western blotting.

7.3.7. Recognition of denatured active, inactive PAL protein and active PAT protein in bean and other system using immunoprecipitation with polyclonal PAL antiserum:

Active and inactive PAL (containing traces of PAT) and active PAT protein were immunoprecipitated with polyclonal PAL antiserum. The polypeptide pattern of each fraction was obtained on denaturation of immunoprecipitates. Results confirmed molecular weights of PAL obtained at low protein concentrations (Chapter 6, section 6.3) and those of native PAT as being different from native PAL protein (Fig. 87, tracks 3, 4, 5 and 6). This pattern of recognition was also

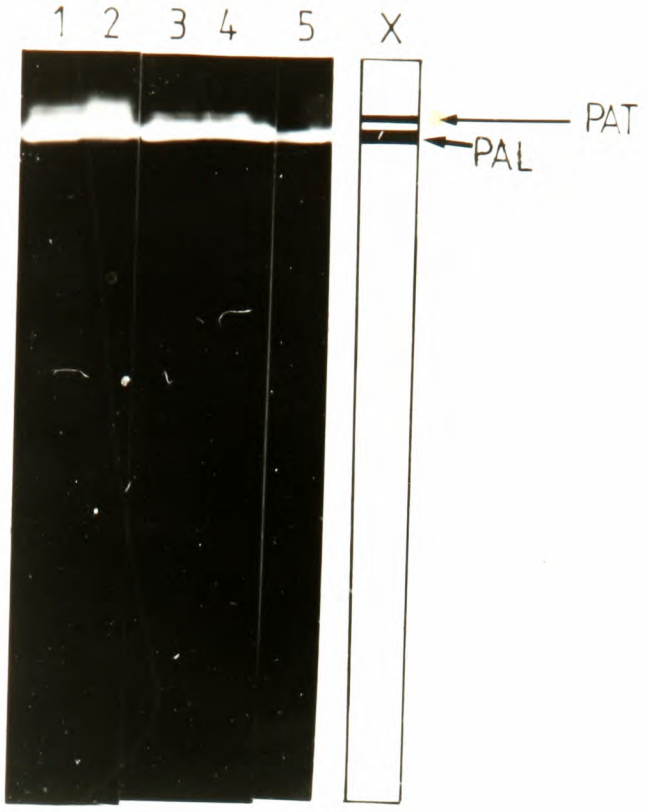
Fig. 83 Preparation of polyclonal antiserum to PAL: recognition of native PAL and PAT using crude polyclonal antiserum to PAL by Western blotting. anodic-PAGE : 10%T. Tracks: X, facsimile of native PAL and PAT protein; 1, crude extract; 2, $(NH_4)_2SO_4$ precipitate; 3, Sephacryl S-200 eluate x 1 protein; 4, Sephacryl S-200 eluate x 2 protein; 5, affinity-purified PAL eluate.

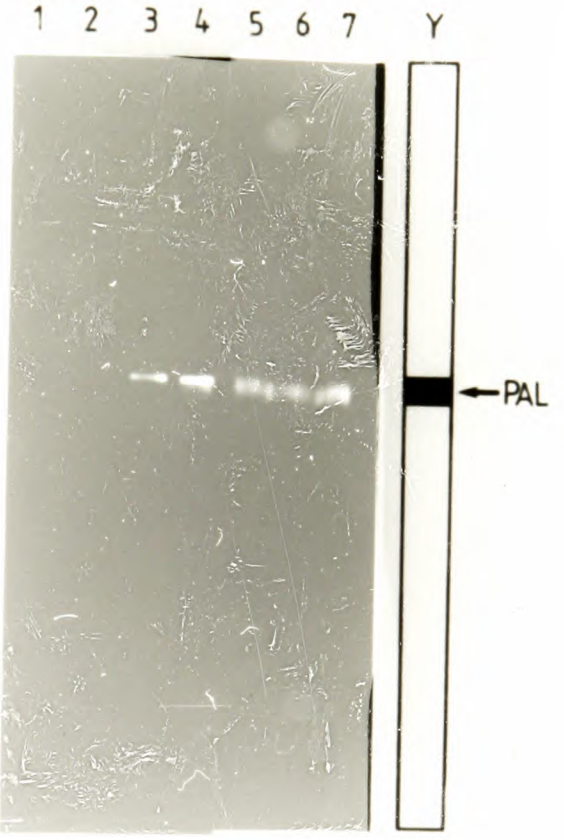
Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 84 Preparation of polyclonal antiserum to PAL: recognition of native affinity-purified PAL using IgG of polyclonal antiserum to PAL by Western blotting. anodic-PAGE : 5%T. Tracks: Y, facsimile of native PAL protein; 1, x 1 protein; 2, x 2 protein; 3, x 3 protein; x 4 protein; 5, x 5 protein; 6, x 5 protein; 7, x 5 protein.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 84 Preparation of polyclonal antiserum to PAL: recognition of native PAL and native PAT by rabbit pre-immune serum and IgG from polyclonal antiserum by Western blotting. anodic-PAGE : 5%T. Tracks: X, facsmile of native PAL and PAT protein; 1 to 3: recognition by pre-immune serum: 1, affinity-purified PAL; 2, Sephacryl S-200 eluate; 3, crude extract; 4 to 6: recognition by IgG from PAL antiserum: 4, affinity-purified PAL; 5, Sephacryl S-200 eluate; 6, crude extract; 7 to 9: recognition by IgG of PAL antiserum purified on a plant-PAT Sepharose 4B column, 7, affinity purified PAL; 8, Sephacryl S-200 eluate; 9, crude extract; 10 to 12: recognition by IgG of PAL antiserum, 10, affinity-purified PAL; 11, affinity-purified PAL; 12, affinity-purified PAL; 13 to 15: recognition by IgG of PAL antiserum purified on a animal (pig-heart-PAT Sepharose 4B column, plant-PAT Sepharose 4B column and rabbit non-immune-PAT-recognising-serum Sepharose 4B column, 13, affinity-purified PAL; 14, Sephacryl S-200 eluate; 15, crude extract; 16 to 17: recognition by IgG of PAL antiserum, 16, affinity-purified PAL; 17, affinity-purified PAL.


1 2 3 4 5
X
PAT
PAL


1 2 3 4 5 6 7
Y
PAL

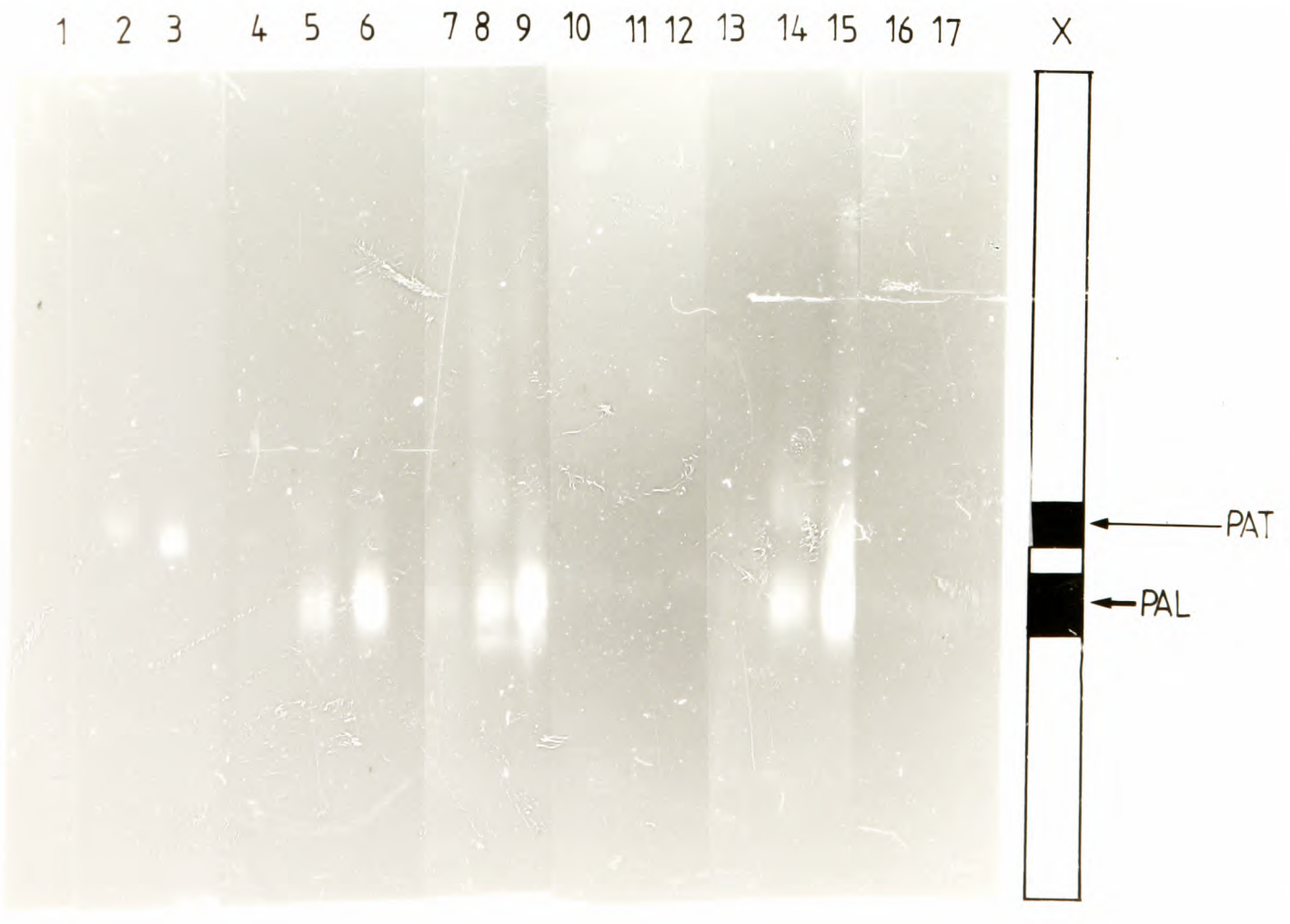
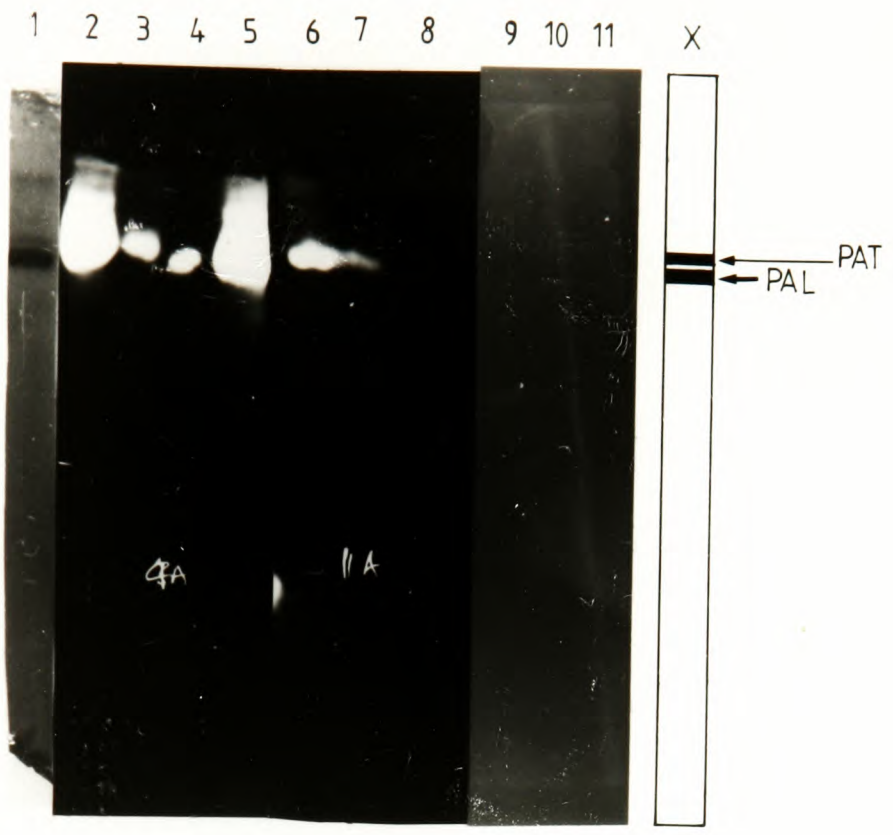

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
X
PAT
PAL

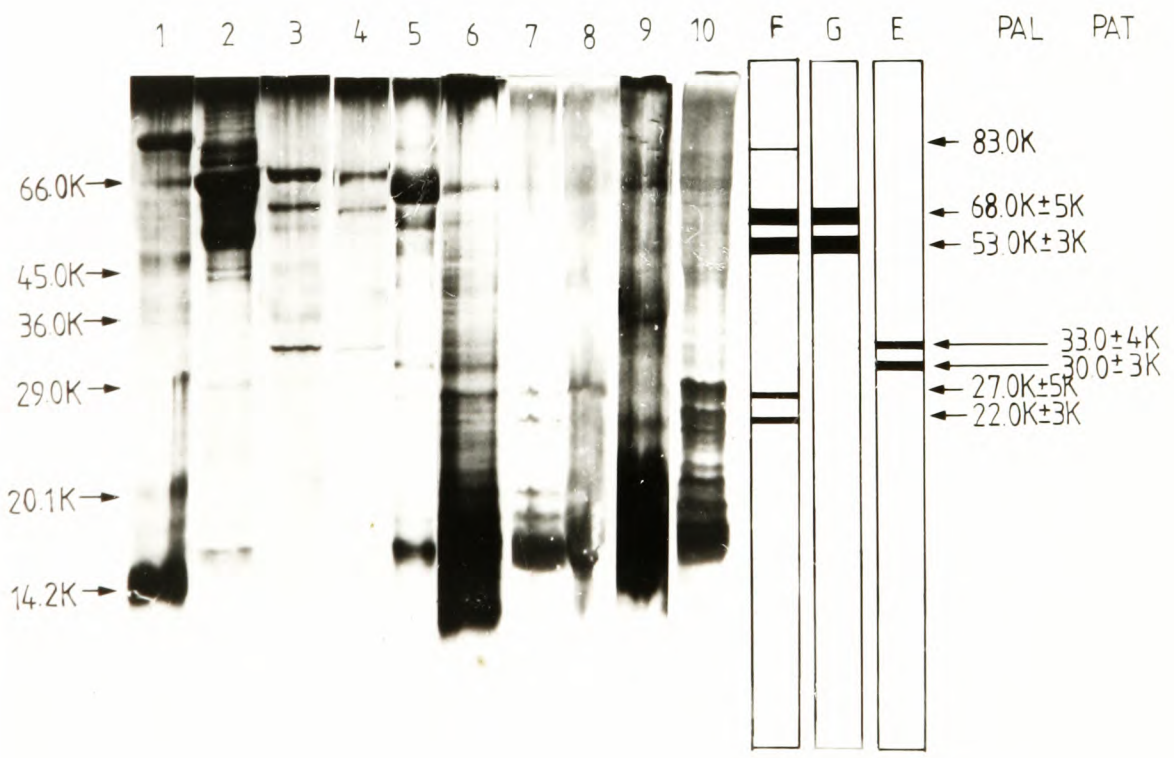
Fig. 84 Preparation of polyclonal antiserum to PAL: recognition of native PAL and native PAT by rabbit pre-immune serum and IgG from PAL antiserum by Western blotting. anodic-PAGE : 5%T. Tracks: X, facsmile of native PAL and PAT protein; 1, Coomasie blue protein stain of crude extract; 2 to 5: recognition by IgG of PAL antiserum, 2, crude extract; 3, PAT; 4, affinity-purified PAL; 5, crude extract; 6 to 8: recognition by pre-immune serum of rabbit 1 in Fig. 1, 6, crude extract; 7, PAT; 8, affinity-purified PAL; 9 to 11: recognition by pre-immune serum of rabbit 2 in Fig. 1, 9, crude extract; 10, PAT; 11, affinity-purified PAL.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.

Fig. 22 Preparation of polyclonal antiserum to PAL: denaturation of immunoprecipitate of active, inactive PAL and active PAT protein in partially purified homogenates of bean and other systems with polyclonal PAL antiserum and rabbit non-immune PAT-recognising serum: SDS-PAGE : 10%T. Tracks: E, facsimile of active PAT protein; F, facsimile of inactive PAL protein; G, facsimile of active PAL protein; 1, MWM; 2, IgG; 3 to 7: PAL and PAT in partially purified bean leaf homogenates: 3, active PAT; 4, active PAL + traces of PAT protein; 5, inactive PAL + traces of PAT protein; 6, inactive PAL + active PAL + active PAT protein; 7, PAL in hypocotyl-derived bean cell suspension; 8, PAL in mung bean seedling tissue; 9, PAL in potato tissue; 10, PAL and PAT in maize seedling tissue.

Individual tracks were cut and placed alongside for reasons of comparison. Each photograph represents a single gel that was run. For further details see 'methods'.


1 2 3 4 5 6 7 8 9 10 11 X
PAT
PAL


1 2 3 4 5 6 7 8 9 10 F G E PAL PAT
66.0K
45.0K
36.0K
29.0K
20.1K
14.2K
83.0K
68.0K±5K
53.0K±3K
33.0±4K
30.0±3K
27.0K±5K
22.0K±3K

obtained with rabbit non-immune-PAT-recognising serum (Fig. 87, track 5).

Polypeptides of native active PAL protein showed near electrophoretic mobility to those of native, active PAT protein (Fig. 87).

Denaturation of protein from various other plant *in vivo* and *in vitro* systems with polyclonal PAL antiserum resulted in similar polypeptide patterns of active, inactive PAL and active PAT protein. Amongst those systems showing inactive and possibly active PAL polypeptides were hypocotyl-derived cell suspension cultures of *Phaseolus vulgaris* L, mung bean seedling tissue, extracts of potato tissue and maize seedlings (Fig. 87, tracks 7,8,9, and 10). Amongst those systems showing active PAT polypeptides were those of maize seedling tissue only (Fig. 87, track 10).

7.4. DISCUSSION:

Polyclonal antisera have been raised to PAL from *Rhodotorula glutinis*, *Phaseolus vulgaris* cultures and *Petroselinium hortense* (Gilbert & Jack 1981, . ; Bolwell *et al.*, 1986). The antiserum raised to PAL from *Phaseolus vulgaris* leaves recognised PAL, similar to that of other antisera raised to PAL.

The recognition of PAT protein (and not other proteins) by the PAL antiserum was both curious and interesting. Antisera raised to PAL from other sources also show recognition for other proteins, most of these unknown. It is possible that one of these proteins is PAT. The specific recognition of PAT protein from Phaseolus vulgaris (and not proteins from several other sources) by the PAL antiserum from Phaseolus vulgaris indicated the presence of common epitopes on both the PAT and PAL protein. It is possible that this epitope is the active site of PAT and PAL protein because of (i) similarities in substrate, L-phenylalanine (ii) similarities in mechanism of action on the substrate, L-phenylalanine by formation of a Schiff's base (Wightman & Forest, 1978; see Hanson & Havir, 1981), (iii) possible occurrence of pyridoxal phosphate cofactors endogenous to the protein (Havir & Hanson, 1973) and (iv) near molecular weights of some PAL and PAT polypeptides (Wightman & Forest, 1978; see Hanson & Havir, 1981). These results also suggested a close ontogenic relationship between the 2 proteins, PAT and PAL.

The recognition of PAT by animal PAT serum obtained in this study was probably illustrative of determinants in the animal serum raised to bacterial aminotransferases possibly as a result of infection. It is interesting that animal systems produce a large amount of PAT protein in the serum on hypersensitive necrosis and other diseases (Bergmeyer, 1965; Decker & Kepppler, 1974).

The existence of PAL as an inactive and active form has been periodically found in the literature (e.g. Altridge & Smith, 1973; Gilbert & Jack, 1981; Faye,

1975; see Chapter 1). These forms have been identified by recognition in the native state by immunotitration or active site labelling (Gilbert & Jack, 1981; Faye, 1975). The antiserum to PAL from Phaseolus vulgaris recognised different polypeptide patterns for active and inactive PAL protein. These polypeptides resembled ligand-free and ligand-bound forms in isolated and affinity-purified PAL preparations (Chapter 6, section 6.3). Such differential recognition of polypeptides made the antiserum a very powerful tool in the estimation of active and inactive forms of PAL protein.

CHAPTER 8

CONCLUSIONS:

The conclusions of this study concerning the initial events leading to the induction of the phytoalexin response in the Phaseolus vulgaris L. - Colletotrichum lindemuthianum L host-pathogen interaction, are summarised in Fig. 88, overleaf and discussed below.

The leaves of the whole (intact) host plant respond to mycelium of the fungal pathogen by induction of necrosis. (reaction A, Fig. 88; Chapter 2, section 2.4).

Areas of the leaf expressing necrosis contain increased quantities of the phenylpropanoid-derived phytoalexin (phaseollin) after pathogen invasion. The concentrations of phaseollin that accumulate are enough to kill both the host and the pathogen, and appear to be responsible for causing the death of the host cell and necrosis (reaction B, Fig. 88; chapter 2, section 2.4).

The increase in phaseollin concentrations is associated with an increase in activity of the first enzyme leading to phenylpropanoid synthesis, L-phenylalanine ammonia-lyase (PAL) (reaction C, Fig. 88; Chapter 2, section 2.4). Standard methods for determination of PAL activities were found to lead to measurements of both phenylalanine amino-transferase (PAT) and PAL activities together. Further, PAT has a higher affinity for L-phenylalanine than PAL. Accurate measurements of PAL activities may be determined when PAT activities are inhibited

by the addition of the specific inhibitor, L-aspartic acid (Chapter 5, section 5.4).

Isolated single cells from the whole (intact) host plant, lacking the middle lamella, were able to respond to the pathogen constituent by induction of PAL activity (reaction C, Fig. 88; Chapter 2, section 2.4). This component was found to be a pathogen cell wall asialoglycoprotein (Chapter 4, section 4.4). This response was not due to wounding, as cells were optimised for viability and intactness (Chapter 3, section 3.4).

A polyclonal antiserum raised to PAL protein purified to homogeneity facilitated further studies on the regulation of PAL activities (Chapter 6, section 6.4 and Chapter 7, section 7.4). The regulation of PAL activities leading to the induction of the phytoalexin response was found to be due to the subsequent activation of de novo synthesised inactive PAL protein. This process was dependent on both substrate supply (by increased activity and de novo synthesis of PAT protein, reaction D, Fig. 88; Chapter 2, section 2.4) and on substrate availability (by a decrease in general protein synthesis, reaction E, Fig.88, Chapter 2,section 2.4). PAL activities were not found to be regulated by end product inhibition of t-cinnamic acid.

The inhibition of general protein synthesis during interaction with pathogen cell wall asialoglycoprotein seems to be associated with a depletion of important constituents of protein synthesis (e.g. UDP, reaction F, Fig. 88; Chapter 2, section 2.4). Although at the same

time there is an increase in de novo synthesis of the glycoprotein phytohemagglutinin (PHA, reaction F, Fig. 88; Chapter 2,section 2.4).

The host cell wall phytohemagglutinin was found to combine with the pathogen cell wall asialoglycoprotein to form a phytohemagglutinin-asialoglycoprotein complex (reaction G, Fig. 88; Chapter 2, section 2.4). Inducers of this recognition process appeared to be galactose and N-acetylgalactosamine. The combining process was irreversible and positively cooperative. The rate of turnover of this complex was possibly regulated by the de novo synthesis of enzymes responsible for the degradation of the PHA-asialoglycoprotein complex.

The events leading to the induction of the phytoalexin response required (i) both translation and transcription for as long as 5 hours after addition of the pathogen cell wall asialoglycoprotein and (ii) the presence of the host cell wall.

The decreased expression of the phytoalexin response is associated with the absence of necrosis and is a reflection of `susceptibility' of the host to further pathogen invasion. Susceptibility is due to the absence of induction of PAL activities by inhibition of both substrate supply and substrate availability.

Non-specific inducers of the phytoalexin response, such as abiotic compounds and continuous light periods (or induced `senescence') also induce PAL activity by regulating both substrate supply and substrate

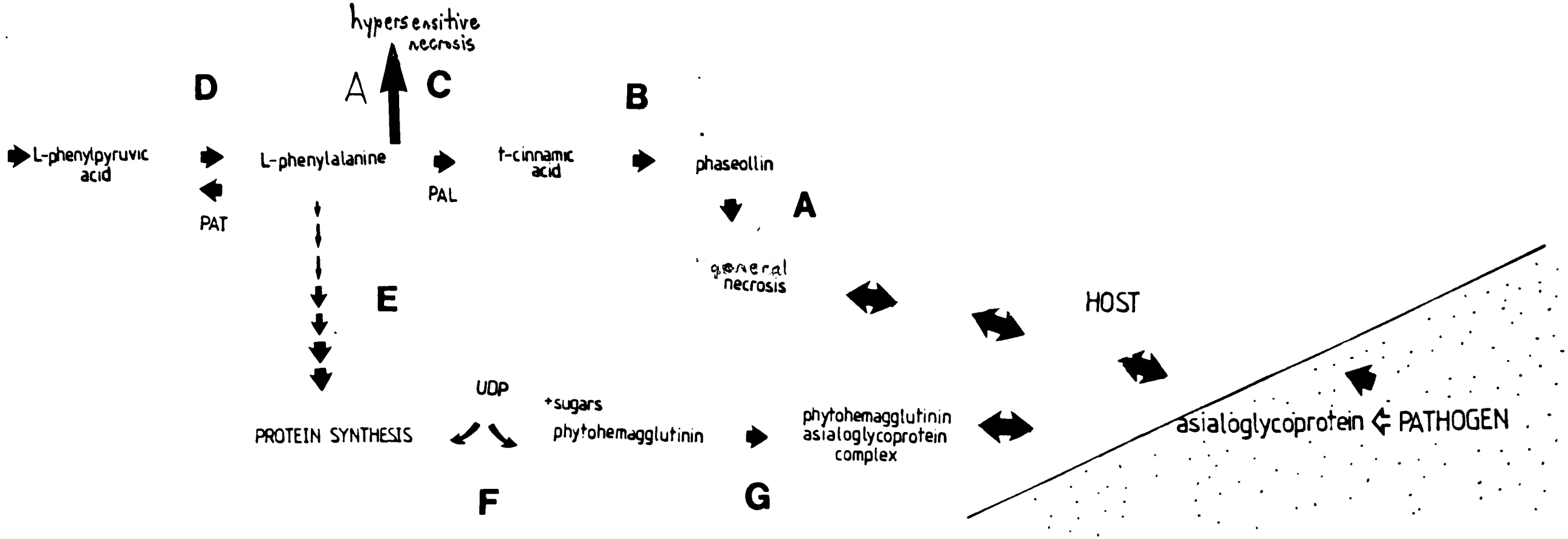


Fig. 62 Induction of general and hypersensitive necrosis and phytoalexin synthesis in the Phaseolus vulgaris L. - Colletotrichum lindemuthianum L. interaction: initial events.

A = induction of general and hypersensitive necrosis
B = induction of phaseollin (phytoalexin) synthesis leads to the induction of general necrosis
C = induction of PAL activity leads to the production of t-cinnamic acid required for phaseollin formation
D = induction of PAT activity leads to the supply of substrate, L-phenyalanine required for PAL activity
E = inhibition of protein synthesis leads to the availability of substrate L-phenylalanine required for PAL activity and possibly hypersensitive necrosis
F = induction of phytohemagglutinin synthesis uses metabolites required for protein synthesis (e.g. UDP)
G = induced synthesis of the phytohemagglutinin-asialoglycoprotein complex.

availability.

The work in this thesis reports the importance of `source´ metabolism and recognition between molecules of the host and pathogen for expression of the phytoalexin response. This thesis confirms the importance of L-phenylalanine ammonia-lyase activities in inducing the phytoalexin response and illustrates the dependence of PAL activity on both the supply and availability of L-phenylalanine governed by the enzyme L-phenylalanine amino-transferase.

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