Expression and characterization of Disproportionating enzyme from *Manihot esculenta* Crantz and *Arabidopsis thaliana* and their production of cycloamyloses

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Abstract

4-α-Glucanotransferase or disproportionating enzyme (D-enzyme, DPE) catalyzes the α-1,4 glycosyl transfer between oligosaccharides. Type I D-enzyme (DPE1) can transfer maltosyl unit from one 1,4-α-D-glucan to an acceptor mono- or oligo-saccharide, which reflects the physiological role of DPE1 in plant starch metabolism. In this study, the genes encoding DPE1 from *Arabidopsis thaliana* (At*DPE1*) and *Manihot esculenta* Crantz cultivar KU50 (Me*DPE1*) were cloned and expressed in *Escherichia coli* and purified to homogeneity. Me*DPE1* encoded 585 amino acid residues, including a 56 residue signal peptide, while At*DPE1* encoded 576 amino acid residues with a 45 residue signal peptide. The molecular mass of both mature enzymes, estimated from deduced amino acid sequence, were the same at 59.4 kDa, with a pI of 5.13. The predicted structures of both enzymes showed the conserved 250's loop and three catalytic amino acid residues, characteristics of disproportionating enzymes in the GH77 glycoside hydrolase family. Biochemical characterization showed that both purified recombinant enzymes were homodimers in solution, with similar optimum pH and temperature for disproportionating activity at pH 6-8 and 37 °C. Using potato amylose as a substrate, AtDPE1 can produce cycloamyloses in the range 16-50 glucose residues, while products from the action of MeDPE1 on the same substrate were in a wider range of 16 to DP>60. These recombinant enzymes are useful tools for elucidation of their functional roles in starch metabolism and for applications in the starch industry.

Keywords:

D-enzyme, GH77 family, *Manihot esculenta* Crantz, *Arabidopsis thaliana*, cycloamylose

Introduction

4-α-Glucanotransferase (EC 2.4.1.25), an enzyme in glycoside hydrolase family 77 (GH77) [1], transfers a sugar moiety from the non-reducing end of an α-1,4-glucan (donor) to the non-reducing end of another α-1,4-glucan (acceptor) chain. This enzyme is known as disproportionating enzyme (DPE), or D-enzyme, in plants [2] and reported as amylomaltase in bacteria [3]. D-enzyme and amylomaltase are both classified in the GH77 family, but differ in their substrate and reaction specificity [3-6]. D-enzyme was first discovered in potato tubers as two isoforms, DPE1 and DPE2, which differ in their structure and function [4, 5, 7]. DPE1 catalyzes an intermolecular transglycosylation reaction by transferring a maltosyl unit from the non-reducing end of a donor α-1,4-glucan, in particular maltotriose, to another acceptor α-1,4-glucan chain. In contrast, whilst DPE2 catalyzes a similar transglycosylation reaction, it transfers single glucosyl residues and can use maltose as a glucosyl donor. DPE2 has been characterized in Arabidopsis [8-10], potato [7] and rice [11]. Arabidopsis DPE2 has been shown to be a versatile biocatalyst, accepting a range of natural and non-natural (e.g. fluorinated) monosaccharide acceptors [12, 13]. The *dpe1* mutant of Arabidopsis accumulated maltooligosaccharides, especially its preferred substrate maltotriose, but does not accumulate maltose [14]. It was proposed that DPE1 plays an important role in the conversion of transitory starch into sucrose in leaves at night [15, 16]. *In vitro* DPE1 from adzuki bean cotyledons can catalyze intermolecular transglycosylation to produce acarviosyl-maltooligosaccharides from acarbose [17] and purified DPE1 from Arabidopsis leaf has been used to produce maltodextrin from maltotriose [6]. Apart from the intermolecular transglycosylation, DPE1 from potato catalyzed intramolecular transglycosylation (cyclization) of amylose to form cyclic α-1,4-glucans (cycloamyloses), with degree of polymerization (DP) ranging from 17 to several hundred [18]. A number of enzymes, such as amylomaltase and cyclodextrin glucosyltransferase, have been reported to be capable of producing cycloamyloses [19].

Cassava (*Manihot esculenta* Crantz) is grown in tropical region (Africa, Asia and the Americas). The high starch content (20-40%) of cassava tubers makes them an important feedstock for use in culinary, animal feed and starch-based products [20]. The Arabidopsis genome has been well studied, with support from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/)[21] and the

Cassava genome is now available on the phytozome genome browser (http://www.phytozome.net/cassava) [22]. D-enzyme genes have been identified in both the cassava and Arabidopsis genomes, but the corresponding recombinant enzymes have not been characterized to date. In this study, we report the cloning and expression of the *DPE1* gene from both plants, along with the characterization of the recombinant enzymes and assessment of their ability to produce useful products, such as cycloamyloses.

1. Materials and methods

1.1 Stock cDNA

Cassava cDNA was obtained from *Manihot esculenta* Crantz KU50 (courtesy of Dr. Yindee Chanvivattana, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand). *Arabidopsis* cDNA stock number U12707, specific to the *DPE1* gene (Genbank accession number AY081744), was purchased from ABRC (Ohio State University).

1.2 Cloning of *DPE1* gene from cassava

The coding region for 4-α-glucanotransferase from cassava (*Manihot esculenta* Crantz: Me*DPE1*) was identified in Phytozome v6.0 (Locus name: cassava4.1_008552m.g). A PCR fragment was obtained using the forward primer 5'– TTC GAA GCA GTT TCT TTA TCC TCT ACC–3' (introduced *Bst*BI site underlined) and reverse primer 5'– GTC GAC CAC CCG CCC ATA CAT TG -3' (introduced *Sal*I site underlined). The forward primer was designed to remove the signal peptide. The PCR program was 2 minutes at 95 °C, 32 cycles of: 8 sec at 98 °C, 20 sec at 70 °C, 105 sec at 72 °C, followed by final elongation for 10 minutes at 72 °C. The PCR reaction was carried out with 1 unit of Phusion High-Fidelity DNA polymerase (Thermo scientific) according to manufacturer instructions, with a final concentration of each primer at 0.2 pmol/µl in 50 µl total volume.

The PCR amplified Me*DPE1* gene was cloned in pTrcHis2c vector (Invitrogen) with two restriction sites (*Bst*BI and *Sal*I) and a C-terminal hexa-histidine tag (amino acid sequence: VDHHHHHH), the plasmid obtained was denoted pTMe*DPE1*. The pTMe*DPE1* was transformed into *E. coli* DH5α by the $CaCl_2$ method and correct

insertion of the *DPE1* gene was confirmed by DNA sequencing.

1.3 Cloning of the *DPE1* gene from *Arabidopsis*

The sequence of the *Arabidopsis thaliana DPE1* gene (At*DPE1*) was identified at locus AT5G64860 in The *Arabidopsis* Information Resource (TAIR) database website. PCR amplification was designed to remove the signal peptide using forward primer 5'- CACC ATG GAG GTC GTT TCG AGT AAT TCC –3' with CACC for directional cloning (underlined) and reverse primer 5' -TCA AAG CCG TCC GTA CAA TGA CAA AAG ATC -3', using the same PCR program as for Me*DPEI.*

The PCR amplified At*DPE1* was cloned in pET151-TOPO vector (Invitrogen) using the Champion™ pET151 Directional TOPO (Invitrogen), which inserts an N-terminal hexa-histidine tag and a V5 epitope beyond a TEV cleavage site, and the plasmid obtained was denoted pETAt*DPE1*. This plasmid was transformed into the expression host BL21 Star™ (DE3) One Shot® (Invitrogen) and screening was performed as described for the cloning of Me*DPE1*.

1.4 Expression and purification of DPE1

Transformed *E. coli* cells containing recombinant plasmids pTMe*DPE1* and pETAt*DPE1* from either cassava or Arabidopsis were separately inoculated into 5 ml LB medium containing ampicillin (100 µg/ml) and cultivated overnight at 37 °C. Each culture (1% v/v) was inoculated into 200 ml Auto Induction Media (AIM) containing ampicillin (100 µg/ml). The cultures were grown at 37 °C 250 rpm while OD_{600} reached 0.5-0.6. The temperature was decreased to 16 °C and the cultures were further incubated at 250 rpm overnight (18 hours). Bacterial cells were collected by centrifugation at 5000 x g for 15 minutes. Cell pellets were resuspended in cell lysis solution (50 mM Tris-HCl pH 8.0, 0.5 M NaCl, 30 mM Imidazole, 0.3 mg/ml DTT, 50 µg/ml lysozyme, 1 tablet per 50 ml cOmplete protease inhibitor cocktail (Roche) and 12.5 µg/ml DNase) and lysed with a cell disruptor (constant system) at 25 kpsi. Crude lysate were cleared by centrifugation at 30,000 x g for 30 minutes at 4 °C.

Crude MeDPE1 and AtDPE1 were applied to 5 ml Ni HiTrap IMAC FF columns (GE Healthcare) washed with 50 mM Tris-HCl pH 8.0, 0.5 M NaCl and 30 mM imidazole, and eluted with a linear gradient of 30 mM to 500 mM imidazole in 50 mM Tris-HCl pH 8.0 containing 0.5 M NaCl. The enzymes were further purified by gel filtration (HiLoad 26/60 Superdex 75 prep grade, GE Healthcare), eluted with

100 mM NaCl in 50 mM HEPES pH 7.5. Single peaks of protein were pooled, concentrated and buffer-exchanged into 20 mM HEPES pH 7.0 with Centrifugal Filter Units of MWCO 30,000 (Millipore, US).

1.5 Disproportionation activity assay

DPE1 assay was modified from that used by Peat *et al.* for the potato enzyme [5]. The reaction mixture, containing 54 μM maltotriose in 100 mM MOPS-NaOH pH 7.0, was incubated with the purified DPE1 at 37 °C for 15 minutes and the reaction was stopped by heating to 95 °C in boiling water for 5 minutes. The amount of glucose released was determined by using the hexokinase-G6P dehydrogenase method [13]. The DPE1 reaction mixture (20 μl) was incubated with hexokinase assay cocktail: 1 mM $MgCl_2$, 0.6 mM Adenosine 5′-triphosphate disodium salt hydrate (ATP, Sigma), 0.44 mM β-nicotinamide adenine dinucleotide hydrate (NAD) and 3.3 unit/ml hexokinase (Roche) in 27.5 mM HEPES pH 7.9 in 178 μl. The starting absorbance (Abs1) at 340 nm was recorded. Two units of glucose-6-phosphate dehydrogenase (G6P-DH, Roche) in 2 μl were added and, after incubation for 10 minutes at 21 °C, the final absorbance (Abs2) at 340 nm was recorded. The change in OD at 340 nm was used to calculate the glucose released, in comparison to a standard curve and one unit of DPE1 activity was defined as the amount of enzyme which released 1 μmol of glucose per minute under the assay conditions. Disproportionation activity was also monitored by iodine staining of non-denaturing polyacrylamide electrophoresis gels containing glycogen. DPE1 preparations were separated by electrophoresis on 0.75 mm thick polyacrylamide gel containing 10% (w/v) acrylamide and 0.3% (w/v) rabbit liver glycogen (Sigma), at 16 mA per slab at 4 °C in a Mini-Gel electrophoresis unit (Bio-Rad). After electrophoresis the gel was incubated in substrate solution buffer (50 mM Tris-HCl pH 8.0, 8 mM EDTA and 0.3% maltodextrin) at room temperature overnight. The gel was rinsed several times with distilled water and stained with iodine solution (0.2% I_2 in 2% KI).

1.6 Characterization of DPE1

The effect of pH and temperature on the disproportionating activity of purified MeDPE1 and AtDPE1 were studied. The enzymes were incubated in 0.1 M buffer at different pH values [sodium acetate buffer (pH 3.0, 4.0, 5.0), sodium phosphate buffer (pH 5.0, 6.0, 7.0), Tris-HCl (pH 7.0, 8.0, 9.0), MOPS (pH 7.0, 8.0), Glycine-NaOH

(pH 9.0, 10.0)]. For optimum temperature measurements, 0.1 M MOPS buffer pH 7.0 was used. The enzymes were incubated in the temperature range 5-60 °C for 15 minutes in the disproportionation activity buffer. Molecular weights of both recombinant enzymes were determined by gel filtration and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(Figure 3C).

For kinetic studies, the initial velocities of the enzymatic reaction were determined by varying the concentration of maltotriose (2-60 mM). Values of the Michaelis constants (K_m and k_{cat}) were calculated.

1.7 Cycloamylose production

Amylose type III from potato (Sigma) was solubilized in water at 2% w/v by boiling for 15 minutes and centrifuged at 5000 x g for 15 minutes to remove the insoluble fraction. The soluble material was dialyzed (MWCO 12000-14000, Cellu-Sep T4, Membrane Filtration Products inc., USA) to eliminate residual glucose and short chain oligosaccharides. The amylose solution was incubated with recombinant MeDPE1 or AtDPE1 (1.2 U/ml) in 5 mM phosphate buffer pH 7.0 (10 ml reactions) at 37 °C for 18 hours and the reaction was stopped by heating to 95 °C for 15 minutes. To remove residual linear glucan amyloglucosidase from *Rhizopus* sp. (Megazyme) was added (1.5 U/ml), incubated at 37 °C and the reaction was stopped as above. The precipitate was removed by centrifugation at 12,000 x g for 30 minutes. The cycloamylose product was precipitated by addition of absolute ethanol (to final concentration 81%), and collected after 2 days by centrifugation at 12,000 x g for 30 minutes. The precipitate was washed twice with absolute ethanol and dissolved at 2 mg/ml in distilled water. Analysis of cycloamylose was performed on high performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD, Dionex-500, USA) equipped with a CarboPac PA-100 column (4x250 mm, Dionex). Elution was performed using a linear gradient of sodium nitrate in 150 mM NaOH (4 to 8% for 0-2 minutes; 8 to 18% for 2-10 minutes; 18 to 28% for 10-20 minutes; 28 to 35% for 20-40 minutes; 35-45% for 40-45 minutes; 45-63% for 55-60 minutes) at a flow rate 1 ml/min. The size of cycloamylose products was compared with standard cycloamyloses, with an average molecular weight of 7000 Da (Ezaki Glico, Japan). The molecular mass spectrum of cycloamyloses was obtained by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-

TOF-MS).

2. Results and Discussions

2.1 *DPE1* gene sequence

Nucleotide sequence analysis of *Arabidopsis thaliana* (At*DPE1* gene, accession number: NP_201291.1) predicted by Chlorop V1.1 showed the presence of an open reading frame of 1731 bp encoding 576 amino acid residues with a 45 amino acid signal peptide at the N-terminus (Figure 2A). For *Manihot esculenta* Crantz (Me*DPE1* gene, Phytozome v6.0: Locus name: cassava4.1_008552m.g), the nucleotide sequence showed the presence of an open reading frame of 1758 bp encoding 585 amino acid residues with 56 amino acid residues as the signal sequence. Molecular weight prediction using Compute pI/Mw Tool from the ExPAsy server [23] indicated that both MeDPE1 and AtDPE1 had the same predicted molecular weight and isoelectric point (59 kDa, pI 5.13). The deduced amino acid sequences of AtDPE1 and MeDPE1 were aligned with potato DPE1 (StDPE1) and bacterial amylomaltase, which are also members of glycoside hydrolase family 77. The BLAST program in GenBank showed plant DPE1 were closely related with approximately 70% identity (Table 1). No signal peptide was present in the sequences of the bacterial amylomaltases [24] (Figure 2A). The members of glycoside hydrolase family 77 all contain 250's loop as a conserved region, which is not present in glycosyl hydrolase with a similar structure. Three catalytic amino acid residues were identified; one glutamic and two aspartic acids (Figure 1, 2C).

StDPE1 from potato (PDB code: 1X1N) was used as template for simulation of three dimensional structures of MeDPE1 and AtDPE1 using I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The models showed a $(\beta,\alpha)_8$ barrel core, 250's loop and three catalytic amino acids in both enzymes (Figure 2B, 2C), supporting the result from amino acid sequence alignment. Further investigation of the crystal structures and ligand binding sites of both MeDPE1 and AtDPE1 are underway.

2.2 Expression and Purification of DPE1

In an initial attempt at heterologous expression, the Me*DPE1* gene was amplified from cassava without the signal peptide, which is likely to interfere with solubility and is not expected to have any catalytic function. This was cloned in to

pET17b, which carries an N-terminal T7 tag and expressed in *E. coli* BL21 (DE3) at 37 °C. However, the protein was expressed in insoluble form and no activity was detected. Expression at lower temperature (16 °C) did not improve the expression of MeDPE1. The Me*DPE1* gene was subsequently cloned into the pTrcHis2C vector (Invitrogen) under the *trc* promoter, as reported for potato *DPE1* [5]. This vector, which inserts 6 histidines at the C-terminal, was employed successfully to express MeDPE1 in *E. coli* DH5α at 16 °C. AtDPE1 was successfully expressed under the T7 promoter using the pET151/D-TOPO^{TM} (Invitrogen) expression vector in *E. coli* BL21 (DE3) at 16 °C.

Transformed *E. coli* strains carrying plasmids for the expression of MeDPE1 and AtDPE1 were grown and induced with lactose at 16 °C. Disproportionating enzymes activity was detected in the soluble cytosolic fraction of *E. coli* cells and the enzymes were purified using Ni affinity chromatography (HiTrap IMAC FF), followed by size exclusion chromatography (HiLoad 26/60 Sephadex 75 prep grade). Chromatograms from gel filtration show single peaks (Figure 3A, 3B) with the calculated molecular weight of the recombinant MeDPE1 and AtDPE1 of 117.03 kDa and 123.17 kDa, respectively. The apparent molecular weights on SDS-PAGE were similar at 66 kDa (Figure 3C), which corresponds well to the molecular weight, calculated from the deduced amino acid sequences of 66 kDa for the monomer. The results suggested both enzymes were homodimers in solution, as previously reported for the potato and pea enzyme [25] while amylomaltases from *Thermus aquaticus* ATCC 33923 [26] and *T. brockianus* [27] were reported to be monomers.

2.3 Characteristics of recombinant MeDPE1 and AtDPE1

Native polyacrylamide gel electrophoresis of MeDPE1 and AtDPE1 on gels containing glycogen gave a positive iodine stain on addition of the transglycosylation substrate maltotriose, confirming the transglycosylation activity of both proteins [14]. Plant DPE1 differs from other GH77 family enzymes, such as amylomaltase and DPE2, in its ability to transfer two glucose units in the transglycosylation reaction rather than one [12]. MeDPE1 and AtDPE1 showed similar optimum pH and temperature for disproportionating activity to other plant D-enzymes (Table 1) which correlated to wild type Arabidopsis DPE1 from leaf at pH 6.5 [6]. In contrast, the characterized bacterial amylomaltases are more tolerant to high temperature and low pH. The kinetic parameters for disproportionating activity of both MeDPE1 and

AtDPE1 were determined with maltotriose as the substrate. The K_m and k_{cat}/K_m were 69.2 ± 10 mM and 12.9 ± 0.2 $mM^{-1}s^{-1}$ for MeDPE1 and 27.5 ± 6 mM and 21.6 ± 3 $mM^{-1}s^{-1}$ for AtDPE1, respectively. This indicates that AtDPE1 is approximately twice as active toward maltotriose as MeDPE1. Crucially transitory starch degradation occurs daily in leaves and AtDPE1 is proposed to be involved in the process [14]. On the other hand, starch synthesis is far more prominent than degradation in cassava tubers. Thus, the observed differences in kinetic properties between the *Arabidopsis* and cassava enzymes may reflect the different physiological contexts in which they operate.

2.4 Identification of reaction products

The ability of MeDPE1 and AtDPE1 to form cycloamyloses when fed long chain amylose was also investigated. Previously, cycloamylose production were reported mostly in bacteria such as *T. aquaticus* ATCC 33923 [3] and *Pyrococcus kodakaraensis* KOD1 [28]. StDPE1, the potato DPE1, is the only plant enzyme reported to produce cycloamyloses [18]. Whether these reactions occur *in vivo* remains to be established. HPAEC-PAD analysis of the products from reactions of MeDPE1 and AtDPE1 with potato amylose revealed the presence of cycloamyloses from DP16 upwards (Figure 4A). AtDPE1 produced cycloamyloses in the range DP 16 to 50 with maximum intensity at DP 18, while cycloamyloses produced by MeDPE1 showed maximum intensity at DP 19 but range up to DP>60. Products analysis by MALDI-TOF-MS showed molecular masses corresponding to cyclic molecules containing different numbers of glucose units, with regular mass differences between peaks corresponding to the mass of one glucosyl unit (162 Da) in a polysaccharide chain (Figure 4B), and correlated well to the theoretical molecular weight of cycloamyloses previously reported [29].

3.Conclusion

Two plant disproportionating enzyme (DPE1) from *Manihot esculenta* Crantz and *Arabidopsis thaliana* were successfully cloned, over-expressed in *E. coli* and characterized. These enzymes showed similar biochemical characteristics, but with notable difference in their kinetic parameters and their ability to form longer chain cycloamyloses (DP >60). These recombinant enzymes are useful tools for the study of starch metabolism and may find application in the production of cycloamyloses.

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