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Running head

Purification and determination of ADAMTS-4 and ADAMTS-5 activity

Purification and activity determination of ADAMTS-4 and ADAMTS-5 and their domain deleted mutants

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Abstract

A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs-4 (ADAMTS-4) and ADAMTS-5 are zinc-dependent metalloproteinases that are involved in the maintenance of cartilage extracellular matrix (ECM) and are currently considered to be the major aggrecanases in the development of osteoarthritis. In this chapter we describe the establishment and cultivation of cell lines expressing ADAMTS-4,-5 and their domain deletion mutants; the collection of medium containing expressed ADAMTS-4,-5; the subsequent purification of this medium through anti-FLAG affinity chromatography; and the characterisation of ADAMTS-4,-5 activity using synthetic Förster resonance energy transfer (FRET) peptide substrates.

Key words

ADAMTS, proteinase purification, affinity chromatography, aggrecanase probes, FRET substrates, active-site titrations

1. Introduction

A disintegrin-like and metalloproteinase with thrombospondin type-1 motifs-4 (ADAMTS-4) and ADAMTS-5 are metzincin metalloproteinases[1] that play a prominent role in tissue

growth and upkeep[1], vascular biology[2], human osteoarthritis progression[3,4] and are increasingly being studied for their function in the central nervous system[5]. Structurally, the active form of these enzymes is comprised of catalytic, disintegrin, thrombospondin type-I, cysteine-rich and spacer domains (Figure 1)[1]. Functionally however, the catalytic and disintegrin domains form the proteolytic unit, whereas the other domains are considered ancillary[1].

Deletion of ancillary domains in both metalloproteinases influences their aggrecanase activity. For example, removal of the C-terminal spacer domain of ADAMTS-4 (Figure 1, TS4-2) led to more effective proteolytic cleavage of bovine aggrecan in both the interglobular domain and the second chondroitin sulphate region[6]. In contrast to this, deletion of the C-terminal thrombospondin type-I motif in ADAMTS-5 (Figure 1, TS5-2) led to an increase in general bovine aggrecan-degrading activity[7]. C-terminal truncation of both ADAMTS-4 and ADAMTS-5 could be part of a regulatory system controlling the overall level of proteinase activity[8]. Domain deleted mutants of ADAMTS-4 and ADAMTS-5 thus provide a useful means to study such processes.

The methodologies that are described herein focus on the expression, purification and activity validation of ADAMTS-4 and ADAMTS-5 and their domain deleted mutants. The presence of multiple disulphides in the domains of both aggrecanases[9] coupled with five predicted glycosylation sites in ADAMTS-5[7] makes a mammalian expression system the most suitable for obtaining these proteinases. Human embryonic kidney 293 (HEK293)-Epstein-Barr Virus Nuclear Antigen (EBNA) cells are thus transfected (*via* FuGENE6 lipofection) with the pCEP4 vector containing the circular DNA (cDNA) sequence encoding the full-length or domain deleted forms of FLAG-tagged ADAMTS-4 or ADAMTS-5[6,7]. The sequences for full-length and truncated ADAMTS-4 and ADAMTS-5 are described elsewhere[6,7]. Once stable cell lines are established, they are treated with Hygromycin B to

favour strong expressors of either proteinase (although for some domain deleted mutants this is balanced against the propensity of cells to detach during long-term tissue culture). The culture medium is replaced with serum-free medium supplemented with lactalbumin enzymatic hydrolysate (LEH) and subsequently collected every 3-7 days. It is then centrifuged, filtered and sodium azide added prior to purification through affinity chromatography (section 3.2).

The latter procedure involves the separation of FLAG-tagged ADAMTS proteinases from untagged contaminants on the basis of non-covalent interactions with a stationary phase consisting of anti-FLAG monoclonal antibody bound to agarose beads. Initially, the anti-FLAG affinity gel is packed into a column equilibrated with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ buffer containing 0.02% (w/v) sodium azide (NaN_3). ADAMTS-4/-5 medium is loaded onto the column and non-specifically bound proteins are washed away with glycine buffer (pH 6.0 and pH 5.0) or high salt wash buffer (50 mM Tris-HCl pH 7.5, 1 M NaCl, 10 mM $CaCl_2$ buffer with 0.02% (w/v) NaN_3) before eluting with either glycine buffer (pH 3.0), or a solution containing an excess of FLAG (DYKDDDDK) peptide. If FLAG peptide is used for elution, a further gel filtration step is required to separate the peptide from the ADAMTS proteinase. If acidic elution is used, the eluate is immediately neutralised with 2 M Tris pH 7.5 and the column washed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ buffer containing 0.02% (w/v) NaN_3 to ensure that the antibody is not denatured further under these conditions (section 3.2).

The amount of purified ADAMTS-4/-5 proteinase is initially computed from the equation for the Beer-Lambert-Bouguer law[10] using an absorbance measurement at 280 nm (A_{280}) and the appropriate molar absorption coefficient (Table 1). Commercially available Förster resonance energy transfer (FRET) substrates are then used to determine whether the proteinase is active after purification (section 3.3). These substrates comprise a fluorophore

and quencher on either side of a cleavable peptide sequence[11]. When the substrate is intact, the proximity of the fluorophore to the quencher means its fluorescence is absorbed. Upon proteolysis, the separation of the quencher from the fluorophore allows the fluorescence to be measured[11]. For ADAMTS-4, the FRET substrate is 5-carboxyfluorescein-Ala-Glu~Leu-Gln-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys-N,N,N',N'-tetramethyl-6-carboxyrhodamine (5-FAM-AE~LQGRPISIAK-TAMRA)[12], while for ADAMTS-5 it is ortho-aminobenzoyl-Thr-Glu-Ser-Glu~Ser-Arg-Gly-Ala-Ile-Tyr-(*N*-3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Lys-Lys-NH_2 (Abz-TESE~SRGAIY-Dpa-KK-NH_2)[13] (where "~" denotes the cleavage site). The Michaelis constant (K_m) for 5-FAM-AE~LQGRPISIAK-TAMRA cleavage by the ADAMTS-4 proteinase is 15 μM[12], whilst for ADAMTS-5 cleavage of Abz-TESE~SRGAIY-Dpa-KK-NH_2 it is 76 μM[13]. To more accurately determine the amount of purified proteinase, an active-site titration with a known concentration of endogenous or small-molecule inhibitor is performed. These titrations are based on reversible tight-binding inhibition kinetics[14], where the concentration of an unknown amount of proteinase can be determined by plotting a series of known concentrations of inhibitor against the enzyme activity expressed as a percentage of the activity in the absence of inhibitor[15]. If the initial enzyme concentration is more than two-fold higher than the reported K_i value of the inhibitor[16], this data is fitted to the equation described by Bieth[15] and extrapolation of the linear portion of the curve to the x-axis yields the concentration of active enzyme[16] (section 3.4). At lower enzyme concentrations, the same curve can be used to calculate the inhibition constant (K_i) of the proteinase[13] (section 3.5).

2. Materials

2.1. Culture of ADAMTS-4 and ADAMTS-5 expressing cells

1. Multi-well culture plates (6/12-well).
2. Intermediate-sized tissue culture dishes (10 x 2 cm).

3. Large tissue culture dishes (15 x 2.5 cm), cell factory or bioreactor.
4. Cell culture medium: Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose and 0.584 g/l L-glutamine, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin.
5. Hygromycin B solution: 50 mg/ml in sterile-filtered phosphate-buffered saline (PBS), pH 7.2 (*see* **Note 1**).
6. Serum-free medium supplemented with lactalbumin enzymatic hydrolysate (LEH): DMEM with 4.5 g/l glucose and 0.584 g/l L-glutamine, 0.2% (w/v) LEH (10% (w/v) stock, 0.45 μm sterile-filtered), 1% (v/v) penicillin-streptomycin.
7. Heparin (*see* **Note 2**).
8. Plastic or glass container for storage of collected medium (*see* **Note 3**).

2.2. Purification of ADAMTS-4 and ADAMTS-5 by FLAG affinity chromatography

1. Anti-FLAG M2 affinity gel: 50% solution of beads in 50% (v/v) glycerol with 10 mM sodium phosphate, 150 mM sodium chloride (NaCl), pH 7.4, with 0.02% (w/v) NaN_3. When not in use, this solution should be stored at -20 ºC in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ buffer, with 50% (v/v) glycerol and 0.02% (w/v) sodium azide (*see* **Note 4**).
2. Protein purification column for low-pressure/0.5 mPa purification.
3. A column-pump system with flow rates up to 20 ml/min and a pressure limit up to 0.5 mPa.
4. Equilibration buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% (w/v) NaN_3.
5. High salt wash buffer: 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM $CaCl_2$, and 0.02% (w/v) NaN_3.
6. Disposable polystyrene/polypropylene tubes or containers for sample collection.

7. Protein spin concentrators (≤ 50 ml, molecular weight cut-off (MWCO) = 3 kDa and 5 kDa).
8. Spectrophotometer that can measure absorbance at a wavelength of 280 nm (A_{280}).

2.2.1. Elution of ADAMTS-4 and ADAMTS-5 using FLAG peptide

1. ADAMTS-4 FLAG peptide elution buffer: 100 μg/ml FLAG peptide in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ with 0.02% (w/v) NaN_3.
2. ADAMTS-5 FLAG peptide elution buffer: 200 μg/ml FLAG peptide in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ with 0.02% (w/v) NaN_3.
3. Gel filtration equilibration buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% (w/v) NaN_3.
4. Gel filtration column: S-200 Sephacryl for removal of FLAG tag.
5. Brij-35 30% (w/v) solution.
6. Glycerol (80% solution in de-ionised water).

2.2.2. Elution of ADAMTS-4 and ADAMTS-5 under acidic conditions

1. Wash buffers: 0.2 M glycine-HCl pH 6.0 and 0.2 M glycine-HCl pH 5.0 filtered through a 0.22 μm filter and stored at 4 °C.
2. Elution buffer: 0.2 M glycine-HCl, pH 3.0 filtered through a 0.22 μm filter and stored at 4 °C.
3. Neutralisation buffer: 2 M Tris-HCl, pH 7.5.
4. Equilibration buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% (w/v) NaN_3.
5. Buffer exchange column: pre-packed Sephadex G-25 desalting column.
6. Brij-35 30% (w/v) solution.
7. Glycerol (80% solution in de-ionised water).

2.3. Activity assays for ADAMTS-4 and ADAMTS-5

1. Microplates: 96-well, polystyrene plates with a flat bottom and a clear cover with condensation rings (200 μl maximum volume).
2. Assay buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ 0.05% (v/v) Brij-35 and 0.02% (w/v) NaN_3 (*see* **Note 11**).
3. ADAMTS-4 FRET substrate: 5-carboxyfluorescein-Ala-Glu~Leu-Gln-Gly-Arg-Pro-Ile-Ser-Ile-Ala-Lys-*N*,*N*,*N'*,*N'*-tetramethyl-6-carboxyrhodamine (5-FAM-AE~LQGRPISIAK-TAMRA).
4. ADAMTS-5 FRET substrate: ortho-aminobenzoyl-Thr-Glu-Ser-Glu~Ser-Arg-Gly-Ala-Ile-Tyr-(*N*-3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Lys-Lys-NH_2 (Abz-TESE~SRGAIY-Dpa-KK-NH_2).
5. Endogenous aggrecanase inhibitor (TIMP-3).
6. Fluorescence microplate reader: excitation wavelength λ_{ex} = 485 nm, emission wavelength, λ_{em} = 538 nm (ADAMTS-4 substrate); λ_{ex} = 300, λ_{em} = 430 nm (ADAMTS-5 substrate).
7. Microcentrifuge tubes (0.5 ml).

3. Methods

All protocols are performed at room temperature unless stated to the contrary.

3.1. ADAMTS-4 and ADAMTS-5 expressing cells

1. Incubate cells with 100 μg/ml (ADAMTS-4) or 800 μg/ml (ADAMTS-5) hygromycin B in multi-well culture plates for at least two weeks before passaging to intermediate and then large tissue culture dishes (*see* **Note 1**). Cells should be cultured in a standard incubator at 37 °C with 5% CO_2 atmosphere.
2. Change medium to serum-free DMEM with 0.2% (w/v) LEH and 1% (v/v) penicillin-streptomycin.
3. Add 100 μg/ml heparin if needed (*see* **Note 2**) and return to the culture incubator.

4. Collect medium every three or four days until cells no longer adhere to plates (*see* **Note 3** and **Note 5**).
5. Centrifuge the collected medium (1250 *x* g, 10 mins, 20 °C).
6. Separate medium from the cell pellet by decanting and filter off any remaining cell debris through a 0.22 μm filter into a plastic or glass container.
7. Load the filtered medium directly onto the FLAG affinity column at 4 °C (section 3.2.) or leave at 4 °C overnight prior to loading. If longer term storage is required, add 0.02% (w/v) NaN_3 and store at -20 °C.

3.2. Purification of ADAMTS-4 and ADAMTS-5 by FLAG affinity chromatography

1. Perform all purifications at 4 °C in the cold room.
2. Re-suspend anti-FLAG M2 affinity gel. Remove two column volumes (CVs), where 1 CV = 1.5 ml) of slurry per half-litre of medium and add it to the protein purification column (*see* **Note 4**).
3. Equilibrate the column with 10-15 CVs of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ with 0.02% (w/v) NaN_3 (flow rate of 2.0 ml/min).
4. Load medium (500 ml) onto the column (flow-rate of 2.0 ml/min).
5. Wash the column with 5-10 CVs of equilibration buffer (flow-rate of 2.0 ml/min).
6. Wash the column with 5-10 CVs of high salt wash buffer (flow-rate of 2.0 ml/min).
7. Follow the FLAG peptide (sub-section 3.2.1) or acidic (sub-section 3.2.2) elution protocols. For ADAMTS4-1 and ADAMTS5-1, FLAG peptide elution is recommended.

3.2.1. Elution of ADAMTS-4 and ADAMTS-5 using FLAG peptide

1. Make up a solution of FLAG peptide elution buffer (see sub-section 2.2.1).
2. Load 3-4 CVs of FLAG peptide solution onto the column and leave for at least 30 minutes (*see* **Note 6**).

3. Elute proteinase with 25-30 CVs of FLAG peptide elution buffer.
4. Collect 20 x 1.0 ml fractions (*see* **Note 7**. For ADAMTS4-1 and ADAMTS5-1, see **Note 8**).
5. Take 30 µl of each fraction and analyse by SDS-PAGE and Western blotting. Freeze the rest of the fractions at -20 °C immediately upon collection until analysis by SDS-PAGE and Western Blotting is complete (*see* **Note 9**).
6. Based on the results from SDS-PAGE and Western blotting, thaw the fractions, pool them and concentrate the proteinase in a protein spin concentrator (MWCO = 3 kDa) for 5-10 minutes (1250 *x* g, 20 °C) if necessary.
7. Equilibrate a Sephacryl S-200 gel filtration column with 2-5 CVs (1 CV = 15 ml) of gel filtration equilibration buffer per 1 ml of sample.
8. Elute the column with 2-5 CVs of gel filtration equilibration buffer and collect the fractions.
9. Analyse the fractions by SDS-PAGE.
10. Pool the collected fractions containing the proteinase and concentrate them in a protein concentrator (MWCO = 3 kDa) for 5-10 minutes (1250 *x* g, 20 °C).
11. Add Brij-35 to a final concentration of 0.05% (v/v).
12. Add 80% glycerol to a final concentration of 10% (v/v). The proteinase sample is now ready for long term storage at -80 °C.
13. Measure the absorbance of purified proteinases (A_{280}) using a spectrophotometer with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% (v/v) Brij-35, 10% (v/v) glycerol and 0.02% (w/v) NaN_3 as a blank.

3.2.2. Elution of ADAMTS-4 and ADAMTS-5 under acidic conditions

1. Wash the column with 10-15 CVs of 0.2 M glycine-HCl pH 6.0 (flow rate of 2.0 ml/min) and collect 5.0 ml fractions (*see* **Note 10**).

2. Wash the column with 10-15 CVs of 0.2 M glycine-HCl pH 5.0 (flow rate of 2.0 ml/min) and collect 5.0 ml fractions (*see* **Note 10**).
3. Elute proteinase from the column using 25-30 CVs of 0.2 M glycine-HCl pH 3.0 (flow rate of 2.0 ml/min) and collect 20 fractions (1.0 ml each, *see* **Note 7**). Each fraction should be collected into a container with 10% (v/v) of neutralisation buffer.
4. Take 30 μl of each fraction and analyse by SDS-PAGE and Western blotting. Freeze the rest of the neutralised fractions at -20 °C immediately upon collection until analysis by SDS-PAGE and Western blotting is complete (*see* **Note 9**).
5. Thaw the collected fractions to room temperature, pool them and concentrate the samples in a protein spin concentrator (MWCO = 3 kDa) for 5-10 minutes (1250 *x* g, 20 °C).
6. Equilibrate the pre-packed Sephadex G-25 desalting column with 15-20 CVs of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$ and 0.02% (w/v) NaN_3 (equilibration buffer) and follow the manufacturer's instructions on the volume of proteinase to load, the volume of equilibration buffer to add and the fractions containing proteinase to collect.
7. Follow steps 11-13 as for FLAG peptide elution (section 3.2.1).

3.3. Activity validation of ADAMTS-4 and ADAMTS-5

1. Calculate the concentration of proteinase from the Beer-Lambert-Bouguer law using the measured absorbance reading and theoretical molar absorption coefficient (Table 1).
2. Make a stock solution of 5-FAM-AE~LQGRPISIAK-TAMRA (ADAMTS-4 substrate) or Abz-TESE~SRGAIY-Dpa-KK-NH_2 (ADAMTS-5 substrate) at a

concentration of 10 mM in DMSO. The solution can be stored in a microcentrifuge tube at -20 °C long term.

3. Dilute the 10 mM 5-FAM-AE~LQGRPISIAK-TAMRA or 10 mM Abz-TESE~SRGAIY-Dpa-KK-NH_2 substrate stock solution to 1 mM in DMSO and store in a microcentrifuge tube at -20 °C long term.
4. Turn on the spectrophotometer and allow it to come to temperature (37 °C) for at least 30 minutes.
5. Pipette 160 μl of freshly prepared assay buffer (*see* **Note 11**) into the 96-well microplate in triplicate for each of the three concentrations to be assayed and in triplicate for "substrate-only" control wells (*see* **Note 12**).
6. Dispense 140 μl of assay buffer into EDTA control wells in triplicate.
7. Add 20 μl of 100 mM EDTA into EDTA control wells.
8. Prepare 350μl of substrate solution at a concentration of 5 μM (5-FAM-AE~LQGRPISIAK-TAMRA) or 200 μM (Abz-TESE~SRGAIY-Dpa-KK-NH_2) in assay buffer (see section 2.3).
9. Pipette 20 μl of substrate into the 96-well microplate in triplicate for each of the three concentrations to be analysed and into "substrate-only" and EDTA control wells.
10. Incubate the microplate (lid on) in the microplate reader for 30 minutes at 37 °C.
11. During this time, make up 100 μl of 200 nM, 100 nM and 50 nM ADAMTS-4 or 800 nM, 400 nM and 200 nM ADAMTS-5 in assay buffer.
12. After the 30 min incubation, add 20 μl of assay buffer to "substrate-only" and EDTA control wells in triplicate and 20 μl each of ADAMTS-4 or ADAMTS-5 to microplate wells in triplicate. The final concentration of substrate will therefore be 0.5 μM (5-FAM-AE~LQGRPISIAK-TAMRA) or 20 μM (Abz-TESE~SRGAIY-Dpa-KK-NH_2).

13. In order to ensure that there are no bubbles in the wells, tap the side of the microplate or 'shake' the plate in the microplate reader.
14. Monitor the increase in fluorescence intensity for 1 hour at 37 °C at the appropriate wavelengths (ADAMTS-4 substrate λ_{ex} = 485 nm, λ_{em} = 538 nm; ADAMTS-5 substrate λ_{ex} = 300, λ_{em} = 430 nm) with readings at least twice a minute.
15. Plot the relative fluorescence intensity (expressed in terms of relative fluorescence units per second, RFU s^{-1}) against time (s) to determine the rate at each ADAMTS concentration (*see* **Note 13**, Figure 2a and 2b).

3.4. Active-site titrations of ADAMTS-4 and ADAMTS-5

1. Turn on the spectrophotometer and allow it to come to temperature (37 °C) for at least 30 minutes.
2. Use the concentration of ADAMTS-4 or ADAMTS-5 proteinase determined through the Beer-Lambert-Bouguer law (prior to freeze-down of the sample) as an initial starting concentration (*see* **Note 14**).
3. Make up 200 μl of TIMP-3 inhibitor at each of the following initial concentrations in fresh assay buffer (*see* **Note 11** and **Note 15**): 320 nM, 160 nM, 80 nM, 40 nM, 20 nM, 10 nM and 5 nM.
4. Add 50 μl of each inhibitor concentration as well as a "no inhibitor" control (assay buffer only) to microplate wells in triplicate.
5. Dispense 100 μl of assay buffer into "substrate-only" control wells in triplicate (*see* **Note 12**).
6. Thaw the ADAMTS-4 or ADAMTS-5 proteinase to room temperature.
7. Make up 1.3 ml of 40 nM ADAMTS-4 or ADAMTS-5 proteinase in fresh assay buffer.

8. Dispense 50 μl of 40 nM ADAMTS-4 or ADAMTS-5 proteinase into each well in triplicate (excluding "substrate-only" wells).
9. Add 80 μl of assay buffer to all wells in triplicate.
10. Incubate the microplate wells in the microplate reader (lid on) for 1 hour at 37 °C (*see* **Note 16**).
11. Prepare 650 μl of substrate solution at a concentration of 5 μM (5-FAM-AE~LQGRPISIAK-TAMRA) or 200 μM (Abz-TESE~SRGAIY-Dpa-KK-NH_2) in assay buffer (see section 2.3).
12. Add 20 μl of 5-FAM-AE~LQGRPISIAK-TAMRA or Abz-TESE~SRGAIY-Dpa-KK-NH_2 to each well to give a final concentration of 0.5 μM or 20 μM, respectively.
13. Remove the microplate lid and tap the side of the plate (or 'shake' the plate in the microplate reader) to remove any bubbles.
14. Monitor the increase in fluorescence intensity in the microplate reader for 1 h at 37 °C at the appropriate wavelengths (ADAMTS-4 substrate λ_{ex} = 485 nm, λ_{em} = 538 nm; ADAMTS-5 substrate λ_{ex} = 300, λ_{em} = 430 nm) at least twice a minute.
15. Plot the final concentration of TIMP-3 against the fractional activity expressed as the gradient of the reaction at each inhibitor concentration against the gradient of the reaction in the absence of inhibitor (*see* **Note 17**) by fitting the data with the equation for tight-binding inhibitors using appropriate curve-fitting software[16]:

$$a = \left(1 - \frac{([\mathrm{E}] + [\mathrm{I}] + K_\mathrm{i}) - \{([\mathrm{E}] + [\mathrm{I}] + K_\mathrm{i})^2 - 4[\mathrm{E}][\mathrm{I}]\}^{1/2}}{2[\mathrm{E}]}\right) x\ 100$$

where "a" is the percentage activity (obtained by dividing the activity with inhibitor with the activity without inhibitor and multiplying by 100); [E] the concentration of active enzyme; [I] the concentration of inhibitor and $K_{i(app)}$ the apparent inhibition constant of the inhibitor (see Figure 3a for a representative plot).

16. Extrapolate the linear portion of the final curve to the *x*-axis to determine the concentration of active enzyme (see Figure 3a). Compare this concentration to that determined by A_{280}. A value lower than the A_{280} by more than 2 nM (e.g. 10 nM initial concentration, 8 nM [E] found) may indicate the presence of inactive proteinase in the preparation.

3.5. Determination of K_i value for small-molecule metalloproteinase inhibitors of ADAMTS-4 and ADAMTS-5

1. Make up a stock solution of metalloproteinase inhibitor in DMSO at 10 mM.
2. Dilute this stock solution to 1 mM using DMSO. These solutions can be frozen at -20 °C for long term storage.
3. Make up 200 µl of inhibitor at each of the following initial concentrations in fresh assay buffer (*see* **Note 11**): 250 nM, 125 nM, 62.5 nM, 31.3 nM, 15.6 nM, 7.8 nM and 3.9 nM.
4. Dispense 50 µl of each inhibitor concentration as well as a "no inhibitor" control (assay buffer only) into microcentrifuge tubes in triplicate.
5. Dispense 100 µl of assay buffer into "substrate-only" control microcentrifuge tubes in triplicate (*see* **Note 12**).
6. Thaw the ADAMTS-4 or ADAMTS-5 proteinase to room temperature.
7. Make up 1.3 ml of 5 nM ADAMTS-4 or ADAMTS-5 proteinase in assay buffer (*see* **Note 18**).
8. Dispense 50 µl of 5 nM ADAMTS-4 or ADAMTS-5 proteinase into microcentrifuge tubes in triplicate (excluding "substrate-only" tubes).
9. Add 80 µl of assay buffer to all microcentrifuge tubes in triplicate.
10. Incubate all microcentrifuge tubes in a tube holder for 1 hour at 37 °C in a dry incubator.

11. Prepare 650 μl of substrate solution at a concentration of 5 μM (5-FAM-AE~LQGRPISIAK-TAMRA) or 200 μM (Abz-TESE~SRGAIY-Dpa-KK-NH_2) in assay buffer (see section 2.3).
12. Dispense 20 μl of 5-FAM-AE~LQGRPISIAK-TAMRA or Abz-TESE~SRGAIY-Dpa-KK-NH_2 into each microcentrifuge tube to give a final concentration of 0.5 μM or 20 μM, respectively.
13. Place the tubes into a tube holder and leave the samples in a dry incubator for up to 8 h at 37 °C.
14. Add the solutions from each microcentrifuge tube into microplate wells.
15. Remove the microplate lid and tap the side of the plate (or 'shake' the plate in the microplate reader) to remove any bubbles.
16. Read the end-point fluorescence intensity using the fluorescence microplate reader at the appropriate wavelengths (ADAMTS-4 substrate λ_{ex} = 485 nm, λ_{em} = 538 nm; ADAMTS-5 substrate λ_{ex} = 300, λ_{em} = 430 nm).
17. Plot the final concentration of inhibitor against the percentage activity expressed as the gradient of the reaction at each inhibitor concentration against the gradient of the reaction in the absence of inhibitor (*see* **Note 17** and Figure 3b) by fitting the data with the equation for tight-binding inhibitors (section 3.4) using appropriate curve-fitting software.
18. The apparent $K_{i(app)}$ of the inhibitor is determined from the curve fit (*see* **Note 19**).
19. Use the K_m value for ADAMTS-4 (15 μM)[12] and ADAMTS-5 (76 μM)[13] to find the actual K_i value using the following equation:

$$K_i = \frac{K_{i(app)}}{1 + \frac{[S]}{K_m}}$$

Where [S] is the initial concentration of 5-FAM-AE~LQGRPISIAK-TAMRA or Abz-TESE~SRGAIY-Dpa-KK-NH_2 substrate.

Notes

1. Different concentrations of hygromycin B are used to select for cells which express the various ADAMTS4 and ADAMTS5 constructs studied. A concentration of hygromycin B that is too high would lead to rapid proteinase production that would result in excessive autocatalysis and detachment of cells during long-term culture. A balance in hygromycin B concentration is therefore required, and values reported in the literature are 200 μg/ml and 100 μg/ml for full length and domain deleted forms of ADAMTS-4 and ADAMTS-5, respectively[6,7]. We found that using 800 μg/ml of hygromycin B to select for stronger expression of *adamts4-4* resulted in cells detaching from tissue culture plates within three days of changing to serum-free medium. The TS4-4 proteinase was also found to be significantly degraded upon Western blotting. In contrast, the TS4-5, TS5-5 and TS5-6 proteinases did not detach from tissue culture dishes under the same conditions. Cultivation of TS4-4 using 100 μg/ml hygromycin B and TS5-5 with 800 μg/ml of hygromycin B delivered higher yields of final protein after affinity purification when compared to the reported concentrations of 200 μg/ml and 100 μg/ml hygromycin B, respectively.
2. In the case of TS4-1, TS5-1, TS5-2, and TS5-3 proteinases, heparin (100 μg/ml) should be added to the culture medium in order to release mature proteinase bound to the cell layer[6,7].
3. We recommend shorter collection times (3 days) for longer forms of ADAMTS-4 (TS4-1 to TS4-3) and ADAMTS-5 (TS5-1 to TS5-4), with longer collection times (4 days) for shorter forms (TS4-4, TS4-5, TS5-5 and TS5-6).

4. The binding capacity of anti-FLAG M2 affinity gel is > 0.6 mg/ml. We suggest purification of ADAMTS-4,-5 from 500 ml batches of medium, for which we typically use 1.5 ml (1 CV) of anti-FLAG M2 affinity gel.
5. If medium collection is carried out over several weeks, it is useful to maintain several intermediate tissue culture plates (10 x 2 cm) with cells in serum in order to re-seed the cell factory, bioreactor or large tissue culture dishes (15 x 2.5 cm) in case of cell detachment.
6. This step is to allow enough time for a competitive equilibrium to be set up between the FLAG peptide and the proteinase in order that the latter elutes from the column in a more concentrated form.
7. In general, the majority of the proteinase will elute within the first 20 fractions when loading 500 ml of medium. Further fractions of proteinase may be collected if desired.
8. Owing to the small quantity of TS4-1 and TS5-1 present in the starting material, a cation exchange resin (Macro-Prep 25 S, 300-400 μl) is required to separate out the FLAG peptide[7,17]. Concentrate the fractions in a protein spin concentrator (MWCO = 5 kDa) and dilute with 10 volumes of 20 mM Tris acetate, pH 6.4, 10 mM $CaCl_2$ with 0.02% (w/v) NaN_3 and load onto the column[7,17]. Wash the column using 10-15 CVs of 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 10 mM $CaCl_2$ with 0.02% (w/v) NaN_3[7,17]. Elute TS4-1/5-1 using 25-30 CVs of 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 10 mM $CaCl_2$ with 0.02% (w/v) NaN_3[7,17]. Process the fractions as described from step 5 of section 3.2.2.
9. Storage of ADAMTS-4,-5 proteinases at 4 °C is highly discouraged as breakdown to shorter truncated versions will occur within a week. Fractions should be stored at -20 °C and thawed only when they are to be pooled and frozen down long term at -80 °C.

10. We found that for 500 ml batches of TS4-4, TS4-5, TS5-5 and TS5-6 medium (using a 1.5 ml column), it was sufficient to wash with 20 ml of 0.2 M glycine-HCl pH 6.0 and 0.2 M glycine-HCl 5.0 to remove any non-specific interactions resulting from undesirable contaminants.
11. The assay buffer should be prepared fresh each day by adding Brij-35 to a buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 10 mM $CaCl_2$ with 0.02% (w/v) NaN_3 to a final concentration of 0.05% (v/v). A 0.05% (v/v) Brij-35 containing solution will attract bacterial and/or fungal contaminants.
12. Assay buffer containing only FRET substrate acts as a negative control. Contaminating proteinases (control) and metalloproteinases (EDTA control) will register as a gradual increase in background activity. Should a steady increase in background activity occur, the buffer should be replaced with a fresh one. For each ADAMTS-4/-5 enzyme concentration assayed, the rate of substrate cleavage should increase proportionally with increasing enzyme concentration.
13. The rate of the reaction at each concentration is determined from the gradient of the line when the cleavage of the substrate (expressed in RFU s^{-1}) is linear. For example, for TS4-4 this was typically up to 10 minutes at a final substrate concentration of 0.5 μM. However, in the case of TS5-5 this was over the course of 60 minutes at a final substrate concentration of 20 μM.
14. The endogenous inhibitor of the aggrecanases (TIMP-3) should be used for active-site titration. The initial concentration of ADAMTS-4/-5 proteinase should be at least ten-fold higher than the K_i value of the inhibitor in order that accurate titrations are obtained. The K_i value for TIMP-3 inhibition of ADAMTS-4 and ADAMTS-5 proteinases is approximately 1 nM.

15. The range of inhibitor concentrations to be used in the active-site titration will depend on the K_i value of the inhibitor and the initial concentration of proteinase determined by A_{280}. These values are not fixed and it may be necessary to use inhibitor concentrations on either side of the listed upper (320 nM) and lower (5 nM) limits to obtain a titration curve as in Figure 3a.
16. The 1 hour incubation is to allow sufficient time for the TIMP-3:ADAMTS-4/-5 complex to reach equilibrium.
17. The gradient of the reaction at each concentration should be taken when the reaction rate is still linear.
18. The final concentration of ADAMTS-4/-5 proteinase should ideally be at least 10-fold lower than the K_i value of the inhibitor. We do not recommend final ADAMTS-4/-5 concentrations of lower than 0.4 nM as the activity against the substrates becomes too low to be measured. At this concentration of TS4-4 and TS5-5, substrate hydrolysis is linear for at least 8 h at 37 °C.
19. This assay should be performed using ADAMTS-4,-5 proteinase stored at different temperatures, from different purification batches and at different concentrations and incubation times to ensure that a consistent $K_{i(app)}$ value is obtained.

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Figure Captions

Figure 1. Schematic diagram of ADAMTS-4 and ADAMTS-5 and their domain deletion mutants. The different domains of these metalloproteinases are identified as follows: Cat = catalytic, Dis = disintegrin, TS_1/TS_2 = thrombospondin type-I, CysR = cysteine-rich, Sp = spacer. The numbering of amino acid residues is from the signal peptide.

Figure 2. ADAMTS4-4 and ADAMTS5-5 cleavage of FRET substrates. a) Representative curves for ADAMTS4-4 cleavage of the 5-FAM-AE~LQGRPISIAK-TAMRA substrate; b) representative curves for ADAMTS5-5 cleavage of the Abz-TESE~SRGAIY-Dpa-KK-NH_2 substrate. The rate (v) is expressed in relative fluorescence units per second (RFU s^{-1}) and is shown to increase linearly with increasing enzyme concentration. Each time-point represents ΔRFU [RFU at time x - RFU at time 0] ± standard error of the mean (SEM) for n = 3 readings at 37 °C. The data was collected on a Molecular Devices SpectraMax M5 spectrophotometer and analysed using SoftMax Pro 6.3 software.

Figure 3. Active-site titration kinetics with TIMP-3 and a small molecule metalloproteinase inhibitor. a) Representative active-site titration curve with an endogenous TIMP-3 inhibitor; b) representative curve for the determination of $K_{i(app)}$ for a small-molecule metalloproteinase inhibitor. In both curves, percentage activities are expressed in terms of the rate of substrate cleavage in the absence of inhibitor. Values represent mean activity with errors expressed as ± SEM for n = 3 readings at 37 °C. The data was collected on a Molecular Devices SpectraMax M5 spectrophotometer and analysed using SoftMax Pro 6.3 software.

Proteinase	Molar absorption	1 unit of A_{280} (μM)

	coefficient, M^{-1} cm^{-1}	
TS4-1	80840	12.4
TS4-2	59410	16.8
TS4-3	45835	21.8
TS4-4	27470	36.4
TS4-5	14480	69.1
TS5-1	98510	10.2
TS5-2	81760	12.2
TS5-3	65830	15.2
TS5-4	50765	19.7
TS5-5	29420	34.0
TS5-6	17460	57.3

Table 1. Molar absorption coefficients and micromolar values for a single A_{280} unit for human ADAMTS-4 and ADAMTS-5 proteinases and their domain deletion mutants. Coefficients were predicted using the ExPASy ProtParam tool and are based on absorption at 280 nm assuming cysteine residues are not reduced. Sequence data was obtained from the UnitProtKB database and domain cut-offs from the publications of Kashiwagi[6] and Gendron[7].