Handling Metalloproteinases

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Substrate cleavage by metalloproteinases involves nucleophilic attack on the scissile peptide bond by a water molecule that is polarized by a catalytic metal, usually a zinc ion, and a general base, usually the carboxyl group of a glutamic acid side chain. The zinc ion is most often complexed by imidazole nitrogens of histidine side chains. This arrangement suggests that the physiological pH optimum of most metalloproteinases is in the neutral range. In addition to their catalytic metal ion, many metalloproteinases contain additional transition metal or alkaline earth ions, which are structurally important or modulate the catalytic activity. As a consequence, these enzymes are generally sensitive to metal chelators. Moreover, the catalytic metal can be displaced by adventitious metal ions from buffers or biological fluids, which may fundamentally alter the catalytic function. Therefore, handling, purification, and assaying of metalloproteinases require specific precautions to warrant their stability. © 2016 by John Wiley & Sons, Inc.

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INTRODUCTION

This unit focuses on metalloproteinases (i.e., endopeptidases) classified in clan MA of the MEROPS database (*http://merops.sanger.ac.uk*; Rawlings et al., 2014). Clan MA enzymes bind their catalytically essential zinc ion by a HEXXH sequence (amino acids in single letter code; X stands for any amino acid residue) in their active sites. In the largest group within the MA clan, the metzincins, the metal-binding motif is elongated to HEXXHXXGXXH/D and accompanied by a conserved methionine-containing β-turn (Met-turn) downstream of the zinc site (Bode et al., 1993; Gomis-Rüth et al., 2012a). Most metzincins act as molecular switchmen in a variety of physiological and pathophysiological processes. The various metzincin families differ not only in the substrate-binding properties of their catalytic domains, which cause differing cleavage specificities, but also in having different types and numbers of non-catalytic domains, which likewise may be important for proteinase-substrate recognition. The structures and functions of these enzymes with references are outlined below (see Background Information).

Recent advances in proteinase proteomics have allowed researchers to identify physiologically relevant substrates of proteinases and their cleavage sites starting from minute amounts of biological fluids (Gevaert et al., 2003; Kleifeld et al., 2010). However, in order to unambiguously confirm these primary data, it is necessary to validate the outcome on a molecular and cellular level. To do so, it is generally required to overexpress significant amounts of the designated proteinase, to purify the enzyme, and to set up a suitable assay

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system. Numerous protocols for this purpose have been developed and published over the past decades, and it may appear difficult to select the most appropriate ones. In this unit, protocols are presented for handling the proteinase of interest. Simple procedures are provided for enzyme purification (Basic Protocol 1) as well as activation and assaying of meprins (Basic Protocol 2). In the second part of the unit, more general techniques like the assaying of proteinases with azocasein (Basic Protocol 3) and the activation of matrix metalloproteases (MMPs) are described (Basic Protocol 4). Convenient systems are presented, and suitable equipment for the assaying of metalloproteinases is discussed. Furthermore, the specific needs of metalloproteinases are discussed, and examples of proteinase-adapted buffer systems are provided.

BASIC PROTOCOL 1

PURIFICATION OF MEPRIN α FROM TRANSFECTED Sf21 INSECT CELLS

There are many ways to purify the proteinase of choice. The first question is whether the proteinase is expressed heterologously and purified after cell lysis, or purified from the supernatant of transfected or infected cells. Protocols available for the expression of metalloproteinases in pro- and eukaryotic organisms are listed in Table 21.16.1.

An overview of expression and purification of proteins can be found in Chapter 1 (Strategies of Protein Purification and Characterization), Chapter 5 (Production of Recombinant Proteins), and Chapter 6 (Purification of Recombinant Proteins) in this manual.

Expression in bacteria, e.g., *E. coli* cells, promises high yields of the desired protein within a short time. However, bacterial expression may be problematic if correct disulfide formation, glycosylation, and/or other post-translational modifications are required, which is the case for most eukaryotic metalloproteinases, the majority of which are extracellular proteins. If the desired protein is to be mutated by site-directed mutagenesis or if only a distinct single domain is to be expressed, e.g., the catalytic domain, then the method of choice might be a prokaryotic expression system. Sequences not eventually required for the purpose of the investigation, such as the signal- or pro-domain or even additional domains, which would be indispensable for correct folding or trafficking in a eukaryotic system, can be easily omitted. Even if the overexpressed protein is deposited in inclusion bodies, there are suitable folding protocols that allow one to obtain good yields of recombinant protein including correctly connected disulfide bonds.

Expression in a eukaryotic system benefits from the fact that the original, native-like glycosylation and correct folding can be achieved, depending on the right choice of the host cell line for heterologous expression. Of course, changing the native protein sequence by the addition of sequence tags, insertions, or deletions can lead to disturbed intracellular trafficking or failures during the folding process, which may result in intracellular protein aggregation.

Table 21.16.1 Additional Publications on Proteinase Expression and Purification

Proteinase	Expression system	Purification system	Reference
Full-length MMP9 and MMP9 proteinase domain	*Drosophila* S2 cells	Gelatin-affinity chromatography	Rasch et al. (2010)
Proteinase domain of MMP12	*E.coli*	Size-exclusion chromatography	Parkar et al. (2000)
Full-length ADAMTS1	High Five insect cells	Ion exchange, heparin affinity, and Ni-NTA chromatography	Lind et al. (2006)

Eukaryotic cells are regularly grown in media containing fetal bovine serum, which also contains proteinase inhibitors such as the general inhibitor of endopeptidases, α_2-macroglobulin, in micromolar concentrations (Sottrup-Jensen, 1989). α_2-Macroglobulin is able to bind most metalloproteinases even during the expression period (provided that the proteinase is produced in its active form) and can dramatically lower the output of active proteinase after the purification process.

An alternative way to obtain the proteinase of interest is its purification from natural tissue. This method is often limited by slow in vivo expression rates or low abundance of the proteinase in the sample. Good sources of many proteinases are serum or other body fluids. In addition, proteinases, which are expressed in high levels by certain cell types, can be purified directly from cultured cells. Besides the classical purification methods like ion-exchange, hydrophobic-interaction, or size-exclusion chromatography, various forms of affinity chromatography based on specific proteinase inhibitors can be employed. However, if the binding affinity is too strong, this might cause problems during the elution of the proteinase. In an ideal setting, the interaction is only moderate, e.g., in the micromolar range.

Here we have picked a straightforward example of inhibitor chromatography for metalloproteinases, which was originally introduced by Moore and Spilburg (1986) using the tripeptide Pro-Leu-Gly-hydroxamate (PLG-NHOH) as a bait for human collagenases. In this setting, the reversible substrate analog inhibitor is covalently coupled to the stationary phase via its amino-terminal imino group using a spacer provided by the CH-Sepharose-4B support. The carboxy-terminal hydroxamate group acts as a metal-chelating warhead, and the short tripeptide moiety binds to specific sub-sites of the substrate recognition region, providing selective specificity. With this substrate analog inhibitor, it is possible to purify several collagen- and gelatin-cleaving matrix metalloproteinases and other proteinases that prefer hydrophobic residues in the positions amino-terminal to the scissile peptide bond. The elution process is usually initiated by changing the pH by applying a solution of 0.1 M non-buffered Tris base, pH 10 (Reyda et al., 1999; Köhler et al., 2000) or by lowering the pH to 6.5 (Moore and Spilburg, 1986). In principle, this method is applicable with any reversible inhibitor of moderate affinity, though one has to be aware that only active proteinases can be purified with this technique.

Materials

Ammonium sulfate
Promeprin α conditioned medium from infected insect cells (Köhler et al., 2000; see Support Protocol)
50 mM HEPES/NaOH, pH 7.5, pH 3.5, and pH 10.4
Sephacryl S-300 (GE Healthcare)
Pro-Leu-Gly-hydroxamate (PLG-NHOH)
CH-Sepharose-4B (GE Healthcare)

Centrifuge (cooled)
10,000 MWCO dialysis membrane
240-ml, 3.5-cm chromatography column
Chromatography system (e.g., ÄKTA Prime, GE Healthcare)
10 ml polypropylene column (Thermo Scientific, cat. no. 29924)

Additional reagents and equipment for dialysis (*APPENDIX 3B*; Zumstein, 1995), activation of promeprin α (Basic Protocol 2, step 1), measurement of proteolytic activity using FRET substrate (Basic Protocol 2, step 3) or azocasein (Basic Protocol 3), immunoblotting (*UNIT 10.10*; Gallagher, 1996; also see Dumermuth et al., 1993), and SDS-PAGE (*UNIT 10.1*; Gallagher, 2012)

Precipitate promeprin α from conditioned medium

1. Add stepwise (using a spatula) 39 g of solid ammonium sulfate per 100 ml conditioned medium (60% saturation), and stir gently overnight at 4°C
2. Centrifuge 60 min at 11,000 × g, 4°C, and discard the supernatant
3. Redissolve the protein pellet carefully in a volume of 50 mM HEPES/NaOH-buffer, pH 7.5, that is equal to 20% of the initial volume of conditioned medium used.
4. Remove residual ammonium sulfate by dialysis (*APPENDIX 3B*; Zumstein, 1995) overnight using a 10,000 MWCO dialysis membrane with at least three changes of buffer against 50 mM HEPES/NaOH, pH 7.5

Perform size-exclusion chromatography

5. Further separate the protein concentrate on a Sephacryl S-300 column (volume 240 ml, diameter 3.5 cm) at a flow rate of 0.4 ml/min using 50 mM HEPES/NaOH, pH 7.5, as running buffer.

   For use in gel filtration, the buffer has to be filtered through a 0.22-μm filter and degassed under vacuum immediately before use.

   Promeprin α elutes between 220 and 360 min, with a peak concentration at about 280 min.

   For further purification, promeprin α has to be activated (Grünberg et al., 1993; also see Basic Protocol 2, step 1) and the main elution fractions have to be determined.

6. Activate promeprin α according to Basic Protocol 2, step 1.

7a. Measure proteolytic activity with a FRET substrate (see Basic Protocol 2, step 3) or alternatively with azocasein (see Basic Protocol 3).

7b. Alternatively, check the meprin α abundance by immunoblotting using specific antibodies (*UNIT 10.10*; Gallagher, 1996; also see Dumermuth et al., 1993).

8. Pool the fractions with the highest proteolytic activity (step 7a) or meprin α abundance (step 7b).

PLG affinity chromatography

This method is selective for meprin α. The sister subunit meprin β is inhibited less strongly by PLG-NHOH, and therefore cannot be affinity purified with this inhibitor (Kruse et al., 2004).

9. Immobilize 500 mg Pro-Leu-Gly-NHOH on 5 g CH-Sepharose-4B according to the manufacturer's instructions.
10. Pour the material into a 10-ml polypropylene column and equilibrate with 50 mM HEPES/NaOH, pH 7.5.
11. Apply the chosen fractions from the size-exclusion chromatography (step 8) to the affinity column and wash several times, each time with 3 ml of 50 mM HEPES/NaOH, pH 7.5, until the OD_{280} drops under 0.01.
12. Prepare vials with 100 μl 50 mM HEPES/NaOH, pH 3.5.
13. To elute the bound meprin α, apply 3 ml of 50 mM HEPES/NaOH, pH 10.4, and collect 100 μl of active meprin α samples in the prepared vials.

    A typical elution profile is shown in Figure 21.16.1.

    pH 3.5 and pH 10.4 are outside the buffer capacity of HEPES, but with the volumes indicated, the elution works without changing the buffer system.

14. Check meprin α purity by SDS-PAGE (*UNIT 10.1*; Gallagher, 2012).

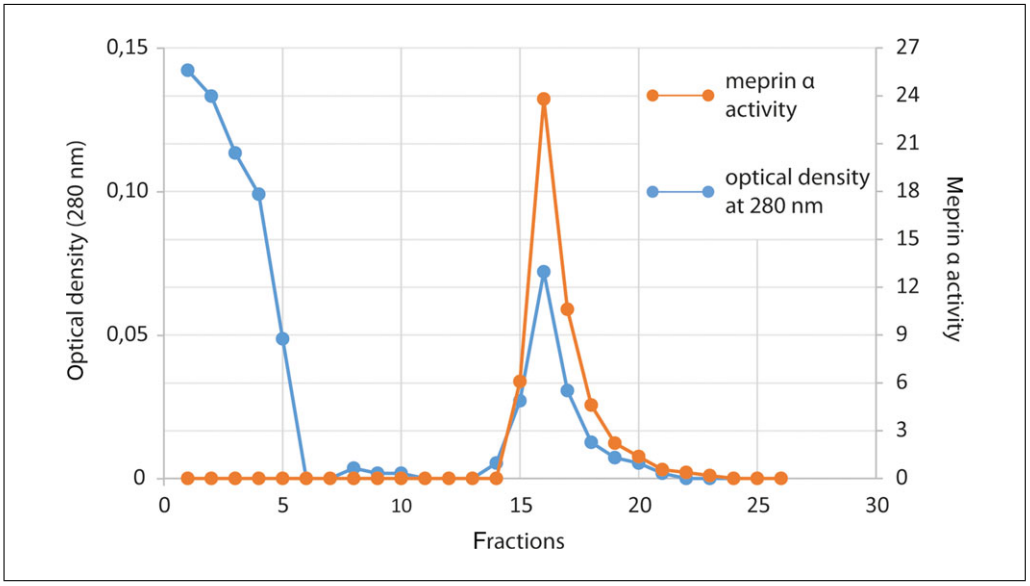

Figure 21.16.1 Elution profile of activated recombinant meprin α from immobilized PLG-NHOH Sepharose. Optical density and meprin α specific activity is indicated for each fraction. For the determination of specific meprin α activity, N-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (PABA-peptide) was used in a colorimetric assay (Köhler et al., 2000).

CULTIVATION AND INFECTION OF INSECT CELLS

SUPPORT PROTOCOL

Materials

BTI-TN-5B1-4 insect cells (High Five; Thermo Fisher, cat. no. B855-02)
Express Five serum-free medium (Thermo Fisher, cat. no. 10486-025)
Penicillin/streptomycin mix (Thermo Fisher, cat. no. 15140-122)
Recombinant baculovirus containing DNA construct of interest

Incubator-shaker
Spinner flask (250 ml)
Fernbach flask (2800 ml)
Centrifuge

Additional reagents and equipment for counting cells (*APPENDIX 3C*; Phelan, 2006)

1. Culture BTI-TN-5B1-4 insect cells (High Five) in serum-free Express Five medium supplemented with 100 U/ml penicillin-streptomycin mix.
2. Count the cells (*APPENDIX 3C*; Phelan, 2006) and seed them at a density of 2.5×10^5 cells/ml in a final volume of 100 ml Express Five medium in a spinner flask. Incubate in an incubator-shaker at 27°C with 60 rpm shaking at environmental humidity and CO_2 concentration.
3. For maintaining the cells repeat step 2 every 3 days in a new spinner flask
4. For infection, seed cells at a density if 2.5×10^5 ml in a final volume of 400 ml Express Five medium in a 2800-ml Fernbach flask and incubate in an incubator-shaker at 27°C with 60 rpm shaking at environmental humidity and CO_2 concentration.
5. At a cell density of 1.8×10^6 cells/ml, infect the cells with a multiplicity of infection (MOI) of 2 to 3 with the recombinant baculovirus.
6. Incubate the cells for 96 hr at 27°C with 60 rpm shaking at environmental humidity and CO_2 concentration.

7. To separate the cells from the supernatant, centrifuge the solution 30 min at 400 × *g*, 4°C.

The supernatant contains the recombinant protein.

BASIC PROTOCOL 2

ACTIVATION OF MEPRIN α AND FRET ASSAY

To characterize a proteinase of interest, it is essential to know whether the proteinase is active. Besides the necessity for catalytic function, the activity is also proof of correct folding of the purified proteinase. For this purpose, a variety of assays are available.

A commonly used method for testing endopeptidase activity is based on fluorescence resonance energy transfer (FRET). The FRET assay uses the principle of resonance energy transfer described by Förster (1948). A donor fluorophore, and an acceptor whose excitation spectrum overlaps with the donor's emission spectrum, are linked by a sequence of amino acid residues susceptible to proteolytic cleavage. In the non-cleaved substrate, the donor is excited, but the energy is not released via fluorescence; instead, radiationless energy transfer to the acceptor occurs, leading to donor quenching. Upon proteolytic cleavage of the peptide sequence, donor and acceptor become separated from each other and the donor-emitted light can be detected. The change in fluorescence intensity with time is proportional to the catalytic activity of the proteinase. One must keep in mind that this only holds true at low substrate concentrations. At high substrate concentrations, there will be additional intermolecular quenching effects between cleaved and non-cleaved substrate, which will reduce the fluorescence yield. Hence, if Michaelis-Menten-type substrate dependence of catalysis is to be determined, the fluorescence yield at each substrate concentration needs to be normalized to the corresponding maximum fluorescence after complete turnover of substrate to product.

Suitable substrates are commercially available—either specific for distinct metalloproteinases, or suitable for entire families of proteinases with similar cleavage specificities. For example, mammalian astacins prefer an acidic, in most cases an aspartate residue, in their P_1' position (Becker-Pauly et al., 2011), whereas matrix metalloproteinases prefer small hydrophobic amino acids in their S_1' pocket (nomenclature according to Schechter and Berger, 1967; the amino acid residues amino- and carboxy-terminal to the scissile peptide bond are termed "P positions" and "P′ positions," respectively; the subsites harboring the substrate's side chains in the active site of a proteinase are correspondingly termed S and S′; cleavage occurs between P_1 and P_1').

FRET assays can be performed conveniently in thermostatted multiwell fluorometric readers, whereby different concentrations of proteinase, substrate, and/or inhibitors can be tested simultaneously. The following evaluation can be performed via commercially available software packages for enzyme kinetics (e.g., Grafit, Erythacus Software). FRET assays are sensitive and fast, and can be implemented in high-throughput approaches. Nevertheless, the tailored substrates can be expensive and good equipment is not a standard in every laboratory.

Materials

1 μM recombinant promeprin α or β (meprin α, R&D Systems, cat. no. 3220-ZN-010; meprin β, R&D Systems, cat. no. 2895-ZN-010) in 50 mM HEPES/NaOH, pH 7.5

0.54 μM bovine trypsin in 50 mM HEPES/NaOH, pH 7.5

400 mM Pefabloc (e.g., Sigma-Aldrich) in distilled H_2O (since Pefabloc has a limited half-life of only 2 hr in aqueous solutions, it must be prepared fresh each time before use.)

Dimethylsulfoxide (DMSO)

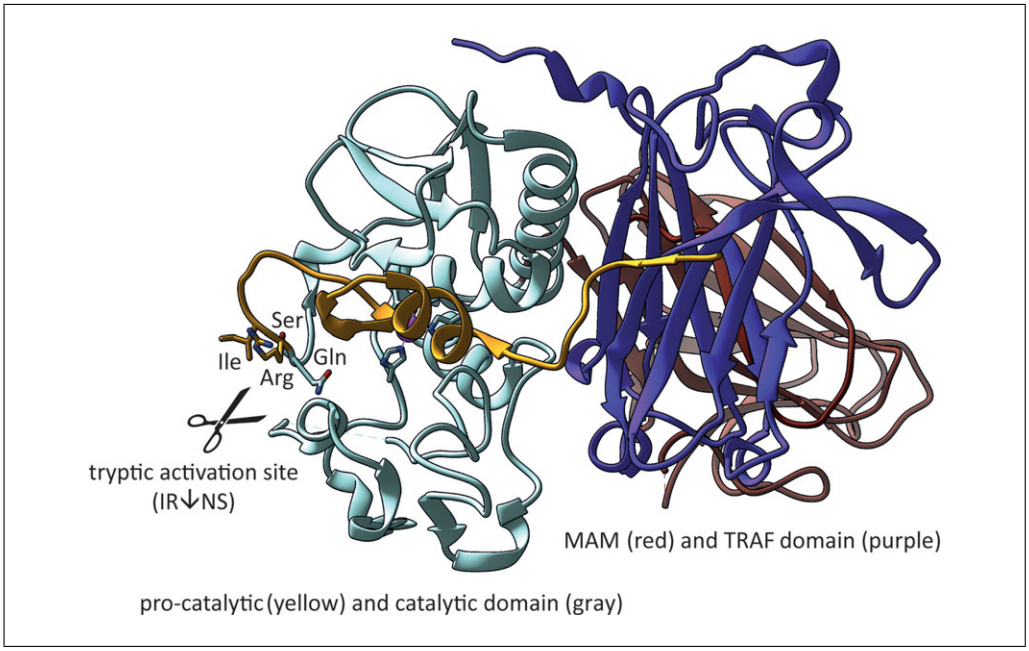

Figure 21.16.2 Structure of promeprin β (4GWN; Arolas et al., 2012) in standard orientation according to Gomis-Rüth et al. (2012b). The pro-peptide (yellow) blocks the active site in the catalytic domain (gray). The protease is activated by trypsin-like proteases by a cleavage between R(61) and N(62). The non-catalytic C-terminal MAM domain is depicted in red and the TRAF domain in purple.

Fluorogenic substrate: Ac-R-E(Edans)-DR-Nle-VGDDPY-K(Dabcyl)-amide [Ac = acetyl; EDANS = (5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid); Dabcyl = 4-((4-(dimethylamino)phenyl)azo)benzoic acid)]; store protected from light at up to several months at –20°C
50 mM HEPES/NaOH, pH 7.5 (Biosyntan)
25 mg/ml proteinase K in 50 mM HEPES/NaOH, pH 7.5, for complete substrate turnover

96-well black microtiter plate (Thermo Scientific, cat. no. 237105)
Varioskan Flash 3001 spectral plate reader (Thermo Scientific)

Activate promeprin

Prior to the FRET assay, promeprin has to be activated. Therefore, the tryptic cleavage site between the pro-peptide and the catalytic domain has to be cleaved (Fig. 21.16.2)

1. Mix 95 µl of 1 µM promeprin with 2.5 µl 0.54 µM bovine trypsin at 37°C (molar ratio: 70 to 1). After 45 min, add 2.5 µl 400 mM Pefabloc and incubate for another 10 min to inhibit trypsin
2. Store the activated meprin solution on ice.

   The meprin concentration is now 950 nM.

Set up the measurement

For the correct calculation of the kinetic parameters, it is necessary to measure enzymatic velocity under steady-state conditions. Therefore, the measurement has to take place immediately after the mixing of proteinase and substrate. To ensure a fast start, the substrate and all other components must be prepared separately before mixing. In order to catch the initial velocity of reactions using these methods, it may be necessary to try a number of concentrations of enzyme to find a condition where the rate of cleavage is

Table 21.16.2 Pipetting Scheme for Activity Measurement of Meprins

Desired substrate concentration (working solution)	250 μM	150 μM	100 μM	50 μM	25 μM	10 μM	5 μM	2.5 μM
Volume substrate stock solution (250 μM)	50 μl	30 μl	20 μl	10 μl	5 μl	2 μl	1 μl	0.5 μl
Volume DMSO (5% v/v)	-	20 μl	30 μl	40 μl	45 μl	48 μl	49 μl	49.5 μl
	Premix 10 μl of active meprin (2.5 nM) and 50 μl of 50 mM HEPES/NaOH, pH 7.5, for each assay. Then, add 40 μl of the prepared substrate solution (see above) to start the reaction in a total assay volume of 100 μl.							
Final substrate concentration (in the assay)	100 μM	60 μM	40 μM	20 μM	10 μM	4 μM	2 μM	1 μM

not too fast. This is especially true when using a multicuvette system, as the cycle time between cuvettes may result in missing an important region of the reaction

3. Dissolve the substrate in 100% DMSO to a final concentration of 20 mg/ml.

   Always keep the substrate protected from light.

4. Dilute the substrate with 50 mM HEPES/NaOH, pH 7.5, to a concentration of 250 μM. For a series of concentrations from 1 μM to 100 μM, see Table 21.16.2c (since the substrate and the proteinase are mixed afterwards). For each sample, 40 μl are needed, so prepare a little more, e.g., 50 μl. To ensure the same concentration of DMSO in all samples, predilute DMSO to 5% v/v with 50 mM HEPES/NaOH, pH 7.5, and add according to Table 21.16.2.

5. Dilute the activated meprin solution (step 2) to a concentration of 2.5 nM with 50 mM HEPES/NaOH, pH 7.5.

   For each reaction, 10 μl of the diluted meprin is required.

6. Mix 10 μl of 2.5 nM activated meprin with 50 μl of 50 mM HEPES/NaOH, pH 7.5, in each sample well of a 96-well black microtiter plate. If inhibitors or other substances are to be analyzed replace the buffer with the respective analyte solution.

Perform the measurement

7. Place the 96-well plate with meprin solution in the Varioskan Flash 3001 spectral plate reader and incubate the reader at 37°C for 10 min.

8. At the same time, place the substrate solutions in a water bath and incubate at 37°C for 10 min.

9. Add 40 μl of the substrate solution to all desired wells of the 96-well plate simultaneously.

10. Start the measurement and monitor the fluorescence intensity at 520 nm, with the excitation wavelength at 350 nm, for 6 min.

11. After these 6 min, add 1 μl of 25 mg/ml proteinase K to reach total turnover of residual substrate, and monitor the reaction for another 15 min.

12. For calculation of the rate of substrate turnover use the following formula:

$$v = \frac{[S] * m}{\Delta F}[M * sec^{-1}]$$

where [S] represents substrate concentration, m represents the slope of substrate turnover from 0 to 360 sec, M represents molarity (mol/liter), and ΔF represents maximum fluorescence intensity of totally hydrolyzed substrate.

USING AZOCASEIN TO MEASURE PROTEINASE ACTIVITY

BASIC PROTOCOL 3

A simpler method to determine the proteolytic activity of a proteinase is the use of chromogenic substrates. These substrates change color after cleavage, which can be detected by a simple spectrophotometer. Compared to the fluorogenic assay, it has some limitations with low proteinase concentration or low affinity towards the substrate. A special type of this assay is the azocasein assay (Iversen and Jörgensen, 1995). Here, an azo dye–coupled casein is cleaved by the proteinase of choice. After the desired incubation time, TCA is added to the sample, which leads to the inactivation of the proteinase and the precipitation of non-cleaved azocasein. The cleaved peptide fragments stay in solution, and the non-cleaved protein can be removed by centrifugation. After this, the amount of cleaved azocasein is proportional to the absorption of the solution at a certain wavelength. Azocasein is inexpensive compared to other substrates. However, due to the additional working steps (incubation, precipitation, centrifugation, and analysis), individual errors can accumulate and render results less reproducible. Therefore, special attention has to be paid to every step of the analysis. In particular, all volumes of buffer, proteinase, substrate, and TCA have to be pipetted precisely to ensure correct concentration of proteinase and substrate. Additionally, make sure that after centrifugation the supernatant is completely free of residual precipitated protein. Remove the cleared supernatant by pipetting to ensure that the pellet does not redissolve.

Materials

12 mg/ml azocasein (sulfanilamide-azocasein; Sigma-Aldrich, cat. no. A2765) in a suitable buffer (azocasein can be dissolved in a broad variety of different buffers; for accurate kinetic measurements, make sure that all azocasein is dissolved; if not, heat the solution to 65°C; centrifuge for 5 min at maximum speed to remove non-dissolved protein)
Suitable buffer for the particular proteinase under study (see recipes in Reagents and Solutions)
Proteinase of interest
10% glacial acetic acid: 50 g of glacial acetic acid in 227 ml of H_2O

Thermostatted water bath
Centrifuge (cooled)
Spectrophotometer

Set up the experiment

1. Dissolve azocasein at a concentration of 12 mg/ml in a suitable buffer for the proteinase of interest.

   For each sample, 200 μl of the azocasein solution is needed.

2. Shake until all azocasein has completely dissolved. If necessary heat to 65°C.

3. Prepare the proteinase of interest at the desired concentration with or without inhibitors or other analytes in a final volume of 50 µl.
4. *Prepare a negative control:* Incubate one sample without protease. Use buffer instead to achieve the same concentration of azocasein.

Perform the experiment

5. Add 200 µl of azocasein solution to each sample of proteinase.
6. Incubate at 37°C for the designated period of time.

   Longer incubation times, up to several days, are possible if the proteinase activity is low. Perform a pilot test to determine the period of time required to attain an absorbance of 1. For proper kinetic measurements, the absorbance should not exceed a value of 1.

7. After the designated period of time, add 250 µl of 10% glacial acetic acid.
8. Incubate for 15 min on ice.
9. Centrifuge for 5 min at 16,000 × *g*, 4°C, to precipitate non-cleaved azocasein.
10. Pipet the supernatant into a new reaction tube and discard the pellet.
11. Measure the supernatant's absorbance at a wavelength of 340 nm.
12. Subtract the negative control from the samples.

    The absorbance is proportional to the amount of cleaved azocasein.

ALTERNATE PROTOCOL

ZYMOGRAPHY TO MEASURE PROTEINASE ACTIVITY

Another method to test a proteinase for its activity is zymography. In this technique, a substrate, usually gelatin or casein, is added to a polyacrylamide gel. After non-reducing SDS electrophoresis (*UNIT 10.1*; Gallagher, 2012), the proteinase can be renatured in a suitable buffer. This method works well with matrix metalloproteinases and some astacins. The limiting factor is the ability of the proteinase to refold in the renaturing buffer and to cleave the embedded substrate. The proteinase can be visualized as clear bands after staining with Coomassie Brilliant Blue (*UNIT 10.5*; Echan and Speicher, 2002). The advantages of zymography are sensitivity and simplicity. With a well-cleavable substrate, proteinases can be detected in as little as picogram amounts. Due to the use of different incubation times, the sensitivity can be adjusted every time for different concentrations and different activities of the investigated proteinase. However, not all proteinases can be refolded after SDS electrophoresis. In this case, native electrophoresis in the absence of SDS and reducing agents is a possible solution, depending on proteinase's isoelectric point. The calculation of kinetic parameters is possible in principle. However, compared to other methods, it is time consuming and requires considerable amounts of protein. For detailed information on zymography see *UNIT 21.15* (Troeberg and Nagase, 2004)

BASIC PROTOCOL 4

ACTIVATION OF pro-MMPs WITH AMINO-PHENYL-MERCURIC ACETATE (APMA)

Metalloproteinases can be post-translationally regulated in vivo at several levels. They are generally expressed as latent zymogens, in which a pro-peptide blocks the access of the substrate to the active center. The pro-peptide needs to be cleaved off in order to gain proteolytic activity. This can occur via furin-like pro-protein convertases on the secretory pathway or later in the extracellular space in a complex proteolytic network. Afterwards, active proteinases underlie the regulation by tissue specific inhibitors with different affinities in vivo. However, heterologous overexpression of a native proteinase

often results in incompletely activated enzymes, which may require additional activation in vitro.

In vitro activation can be achieved in various ways depending on the type of proteinase. Astacins often are activated by trypsin like-proteinases, but auto-activation has also been observed under certain conditions (Yiallouros et al., 2002). For activation with trypsin-like proteinases, a nanomolar to micromolar concentration of trypsin or plasmin is incubated with the designated metalloproteinase for a short period of time, so that only the activation site is cleaved (since it is more exposed and easier to access than other putative cleavage sites) but the metalloproteinase is not degraded. To diminish the unwanted side effects of the activating proteinase, it must be either removed or inhibited by irreversible serine proteinase inhibitors such as Pefabloc (i.e., AEBSF, 4-(2-amino-ethyl)-benzene-sulfonyl-fluoride or PMSF (phenylmethylsulfonyl fluoride).

A convenient in vitro procedure for the activation of pro-MMPs and ADAMs is the use of amino-phenyl-mercuric acetate (APMA). This chemical oxidizes the cysteine of the cysteine switch, which blocks the active site zinc ion in the latent pro-proteinase.

Materials

Amino-phenyl-mercuric acetate (APMA; Sigma-Aldrich, cat. no. A9563)
Dimethyl sulfoxide (DMSO)
MMP in suitable buffer, e.g., MMP buffer, BTP buffer, ADAM buffer, or ovastacin buffer (see recipes for these buffers in Reagents and Solutions)
Thermostatted water bath

NOTE: Since APMA has a limited half-life, it has to be prepared fresh each time before use. Due to its poor solubility in water, addition of any kind of salt will lead to precipitation of APMA in the stock solution.

Alternative 1: Activation with APMA dissolved in DMSO

1a. Dissolve APMA to a final concentration of 50 mM in DMSO and vortex gently.

2a. To 38 µl MMP solution, add 2 µl of 50 mM APMA (final concentration 2.5 mM).

3a. Incubate at 37°C for 1 hr.

If DMSO affects the experiment, APMA can also be dissolved in distilled water instead (see steps 1b to 3b below)

Alternative 2: Activation with APMA dissolved in distilled H_2O

1b. Dissolve APMA to a final concentration of 5 mM in distilled water and mix gently.

2b. To 40 µl MMP solution, add 10 µl of 5 mM APMA (final concentration 1 mM).

3b. Incubate at 37°C for 2 hr.

REAGENTS AND SOLUTIONS

Use Milli-Q-purified water or equivalent (double-distilled H_2O) in all recipes and protocol steps. Metalloproteinases differ in their specific requirements of ions like zinc, calcium, sodium, chloride and others. Either their catalytic domains or their additional carboxy-terminally attached domains may bind other ions, which confer to the structural stability and function of these enzymes. Listed here are specific requirements for MMPs, ADAMs, BTPs, meprins, and ovastacin. Table 21.16.3 lists a series of recipes for the routine use with metalloproteinases.

ADAM buffer

25 mM Tris·Cl, pH 9.0 (*APPENDIX 2E*)
0.005% Brij-35
Store up to 2 weeks at 4°C

Table 21.16.3 List of Enzyme-Substrate Combinations for Routine Use

Proteinase	Sequence	Excitation (nm)	Emission (nm)	Buffer	Manufacturer
ADAM 10 (20 nM)	Mca-PLAQAV-Dpa-RSSSR-NH_2 (12 μM)	320	405	ADAM buffer[a]	R&D Systems Europe Ltd.
MMP 2, 8, 9 (20 nM)	Dnp-PLGLWA-Dnp-R-NH_2 (50 μM)	280	350	MMP buffer[a]	Enzo Life Sciences GmbH
MMP 2, 9 (20 nM)	Mca-PLA-Nva-Dap-AR-NH_2 (50 μM)	328	393	MMP buffer[a]	Bachem
Meprin α, β (0.25 nM)	Ac-R-E(Edans)-DR-Nle-VGDDY-K(Dabcyl)-NH_2 (20 μM)	350	550	50 mM HEPES/NaOH, pH 7.5	Biosyntan
BMP-1 (10 nM)	Ac-R-E(Edans)-DR-Nle-VGDDY-K(Dabcyl)-NH_2 (25 μM)	350	550	BTP buffer[a]	Biosyntan
Ovastacin (300 nM)	Ac-R-E(Edans)-DR-Nle-VGDDY-K(Dabcyl)-NH_2 (20 μM)	350	550	Ovastacin buffer[a]	Biosyntan

[a]See recipe in Reagents and Solutions.

BTP buffer

50 mM HEPES/NaOH, pH 7.4
150 mM NaCl
5 mM $CaCl_2$
Store up to 2 weeks at 4°C
0.02% octyl-β-D-glucopyranoside
Store up to 2 weeks at 4°C

MMP buffer

50 mM Tris·Cl, pH 7.4 (*APPENDIX 2E*)
150 mM NaCl
10 mM $CaCl_2$
0.005% Brij-35
Store up to 2 weeks at 4°C

Ovastacin buffer

50 mM Tris·Cl, pH 7.4 (*APPENDIX 2E*)
150 mM NaCl
Store up to 2 weeks at 4°C

COMMENTARY

Background Information

The mammalian metzincins can be classified into four major families, termed ADAMs (a disintegrin and metalloproteinase), ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs), MMPs (matrix metalloproteinases), and astacins (Stöcker et al., 1995. They are generally translated as inactive pro-enzymes, which are proteolytically activated during or after secretion and regulated by extracellular inhibitors and enhancers (Ra and Parks, 2007; Guevara et al., 2010).

ADAMs are always membrane associated and are composed of an amino-terminal propeptide, a proteinase domain, an additional disintegrin-like domain, a cysteine-rich domain, a transmembrane domain, and a cytosolic tail (Seals and Courtneidge, 2003; Klein and Bischoff, 2011). The disintegrin domain is able to bind and interact with cell surface integrins, which implies additional roles in signaling and cell adhesion. In fact, only 13 of the 21 expressed ADAMs have an active proteinase domain (Edwards et al., 2009). The most prominent proteinases of this family are ADAM10 and ADAM17, also known as α-secretase and TACE (tumor necrosis factor α, TNF-α, converting enzyme), respectively. Both are able to shed a large variety of cell surface-associated proteins off the plasma membrane, resulting in activation and/or inactivation of various cell-signaling pathways. Both have impact on notch signaling, and they prime proteins for cleavage by intramembrane proteinases (Blobel, 1997).

ADAMTSs share the general composition of ADAM proteinases. In addition, they contain multiple thrombospondin-like repeats and other C-terminal domains, and they are soluble due to the lack of a membrane anchor (Takeda, 2009). While, in ADAM proteinases, only the Cys-rich domain contains a hyper-variable region contributing to substrate selectivity, in the ADAMTS proteinases both the Cys-rich domain and the disintegrin domain fold in front of the active site and control its accessibility (Gerhardt et al., 2007). ADAMTS2, 3, and 14 are the procollagen N-proteinases, which act in concert with BMP1 during procollagen maturation (Hojima et al., 1989; Colige et al., 1997). ADAMTS4 and ADAMTS5 are also known as aggrecanases (Tortorella et al., 2001), and ADMTS13 cleaves von Willebrand factor (Wu et al., 2006). The function of many other ADAMTS proteinases is less clear. Knockout studies reveal that several ADAMTS proteinases are involved in organogenesis and embryonic development (Dubail and Apte, 2015).

MMPs typically comprise a pro-domain, a catalytic domain, and a C-terminal hemopexin-like domain. The latter accounts for protein-protein interactions with substrates or inhibitors (Knäuper et al., 1997). Historically, MMPs have been designated as collagenases, gelatinases, or stromelysins according to their then-known preferred substrates (Ra and Parks, 2007; Sternlicht and Werb, 2009). The collagenases MMP1, MMP8, and MMP13 cleave triple-helical collagen at a conserved site, resulting in fragments that are three-fourths and one-fourth of the full length, which then depolymerize ("melt") into gelatin that is prone to degradation by other proteinases. Besides the classical collagenases, also the membrane-bound MT1-MMP (MMP14) is able to cleave triple-helical collagen. The gelatinases A and B (i.e., MMP2 and MMP9) cleave denatured collagen (gelatin) and many cytokines and chemokines and have some unique structural features such as three inserted fibronectin-like subdomains inside their proteinase domains. Stromelysins are involved in the cleavage of several different extracellular matrix proteins, but are unable to cleave collagen in either its triple-helical or denatured form. The MT-MMPs are membrane bound through either a transmembrane domain C-terminally attached to their hemopexin domain or via a glycosylphosphatidylinositol (GPI) anchor (Visse and Nagase, 2003; Sternlicht and Werb, 2009).

Mammalian astacins are grouped into subfamilies termed BMP1/tolloids (BTPs), meprins, and ovastacin (Gomis-Rüth et al., 2012a). BMP1 stands for bone morphogenetic protein-1, although it is different from TGF-β-like BMPs (Wozney et al., 1988). In lower vertebrates and invertebrates, the astacin family is much more heterogeneous and versatile, including, for example, embryonic hatching enzymes and proteinases with toxin domains (Möhrlen et al., 2006). BTPs are characterized by CUB and EGF-like domains located C-terminally to the proteinase domain. These domains are responsible for dimerization of the proteinase and for substrate recognition (Berry et al., 2009). BTPs cleave growth-factor antagonists during embryonic dorsoventral axis formation in both vertebrates and invertebrates (Piccolo et al., 1997; Scott et al., 1999). In adult vertebrates, they

process precursor proteins for proper assembly of the extracellular matrix. A significant example is BMP1, also termed the procollagen C-proteinase (PCP), which removes the C-terminal pro-peptides from fibrillar procollagens (Hojima et al., 1989). Meprins possess additional MAM, TRAF, intervening, EGF-like, and transmembrane domains downstream of the proteinase domain. The MAM domain facilitates formation of disulfide-bonded dimers, as seen in meprin β; in the case of meprin α, disulfide-bonded dimers can join into huge homo-oligomers in the megadalton range (Becker et al., 2003; Arolas et al., 2012). Both meprin subunits are translated with a transmembrane anchor. In contrast to meprin β, the α subunit loses its anchor due to furin cleavage in the trans Golgi and becomes soluble if not co-expressed with meprin β (Tang and Bond, 1998). Meprins have multiple functions as cell surface sheddases and as processing enzymes for extracellular matrix components (Broder and Becker-Pauly, 2013; Prox et al., 2015). Ovastacin consists of a pro-domain, a catalytic domain, and a C-terminal domain of unknown function. Ovastacin is expressed in the mammalian oocyte, from where it is released and activated during sperm-egg fusion to protect the embryo from penetration by subsequent sperm cells (Quesada et al., 2004; Stöcker et al., 2014). In the latent pro-forms of MMPs and ADAMs, the thiol group of a conserved cysteine residue of the pro-peptide blocks the catalytic zinc ion. This cysteine switch needs to be released to activate the proteinase (Van Wart and Birkedal-Hansen, 1990), which is achieved in vivo by furin-like pro-hormone convertases on the secretory pathway, by other serine proteinases and/or metalloproteinases at the cell surface, or in the extracellular space. The MMPs not bound to membranes are involved in complex networks of activating proteinases and scaffolding proteins, which are unique for each MMP (Ra and Parks, 2007). In addition, MMPs can be activated chemically by oxidation. Macrophage-derived reactive oxygen species (ROS) are able to oxidize the latency mediating cysteine in the pro-domain, resulting in an activation of the proteinase (Ra and Parks, 2007). In vitro, ADAMS and MMPs can also be activated by sulfur-attacking organomercurials like *p*-amino-phenyl-mercuric-acetate (APMA). However, this does not work with ADAMTS proteinases and astacins. In the latter, a conserved aspartate of the pro-sequence confers latency on the pro-enzyme, in analogy to the cysteine switch (Van Wart and Birkedal-Hansen, 1990; Guevara et al., 2010). Most astacins are activated by different trypsin-like serine proteinases at a conserved cleavage site 9 amino acid residues upstream of the first tryptophan in the mature chain (Guevara et al., 2010; Gomis-Rüth et al., 2012a). In a few cases, auto-activation by internal cleavage may occur, as observed in astacin (Yiallouros et al., 2002).

After activation, metzincins underlie the control by natural enhancers and/or inhibitors. The procollagen C-proteinase activity of BMP1 (i.e., PCP) is enhanced by the procollagen C-proteinase enhancer protein (PCPE) by at least one order of magnitude, in a strictly substrate-dependent manner (Adar et al., 1986; Kronenberg et al., 2009). The most versatile physiological scavenger of metalloproteinases is the nonspecific inhibitor α_2-macroglobulin, which is present at concentrations up to 2.5 mg/ml in blood plasma. Proteolytic cleavage within the inhibitor's so-called bait region induces a conformational change, which entraps the proteinase in a cage formed by the tetrameric α_2-macroglobulin oligomer. Additionally, an isopeptide bond between a lysine side chain of the proteinase and α_2-macroglobulin is formed due to a highly reactive thioester within α_2-macroglobulin. In a third step, the receptor-binding domain of α_2-macroglobulin is presented to be recognized by macrophages and the complex endocytosed (Sottrup-Jensen, 1989; Marrero et al., 2012). However, large proteinases like the meprins are not capable of cleaving the bait region, presumably due to steric hindrance, and, therefore, are resistant to α_2-macroglobulin. The most important specific MMP inhibitors are the tissue inhibitors of metalloproteinases (TIMP1 to TIMP4), which can block MMPs with different affinities down to the pM range (Baker et al., 2002). Selective inhibitors of distinct MMPs, ADAMs, or ADAMTSs are rare. However, TIMP3 is able to inhibit ADAMTS4, ADAMTS5, and all ADAMs except ADAM8, ADAM28, and ADAM33 (Nagase et al., 2006; Edwards et al., 2009). Furthermore, TIMP1 inhibits ADAM10 (Schelter et al., 2011). BTPs are potently inhibited by the protein sizzled, from the clawed frog *Xenopus*. Sizzled is a soluble version of the WNT-ligand-binding domain of frizzled receptors (Ploper et al., 2011). However, the homologous sizzled-related proteins from mammals do not inhibit mammalian BTPs (Bijakowski et al., 2012). The only specific inhibitors of meprins and ovastacin are fetuin

plasma proteins of cystatin superfamily expressed in the liver, which are present in extracellular fluids in micromolar concentrations (Hedrich et al., 2010; Dietzel et al., 2013; Stöcker et al., 2014).

Critical Parameters

Metalloproteinases have several structural and functional features in common. They all contain a catalytic zinc ion, which is complexed, with a few exceptions, by histidines and a glutamic acid–bound water molecule. However, there is also considerable variability between the different families of metalloproteinases due to their different multidomain structures. For example, some of them contain additional calcium-binding domains, or their catalytic domains may accommodate other structurally important metal ions, which may be accessible to chelation by certain buffers. Chelating agents like EDTA can bind both transition metals, such as zinc, and alkaline earths, such as calcium. Other chelators like 1,10-phenanthroline only bind transition metals. Therefore, it is generally recommended to avoid chelating buffer components like EDTA, 1,10-phenanthroline, or citrate when characterizing metalloproteinases. On the other hand, various other bivalent metal ions, of iron, manganese, cobalt, copper, and mercury, to name just a few, can replace the catalytically essential zinc and thereby inactivate metalloproteinases or at least modulate their activity. For more detailed information on metallobiochemistry see, e.g., Riordan and Vallee (1988).

Nearly all metalloproteinases are membrane bound or secreted in the extracellular space. During their intracellular trafficking, they undergo several steps of post-translational processing including disulfide bridge formation. If the proteinase of interest is expressed in a bacterial system (e.g., *E. coli*), it is necessary to form these disulfide bridges afterwards in a refolding process, which can be very challenging (Reyda et al., 1999). If proteinases are expressed in a eukaryotic system, this procedure is not necessary; however, the yield is often limited.

If the proteinase is expressed and purified as the pro-enzyme, it can be stored in its inactive state for a long period of time up to multiple years at −20 or −80°C. Astacin as the metzincin prototype can even be lyophilized in its active form and stored nearly indefinitely.

The assay buffer requirements for each proteinase are as diverse as the different proteinases. However, it was revealed that mild detergents like Brij-35 or octyl-beta-D-glucopyranoside (0.05 to 0.1% w/v) have a positive influence on their activity as well as their stability and solubility in aqueous solutions (Hojima et al., 1989; Bijakowski et al., 2012).

Troubleshooting

To avoid any source of trouble in advance, make sure you are working with absolutely clean water without any contaminants from metal ions (e.g., water supply system with metal pipes must be avoided). In addition, some proteinases, once activated, are very unstable, which might lead to decreased proteolytic activity. Be sure to work quickly and always on ice. Table 21.16.4 provides a list of common problems regarding the presented methods as well as their origin and solution.

Anticipated Results

The yield of purified proteinase from conditioned insect cell medium varies depending on the used cell line, quality of virus, and expression time. In general, between 1 and 10 mg per liter can be achieved using the described purification method.

The detection of proteinase activity with a FRET assay is a relatively sensitive method. With appropriate substrates, proteinases and inhibitors can be characterized even at picomolar concentrations. For example, a standard assay with meprin α and Ac-R-E(Edans)-DR-Nle-VGDDY-K(Dabcyl)-NH_2 can be performed with a proteinase concentration of 0.25 nM and below. Metalloproteinase activation should be completely abolished by the addition of 5 mM 1,10-phenanthroline.

The azocasein assay is a less specific assay, since a variety of different proteinases are able to cleave casein. Using a highly active proteinase will result in visible degradation of azocasein in minutes (valuable after TCA precipitation and measurable by VIS spectroscopy). However, slow-acting proteinases will need to incubate for a longer period of time. Sometimes an incubation time of several hours or even an overnight digestion will be necessary to detect cleaved azocasein.

The activation of MMPs, ADAMs, and ADAMTs proteinases with APMA is a reliable method. After 1 to 2 hr, nearly all proteinase should be activated. The success of activation can be measured using a suitable activity assay.

Time Considerations

All protocols except Basic Protocol 1 can feasibly be completed within 1 day of work.

Table 21.16.4 Troubleshooting Guide for Working with Metalloproteinases

Problem	Possible cause	Solution
Recombinant proteinase does not precipitate with 60% ammonium sulfate	Amount of ammonium sulfate is too low to precipitate the desired protein	Try different concentrations of ammonium sulfate from 40% to 80% saturation
Recombinant proteinase is not separated by gel filtration	Wrong pore size	Adjust pore size of the solid phase material
	Flow rate is too high or too low	Adjust flow rate
	Protein aggregates	Try different salt concentrations
Recombinant proteinase does not bind to its inhibitor	Proteinase is not active	Activate proteinase, check amount of active proteinase by an appropriate assay
	Inhibitor binding is not tight enough	Determine inhibition constant by FRET assay
	Insufficient amount of chromatography matrix	Use more matrix
Proteinase does not elute from the affinity column	Inhibitor binding is too tight	Try different inhibitor
		Try different elution procedures: e.g., alter pH of the elution buffer, change concentration of soluble inhibitor, etc.
No activity is observed by FRET assay	Proteinase is not active	Activate proteinase, check amount of active proteinase by a different method
Only low activity is observed by FRET assay	Substrate affinity is too weak	Try different substrate
	Aromatic agents, like Pefabloc, are quenching the substrate	Try to avoid aromatic agents in the sample buffer
	Proteinase denatures during the assay	Try different assay buffers, try to keep DMSO concentration as low as possible
	Concentration of proteinase is too low	Increase proteinase concentration
Substrate precipitates during the assay	Substrate concentration too high	Decrease substrate concentration
	DMSO concentration too low	Increase DMSO concentration
Too much fluorescence detected	Substrate concentration is too high	Decrease substrate concentration
Azocasein does not dissolve in the desired buffer	Solubility of azocasein is too low	Decrease the concentration of azocasein
		Heat the azocasein solution to 65°C

continued

Table 21.16.4 Troubleshooting Guide for Working with Metalloproteinases, *continued*

Problem	Possible cause	Solution
No activity is observed by the azocasein assay	Azocasein is not cleaved by the proteinase	Check the cleavability of azocasein and the proteinase by SDS-PAGE
	Incubation time is too short	Increase incubation time
	Proteinase is not active	Activate proteinase, check activity by a different method
	Proteinase denatures during the assay	Check proteinase activity at the end of the assay
MMP, ADAM, ADAMTS do not get activated by APMA	APMA is already oxidized	Prepare fresh APMA solution

The purification takes up to 3 days, depending on which steps are performed as overnight steps. Possible overnight steps are the precipitation with ammonium sulfate, the dialysis after the precipitation, and the binding of meprin α to the matrix-coupled Pro-Leu-Gly-hydroxamate.

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