Challenges and solutions for purification of ADAMTS proteases- an overview

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Running Head: ADAMTS purification overview

Abstract

ADAMTS are secreted metalloproteinases implicated in many key biological processes. The 19 different members of this family share an identical domain composition at the level of their amino-terminal portion, whereas the identity and number of the domains forming their carboxy-terminal half are divergent and define distinct ADAMTS subfamilies. Due to their large size, extensive glycosylation, the presence of specific domains, their tendency to form aggregates, their relatively low abundance in tissues and the presence of many disulfide bonds, ADAMTS are very hard to isolate, express and purify, as either native or recombinant active enzymes. This chapter provides an overview of critical steps to take into account when obtaining these proteases for biochemical and functional investigation.

Keywords

ADAMTS, aminoprocollagen peptidase, metalloproteinase, recombinant enzyme, purification

The ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin type 1 repeat) form a family of 19 secreted enzymes. Together with MMPs, ADAMs and Astacins, they belong to the “Metzincin” clan, meaning that they contain a zinc-binding catalytic motif and a conserved methionine following the active site (1). They possess additional specific features such as the presence of a “disintegrin-like” domain and a C-terminal ancillary domain having a characteristic modular structure that contains at least one (and up to 15) ThromboSpondin type 1 repeat (TSR) (2). Subfamilies of ADAMTS have been established based on known substrates and on the composition of their C-terminal ancillary domain. All the ADAMTS share also some common biological and biochemical features (3). They contain several disulfide bonds, are usually highly glycosylated and tend to form aggregates when purified and concentrated. Their affinity for their ligands and substrates largely depends on the final composition of their C-terminal domains (4). They are also subject to cleavages, sometime autocatalytic, affecting the C-terminal ancillary domain and, consequently, having a potential impact on substrate recognition and thus proteolytic specificity (5). Unlike MMPs, the catalytic domain of ADAMTS expressed alone does not typically display activity because the prodomain is required for the correct folding of the enzyme and because the ancillary domains contribute significantly to substrate recognition. Finally, the activity of ADAMTS can be fully and irreversibly inhibited in conditions that do not affect the vast majority of other metalloproteinases such as pH lower than 6.0 or the presence of chemicals able to interact with Ca^{++} or Zn^{++} such as phosphate or imidazole. As a result of these specificities, both purification procedure and enzymatic activity assays have to take into account all these constraints and limitations.

The study of enzymes purified from tissues is mandatory to obtain information about the activity and the biological properties of the native protein. However, production and use of recombinant enzymes is required in several situations such as studies of modified proteins (mutation, truncation, chimeric enzymes …) or when the enzyme is produced at low level or in tissues which are minute or difficult to procure. In the specific case of the aminoprocollagen peptidases (ADAMTS2, 3 and 14) (6, 7), recombinant enzymes have been produced to study the role of the different domains forming the C-terminal half of the enzymes (6, 8), to produce large amounts of human and mouse enzymes for identification of new substrates (9, 10) or evaluating the biological consequences of various mutations detected in patients (11).

I- Production of recombinant enzyme

The approaches described below have been used successfully to produce recombinant ADAMTS2, 3, 12 and 14, but can serve as a starting protocol for the other members of the family.

Step 1. Choice of cells

Bacteria, yeast or insect cells are not suitable for the production of large secreted proteins containing numerous disulfide bonds and for which the 3D structure is critical for their biological functions.

Choosing the most suitable mammalian cell line is the first and perhaps one of the most critical steps since it will strongly influence the entire strategy and chances of success. Several aspects have to be considered regarding:

- The proliferation rate (to rapidly expand the number of cells).
- Cell “immortality” to have long term propagation of cultures.
- The capacity to grow in chemically defined culture media without serum or other supplements rich in proteins in order to increase the purity of the starting material.
- The capacity to express large amount of recombinant material in the long term.
- The absence of endogenous expression of proteins or activities that could mimic, or interfere with, the activity of the recombinant enzyme.
- The capacity to preserve (when required) post-translational modifications occurring in vivo and required for proper activity (proteolysis, glycosylation, etc).

For our studies, several cell lines were tested. HEK293 cells were found to be the best choice since they fulfill all the requirements stated above while many of the other cell lines were not able to produce high amounts of enzyme in the long term. This may result from a weak but significant negative selection by the ADAMTS expression on the proliferation or adhesion of many immortalized or transformed cells.

Step 2. Cell engineering

The cDNA coding sequence is inserted under the control of a strong promoter (such as CMV) in a mammalian expression vector containing a selection gene to allow selection of transfected cells. In situations where cells expressing or not the recombinant protein have to be compared it might be appropriate to use an “inducible” system (such as the pcDNA4/TO vector (Invitrogen) or equivalent) in order to generate cells conditionally expressing the recombinant protein, which represent a much robust and reliable model than comparing different cell lines with and without constitutive expression. In some other experimental contexts, it might be most relevant to produce a version of the enzyme lacking enzymatic activity, which is usually obtained by changing the “Glu” in the catalytic metalloproteinase pocket into a “Ala” (8). An example of such use would be the study of the functions

and binding partners of the most C-terminal ancillary domains which are known to be highly sensitive to autocatalytic cleavage.

Efficient commercial antibodies for ADAMTS are rarely available since most of them were not properly validated, lack specificity or have been raised against epitopes present in the prodomain or in the C-terminal domain which are most of the time cleaved off during the maturation process. Thus these antibodies may not be useful to study the mature active form of ADAMTS. Therefore, tagging the recombinant protein with specific sequences is helpful. However, such sequences have to be introduced in the sequence of the mature enzyme (resulting from the cleavage of the N-terminal propeptide and C-terminal domains) and in a region which can afford such modification without affecting the biological properties of the entire enzyme. In the specific case of ADAMTS2, 3 and 14, a HA tag (YPYDVPDYA) was inserted "in frame" in the spacer domain, without modifying the expression, the maturation or the activity of the recombinant enzymes (8). Although not experimentally determined yet, it suggests that the other ADAMTS could be tagged similarly without affecting significantly their biology.

Detailed procedure:

- Plating of HEK293 cells at 25% confluence (DMEM containing glucose, 5 % fetal calf serum (FCS) and pyruvate.
- 18 hours later, the expression vector possessing the required characteristics and containing the cDNA coding sequence is transfected using Fugene6 or any equivalent method allowing efficient transfection.
- Two days after transfection, the culture medium is replaced by the same medium supplemented with the selection agent appropriate for the resistance gene present in the expression vector.
- The selection culture medium is renewed every 2 days.
- After selection of the resistant cells (10-15 days), subcloning is performed to identify clones displaying the highest expression and the highest enzymatic activities.
- *When an inducible system is used, the absence of expression in the control condition should be verified.*

The selected clones are then amplified and stored in liquid nitrogen until use.

Step 3. Production

Depending on the cell type used, protocols can be slightly different. As an example, we used adherent "regular" HEK293 cells whereas HEK293 cells growing in suspension in chemically defined culture medium are now available. We tried to adapt our cells to this type of medium. The results were very

good in terms of production but the enzymatic activity was very low because of problems related to the maturation and activation of the enzymes. This illustrates that procedures for production have to be carefully set up and monitored before starting large scale production and should not be changed without careful evaluation.

Detailed procedure:

An identical protocol was developed for the production of ADAMTS2, 3 and 14.

- Expansion of the selected clone in DMEM (with glucose and pyruvate, and supplemented with 5 % FCS).
- Ten confluent 10 cm-diameter culture dishes are trypsinized, pooled and divided in a total of 100 new 10 cm-dishes in the same culture medium (10 ml per dish).
- *Multilayer T-flasks providing an expanded surface for cell culture can be used to facilitate handling. However, visual/microscopic examination of cell quality is not possible in these plates and they should be used only when culture conditions are well established. Identical ratios between the number of cells and the volume of culture medium should be kept. Be aware also that medium exchanges are not as efficient in such culture devices as in regular culture dishes.*
- At confluence (after 3 days), the medium is replaced by 10 ml of fresh DMEM (+ pyruvate and glucose) without serum in order to reduce the protein load and to avoid the presence of alpha2-macroglobulin (an irreversible inhibitor of many proteases). This medium is further supplemented with
  - Soyabean Trypsin Inhibitor (40 µg/ml) to prevent proteolytic degradation,
  - $ZnCl_2$ (40 µM) since Zn^{++} is an essential component of the catalytic site of Metzincins, including ADAMTS
  - Heparin (at 50 µg/ml) since we have shown that it favors the release into the culture medium of some ADAMTS proteins which otherwise tend to stay associated with the cell surface.
- After 48 h, the conditioned medium is collected, centrifuged to remove cell debris and store at -80°C until purification.
- *At this step, the enzyme quality and concentration can be evaluated by Western blotting, using anti-tag antibody if applicable. However, its activity cannot be reliably determined accurately because of the presence of heparin which acts as a weak reversible inhibitor.*

Step 4. Purification

The procedure developed for purifying the native enzyme (see chapter “Purification of native or recombinant ADAMTS2, and procollagen I cleavage assay”) can be used and usually provides higher purification grade because of the lower protein load in the starting material. However, neither the native nor the recombinant enzymes are 100 % pure and the nature of the contaminants can be different depending on the origin of the initial sample or extract, which underlines the necessity to use adequate negative controls for any assay evaluating recombinant ADAMTS. A particularly well suited strategy is to develop cell clones conditionally expressing the enzyme of interest upon the presence of an inducer (see “Step 2”). With such model, cultures from a single clone can serve as starting material to purify the enzyme (with inducer) and to generate the negative control (without inducer), the two types of sample being processed following the same purification procedure.

II. Activity

The aminoprocollagen peptidase activity can be measured by using the procedure described in the chapter regarding “Purification of native or recombinant ADAMTS2, and procollagen I cleavage assay”. Besides the fibrillar procollagens initially described as the primary substrate of ADAMTS2, 3 and 14, a recent large scale analysis of their substrate repertoire has been recently performed and has led to the identification of several previously unknown substrates, such as fibronectin, LTBP1, TGFβ-R3 or DKK3 (9).

The cleavage of these substrates have been confirmed by Western blotting experiments using purified recombinant substrates and enzymes. The conditions for cleavage were not optimized and were comparable to those used for the aminoprocollagen peptidase assay. Therefore, the kinetic constants were not determined. While these assays using recombinant proteases and substrates are easy to implement and provide clear results, they do not perfectly reflect the in vivo context and have their own drawback. Some of these caveats are described below:

- ADAMTS are found in vivo as different polypeptides resulting from series of cleavages removing the N-terminal propeptide, but also by cleavages occurring in the C-terminal ancillary domain which is thought to dictate the specificity of the enzyme for different substrates. As a result, the generated fragments can contain the catalytic domain but not the sequences required for substrate recognition while some others, without catalytic activity but containing the interacting domains, can potentially act as competitive inhibitors. Therefore, the use of different purification procedures potentially leading to specific enrichment of some fragments versus others might introduce differences and biases when determining enzyme activity.
- The activity may also be regulated by co-factors or other regulators. It was already reported that the cleavage of the C-propeptide of type I collagen by BMP1 and tolloids (metzincins of

the astacin family) is strongly increased by the presence of PCPCE which grasps the stalk of the C-propeptide trimer to boost its cleavage (12). However, PCPE1 has no effect on the cleavage of other substrates of BMP1, demonstrating a specific co-factor activity. Similarly, it has been shown that CCBE1 increases the cleavage of pro-VEGF-C into active VEGF-C by ADAMTS3 (13), while it has no effect on the cleavage of other substrates. Fibulin-1 was shown to be a co-factor for the action of ADAMTS1 and ADAMTS5 (14, 15). These examples suggest the existence of factors influencing the cleavage of specific substrates by ADAMTS.

As a way to take into account such potential limitations, cell culture models have been developed as an alternative to the use of purified proteins. Cells expressing recombinant ADAMTS proteases can be used in a variety of strategies to measure activity or elucidate substrates and biological effects. For example, if the ADAMTS is impossible to purify, conditioned medium can be used to observe activity, the expressing cells could be co-transfected with a candidate substrate, or the expressing cells can be co-cultured with cells providing a library of substrates as described below.

Substrate cleavages in culture models

In order to develop versatile models for identifying ADAMTS cleavage activity on wide repertoires of substrates, the choice was made to establish co-cultures between cells producing potential substrates (fibroblasts, endothelial cells, etc.) and HEK293 secreting the ADAMTS under study.

- As a prerequisite, the cell line potentially producing substrates should not express the enzyme of interest or any other enzyme that might have similar activity. Cell engineering using the “Crispr/Cas9” technology or use of cells derived from knockout mice (mouse embryo fibroblasts for example) or from patients with inactivating mutations are most useful. Thus, most of the target substrates of the protease should be intact in these cells, maximizing the likelihood of observing differences between cleaved and uncleaved substrates upon treatment with the ADAMTS protease.
- HEK293 cells with inducible expression of the studied enzyme are particularly well suited for such applications. Except for the inducer, they allow the use of identical conditions for both the negative control (absence of inducer) and the condition with enzyme (with inducer) which considerably improves the robustness of the assay.

The following conditions were used to better characterize the substrate repertoire of ADAMTS2, 3 and 14.

- Dermatosparactic fibroblasts[1] and HEK293 cells (able to conditionally express one ADAMTS) are trypsinized, pooled at a 4:1 ratio[2], and seeded in 30 "10 cm-diameter" culture dishes (8 x 10^5 fibroblasts and 2 x 10^5 HEK293 per dish) in 10 ml of DMEM with 5 % FCS.
  - *[1]: Skin fibroblasts derived from a patient with a null mutation in Adamts2 (dermatosparactic type of Ehlers-Danlos syndrome) were used because they do not express active ADAMTS2 and ADAMTS3, while they produce only very low amount of ADAMTS14.*
  - *[2]: Because of differences in proliferation, the initial ratios between the HEK293 cells and the other cells has to be carefully determined in order to obtain homogenous co-cultures with maximal direct contacts between the two cell types (rather than having numerous clusters containing one or the other cell type) since ADAMTS tend to remain associated with the cell layer close to their secretion site.*
- After 18 hours, the medium is replaced by the same medium in 15 dishes (control) and by the same medium but containing 1 µg/ml doxycycline in the 15 dishes where the ADAMTS has to be induced (protease).
- After 24 hours, the culture medium is removed and the cell layers are washed two times with 10 ml DMEM without phenol red and without serum. The same medium (DMEM without phenol red and without serum) is added to the culture dishes and supplemented (induction of synthesis of the recombinant ADAMTS) or not (control condition) with doxycycline at 1 µg/ml.
- After 48 hours, the conditioned media are collected, the control and the "protease" samples are pooled separately, centrifuged to remove cell debris and concentrated (at 2 mg protein/ml) using filtration centrifugal devices with low molecular weight cutoff (3000 MWCO).
- Samples can then be used for Western blotting evaluation or for large scale and high throughput proteomic analyses such as iTRAQ-TAILS (16, 17).

In summary, there is clearly not a 'one size fits all" strategy, since individual ADAMTS proteases that have not been previously expressed may have specific characteristics that will require trial and error, consideration of alternatives and demand patience and flexibility. This overview has presented some approaches and helpful guidelines for purification and use of recombinant protease that will stimulate the reader to consider alternative approaches. Specific purification approaches are presented in other chapters of this volume. In general, however, the use of mammalian cells, overexpression of appropriately engineered plasmids, and selection of an intelligent purification approach based on the general properties of the ADAMTS family, are likely to give a successful outcome.

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