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Purification of an insect derived recombinant human ADAMTS-1 reveals novel gelatin (type I collagen) degrading activities

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Abstract

ADAMTS-1 (A Disintegrin And Metalloprotease with ThromboSpondin repeats) is a member of a family of secreted proteolytic enzymes with a complex modular structure. These enzymes are characterised by an N-terminal metalloproteinase domain, a disintegrin-like domain and a carboxyl terminal region containing variable numbers of a repeat sequence with homology to thrombospondin-1. The expression of the gene for ADAMTS-1 has been associated with inflammation, ovulation, angiogenesis, cellular proliferation and bone formation. ADAMTS-1 can proteolytically process large proteoglycans indicating a potential role in extracellular matrix turnover. In this study, we have tested ADAMTS-1 activity in gelatin zymogram assays. Since previous data demonstrate that ADAMTS-1 is a matrix metalloproteinase (MMP) substrate and is highly unstable in conditioned medium from eukaryotic cell types, we created an insect cell line expressing human ADAMTS-1. We isolated an epitope tagged full-length recombinant ADAMTS-1 from serum free insect cell conditioned medium. The purified protein had aggrecanase activity and appears as two major bands on the silver stained SDS-PAGE corresponding well to a pro-domain on form of 115 kDa and a pro-domain off form of 90 kDa. Using denatured type I collagen in zymographic analysis we demonstrate that ADAMTS-1 has a previously unreported gelatinolytic activity. Also, we notice that processing of its C-terminal region by an apparently autocatalytic process reveals a 27 kDa species with gelatinolytic activity. Furthermore, we show that MMP2 but not MMP13 remove ADAMTS-1 specific gelatin zymopraphic zones. (Mol Cell Biochem **281:** 95–102, 2006)

Key words: adamts, metalloproteinase, zymogram

Introduction

ADAMTS proteins (A Disintegrin And Metalloprotease with ThromboSpondin repeats), are members of a family of secreted multi-domain neutral endopeptidases that is currently composed of 19 members (http://merops.sanger.ac.uk/). Several of these proteins have been associated with physiological functions pertaining to the extracellular matrix (ECM) turnover during development and in pathologies such as rheumatoid arthritis. Currently, genes for two members of the ADAMTS family have been discovered to contain mutations associated with inherited disease phenotypes. The *ADAMTS-2* gene contains mutations which confer a fragile skin phenotype termed Ehlers Danlos Syndrome (VIIC) due to the aberrant processing of pro-collagen [1] and mutations in *ADAMTS-13* have been shown to be responsible for thrombotic thrombocytopenic purpura (TTP), a syndrome characterised by haemolytic anaemia in which cleavage of the Von Willebrand factor is compromised [2]. Both ADAMTS-2 andADAMTS-13 in contrast to other ADAMTS proteins

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contain an RGD sequence in their C-terminal regions suggesting a potential function in cell adhesion.

As far as the range of ECM substrates has been investigated, several themes relating to ADAMTS activity are beginning to emerge. ADAMTS-1 has been shown to cleave the hyalectan proteoglycans aggrecan and versican specifically at glutamyl bonds [3, 4]. This is a property it has in common with ADAMTS-4, -5 and -9 (often termed aggrecanases) [5–7]. ADAMTS-2, -3 and -14 are pro-collagen *N*-propeptidases cleaving the N-terminal pro-peptides from type I and type II collagens [8–10]. ADAMTS-4 can also cleave the hyalectan family proteoglycans brevican and versican which suggests that this enzyme may have roles in proteoglycan turnover in cartilage, brain and the neural system [3, 11].

ADAMTS-1 was identified in 1997 by Kuno *et al.* and is one of the most studied family members [12]. Recently, mice deficient in ADAMTS-1 have been created and these animals although viable show morphological defects in the kidney, lower urogenital tract, adrenal glands and in adipose tissue [13]. In addition, ADAMTS-1 deficient animals show growth retardation indicating a role for this enzyme in bone formation. In line with this it also appears to be a target for parathyroid hormone in bone [14]. Recently, we also showed the presence of ADAMTS-1 protein in rat osteoblasts at sites of bone formation [15].

ADAMTS-1 has also been associated in terms of its gene expression characteristics and the process of ovulation with cathepsin L [16]. Furthermore, ADAMTS-1 protein has been demonstrated to be involved in the inhibition of angiogenesis and shown to bind VEGF and effect the VEGF signalling pathway [17, 18]. Importantly, it was concluded that the anti-angiogenic effect of ADAMTS-1 was dependent on, at least in part, on an intact proteinase domain [19].

Previous work done on ADAMTS-1 showed that it is furin processed in the N-terminus upon secretion and that it is a substrate for a number of MMPs [20]. Notably, Wei *et al.* showed clearly, by expressing metalloproteinase inactivated versions of ADAMTS-1 that it is autolytic, i.e degrade itself [21]. We have therefore created a stable insect cell line expressing ADAMTS-1 and developed a rapid purification system. The purified enzyme was then characterised by aggrecan cleavage and extensive gelatin zymography, a general assay to follow MMP activity.

Materials and methods

Generation of antisera for human ADAMTS-1

A peptide spanning the sequence KKPKHYIDFC corresponding to amino acid #935-944 (of the 951 amino acid protein), of human ADAMTS-1 (gb: AF170084), was synthesised in the Biochemistry Department at the University of Newcastle by Dr Joe Gray *et al.*. This peptide was coupled to KLH and used to immunise rabbits and the generated antisera was purified on protein-A sepharose (Amersham).

Expression of recombinant protein from insect cells

High Five™ cells were routinely cultured on tissue culture plastic and maintained at 27 °C in Ultimate™ serum-free insect cell media (Invitrogen) as per manufacturer's directions. The full-length human ADAMTS-1 open reading frame (bp: 294–3143, gb: AF170084) was PCR amplified from KIAA cDNA clone 1346 (Kazusa DNA Research Institute, Japan) with forward primer (ggagctcaaaATGgggaacgcggagc) and reverse primer (ggactcgag actgcattctgccattgtg) including XhoI restriction sites to facilitate sub-cloning into pIB/V5-His insect cell expression vector (Invitrogen). To assure no mutations had been introduced by the PCR-reaction, it was sequenced using an Applied Biosystems 377 apparatus. The resulting expression construct generated a full-length ADAMTS-1 with a V5- and 6xhistidine-epitope added to its COOH-terminus. The expression plasmid was transfected into High-Five™ insect cells with Lipofectamine (Invitrogen) and plated onto a 35-mm petri dishes. After 48 h, antibiotic selection (100 μg/ml Blastocidin, Invitrogen) was started and continued for 3 weeks. Colonies that survived the selection were manually picked, expanded and maintained in medium containing 5 μg/ml Blastocidin. ADAMTS-1 expression of isolated cell clones was verified by Western blot analysis of conditioned medium using HRP-conjugated anti-V5 monoclonal antibody (Invitrogen).

Purification of ADAMTS-1

Four hundred millilitre of conditioned media from ADAMTS-1 expressing High-Five™ expressing cells was supplemented with inhibitors to cysteine, serine and aspartic protease and cleared by centrifugation at 5000 $\times g$ for 10 min and stored at -20 °C until the start of purification. The conditioned media was complemented with 30 mM Tris–HCl, pH 7.5 before incubation with 3 ml of SP-sepharose (Amersham) overnight at 4 °C. Unbound and weakly bound material was washed away with 10 bed volumes of a buffer containing 300 mM NaCl, 0.01% Brij 35 and 50 mM Tris – HCl, pH 7.5. Tightly bound material was batch eluted twice with two bed volumes of 1.0 M NaCl, 0.01% Brij 35 and 50 mM Tris–HCl, pH 7.5. The pooled eluate was diluted five times before applying to a 1 ml Hi-trap heparin-sepharose column (Amersham). The heparin-sepharose was washed with 10 bed volumes of elution buffer before use to wash away any contaminating proteins bound to the column. The column was then washed with five bed volumes of binding buffer before elution with

an increasing concentration of NaCl ranging from 0.15 to 1.0 M in 0.01% Brij 35 and 50 mM Tris–HCl, pH 7.5. The pooled V5-epitope positive eluate was applied to a chelating column (1 ml Hi-trap, Amersham) saturated with Ni^{2+}. Both the heparin and Ni-column were controlled using a fast protein liquid chromatography instrument (FPLC, Pharmacia). Following binding of the pooled protein onto the chelating column, it was washed with five column volumes of binding buffer (1.0 M NaCl, 0.01% Brij 35, 10 mM imidazole and 50 mM Tris–HCl, pH 7.5). Tightly bound ADAMTS-1 was eluted with an increasing gradient from 10 to 300 mM imidazole buffer also containing 1.0 M NaCl, 0.01% Brij 35 and 50 mM Tris–HCl, pH 7.5. Elution was monitored by in-line UV and conductivity measurements. Eluate of 0.4 ml fractions were collected and tested by dot blot, silver nitrate staining, Western blot and gelatin zymogram analysis as described below. Purified protein was stored in elution buffer on ice and quantified using the protein assay (Bio Rad) with bovine serum albumin as reference protein.

Dot blots

Three microlitre of each fraction was spotted on a nitrocellulose membrane (Protran Schleicher & Schuell) and air dried. An anti-V5 monoclonal antibody (1:5000) conjugated with HRP in Tris-buffered saline, pH 7.5 + 5% (w/v) non-fat milk and 0.1% Tween 20 was then incubated with the membrane for 20 min followed by washing for 2 × 5 min in 250 ml of Tris-buffered saline, pH 7.5 + 0.1% Tween 20 before chemiluminescence processing with ECL reagents (Amersham).

Immunoblots

SDS and beta-mercaptoethanol treated protein samples (10 μl) was heated at 95 °C for 5 min and then separated on 8% SDS-PAGE mini-gel according to Laemmli [23] together with prestained molecular mass markers (Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane using a semi-dry transfer system with 50 mM Tris, 40 mM glycine and 20% methanol at pH 9.2 and constant voltage (80 V/mini-gel) for 40 min, according Bio-Rad manufacturer's manual. After the transfer membranes were blocked by incubation with Tris-buffered saline, pH 7.5 + 5% (w/v) non-fat milk and 0.1% Tween 20 overnight at 4 °C. Horse-radish peroxidase conjugated anti-V5 monoclonal antibody diluted 1:5000 in blocking buffer was then applied for 2 h followed by washing twice for 5 min in Tris-buffered saline, pH 7.5 + 0.1% Tween 20. For the anti-ADAMTS-1, polyclonal blocking was done for 1 h at room temperature followed by primary incubation in blocking buffer overnight at 4 °C and then washed in Tris-buffered saline, pH 7.5 + 0.1% Tween 20 for 30 min. Secondary antibody was a mouse anti-rabbit HRP conjugated (1:10 000) in blocking buffer for 1 h at room temperature followed by a 30 min wash step (Amersham). Membranes were then processed for chemiluminescence with ECL reagents.

Silver staining

Protein samples were treated and run as described in Immunoblots. After separation, the proteins were fixed in methanol/acetic acid/water (45:5:50) for 30 min followed by a 20 min wash in water. The gel was then incubated in 0.02% sodium thiosulfate for 2 min, rinsed with water, incubated in 0.1% silver nitrate at 4 °C in fridge for 20 min followed by two short rinses with water before developing in 0.04% formaldehyde in 2% Na_2CO_3. When sufficient stain was obtained, the development was stopped by incubation in a 1% acetic acid solution [37].

Aggrecanase assay

One microgram of purified bovine aggrecan in 100 μl of 50 mM Tris – HCl, pH 7.6, 150 mM $NaCl_2$ and 5 mM $CaCl_2$ solution was incubated with 0.1 μg recombinant ADAMTS-1 for 16 h at 37 °C. Prior to SDS-PAGE separation, the aggrecan molecule was deglycosylated with chondrotinase ABC (0.01 unit/μg aggrecan) for 2 h in 50 mM Tris – HCl, pH 8.0, 30 mM sodium acetate, 10 mM EDTA, 5 mM PMSF, 0.3 mM pepstatin A and 10 mM ethylmalemide. After SDS-PAGE, the separated proteins were blotted onto nitrocellulose membranes and blocked overnight in PBS + 5% non fat milk and 0.1% Tween 20. To detect aggrecan fragments the blot was incubated with antibody 2-B-6 (anti chondroitin sulphate stub antibody, Seikagaku). As a control for metalloproteinase activity, the addition of 10 mM EDTA inhibited aggrecan processing by ADAMTS-1.

Gelatin zymography

This was performed as described in Hawkes *et al.*'s Methods in Molecular Biology, vol. 151. In brief: samples of 15 μl were diluted with 5 μl 4X sample buffer: 0.187 M Tris–HCl, pH 6.8, 4% (w/v) SDS, 0.04% bromophenol blue and 20% (v/v) glycerol, incubated at 37 °C for 5 min, and run on a 8% SDS-PAGE gel containing 0.1% (w/v) gelatin. The gelatin was prepared by heating type I collagen for 30 min at 55 °C and stored at −20 °C. Eight percent polyacrylamide gels were run in an ice bath at constant power of 120 V for 80 min. The gels were then washed twice for 10 min with 2.5% Triton X-100 in water at room temperature followed by

incubation overnight in a buffer containing 0.05 M Tris–HCl, 5 mM $CaCl_2$, 5 μM $ZnCl_2$ and 0.05% Brij 35, pH 8.8. After incubation, the gel was stained in 0.1% Coomassie brilliant blue R-250 (w/v) in methanol/acetic acid/water (45:10:45) for 3–4 h at room temperature and than destained with the same solution but without the Coomassie brilliant blue R-250, until clear zones revealing gelatin lysis appeared. All clear zones in the zymogram referred to as ADAMTS-1 generated here did not appear in the presence of 10 mM EDTA indicating that a metalloproteinase generated the clear zones.

Matrix metalloproteinase incubation of ADAMTS-1

Recombinant human MMP2 and MMP13 were from R&D Systems and activated with APMA according to manufacturer's instructions before use. Either 0.2 μg of ADAMTS-1, MMP2 or MMP13 was incubated separately (ADAMTS-1) or together as described, in 50 μl of 50 mM Tris, 10 mM $CaCl_2$, 150 mM $NaCl_2$, 0.05% Brij 35, pH 7.5 at 37 °C for 30 min. After the incubation, 15 μl of 4X sample buffer was added and a gelatin zymogram was run as outlined above.

Results

The expression of recombinant human ADAMTS-1 in insect cells

Standard molecular biological procedures were used in order to amplify the ORF from the KIAA1346 cDNA clone encoding ADAMTS-1 (a kind gift of the HUGE database). This fragment was subcloned into an insect cell expression vector and sequenced to confirm no errors had been introduced by the PCR reaction. The vector adds a 6xhistidine and a V5 epitope onto the C-terminus of the protein to facilitate purification and detection, respectively (Fig. 1A). Cells expressing ADAMTS-1 were selected using blasticidin resistance and Western blot analysis of serum free conditioned media showed a single 90 kDa protein using both a polyclonal anti-ADAMTS-1 antibody and a V5 recognising antibody (Fig. 1B). No low molecular weight bands were reacting with the antibodies used in either lane, indicating that the major part of the detected ADAMTS-1 is kept intact. The molecular size of 90 kDa correspond well with the reported size of secreted ADAMTS-1, lacking its pro-domain suggesting efficient processing by insect cell derived furin pro-protein convertases [20].

Purification of ADAMTS-1 from insect cell CM

As first step in the purification procedure we concentrated the recombinant ADAMTS-1 using cation-exchange beads

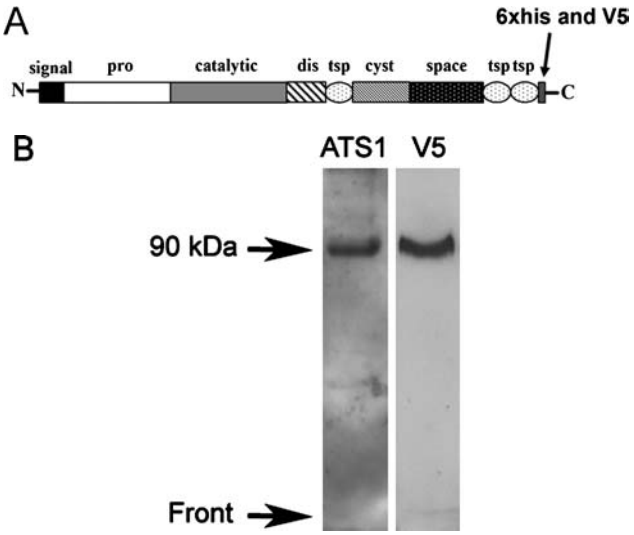

Fig. 1. ADAMTS-1 structure and appearance in insect cell media. Schematic presentation of the domain structure of ADAMTS-1 (A). In this study, a V5 and 6xhistidine epitope was added at its C-terminus to facilitate detection and purification, respectively. Serum free conditioned media from insect cells expressing this ADAMTS-1 construct was separated on SDS-PAGE followed by a transfer of the proteins to a nitrocellulose membrane. The membrane was probed using an anti-ADAMTS-1 antibody (ATS1) or a V5 antibody (V5) followed by processing for chemiluminescence with ECL reagents (B). The major visible protein in both blots fits well with a 90 kDa secreted ADAMTS-1 lacking its pro-domain, already described in the literature. No low molecular weight processed forms are visualised indicating that the major part of the detected ADAMTS-1 in this expression system is in the pro-domain off form. Signal sequence (sig), pro-domain (pro), catalytic domain (catalytic), disintegrin domain (dis), thrombospondin repeat (tsp), cystein rich domain (cyst) and spacer domain (space).

followed by batch elution. Then we took advantage of the known strong heparin binding interaction of the thrombospondin type 1 repeats in ADAMTS-1 [24] and coupled this with the ability of the added histidine tag to bind a Ni-chelating column to design a purification strategy that enabled us to rapidly isolate essentially pure ADAMTS-1. This was necessary since enrichment of this protein induces rapid breakdown and inactivation of its proteinase activity (*unpublished observation*). Online UV measurements from the elution of the Ni-chelating column showed a small protein peak eluting late in the imidazole-gradient well separated from the bulk protein (Fig. 2A). Using a rapid dot-blot protocol, the minor peak was identified as V5-epitope positive (*data not shown*). SDS-PAGE followed by silver staining of fractions covering this minor peak showed presence of two protein bands (Fig. 2B). Importantly, both these bands, a 90 kDa protein together with a 115 kDa protein component, were V5-epitope positive in Western blotting showing that the silver stained protein is most likely the recombinant ADAMTS-1 (Fig. 2C). The molecular size of 115 kDa is in good agreement with the expected sizes of full length ADAMTS-1 still

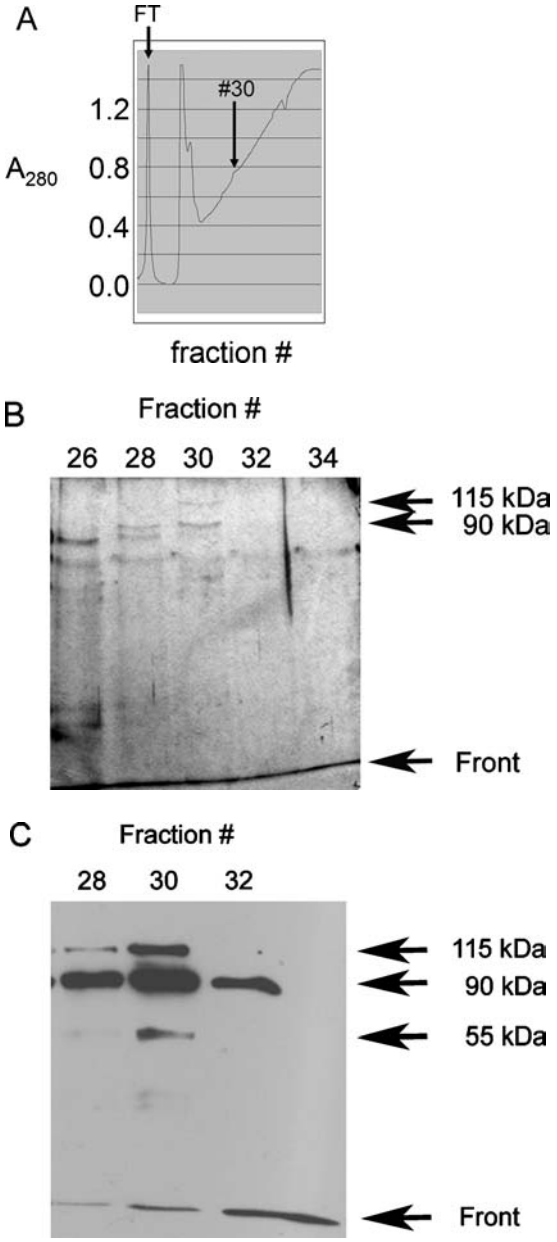

Fig. 2. Nickel chelating chromatography of V5 and 6xhistidin tagged ADAMTS-1. V5 positive proteins from a heparin-sepharose column were applied to a Ni^{2+} loaded chelating column and bound proteins were eluted with an imidazole gradient ranging from 10 to 300 mM. (FT) indicate the proteins not binding to the column and thus flows through (FT). Fractions were collected and online absorbance reading at 280 nm generated a protein profile where the arrow indicates peak V5 positive material (A) according to dot blot experiments (*data not shown*). The fractions covering the V5 positive peak were then analysed with SDS-PAGE followed by silver staining for protein (B) and Western blotting for ADAMTS-1 using the V5 antibody (C). The molecular size of the silver stained proteins in fraction #30 (peak V5 fraction) correspond with ADAMTS-1 species with and without its pro-domain. Western blot of the same fractions show that both the silver stained protein species are positive for the V5 antibody indicating that fraction #30 contains essentially pure ADAMTS-1 protein. Molecular weights of proteins in peak V5 positive fraction are indicated.

containing the pro-domain. Furthermore, concentration of the purified ADAMTS-1 proteins revealed an additional protein species of 110 kDa upon both silver staining and Western blot (Fig. 3A). The 110 kDa size fits well with a pro-domain on form generated from alternate usage of initiating codons present in the beginning of the ADAMTS-1 sequence.

Activity of the ADAMTS-1 protein

To verify that our purified recombinant ADAMTS-1 was enzymatically active, we first confirmed that it could cut the large proteoglycan aggrecan as has previously been reported [4], (Fig. 3B). We then tested if ADAMTS-1 being a metalloproteinase related to the MMP family of endopeptidases could function in zymograms. This was assayed by applying the simple and widely used method to detect MMP activity; gelatin zymography. Denatured type I collagen was incorporated in polyacrylamide gels and the fractions covering the eluted V5-positive protein peak from the Ni-chelating column were assayed in routine gelatin zymography experiments. The results (Fig. 3C) showed that the peak ADAMTS-1 containing fractions displayed gelatinolytic activity corresponding to clear zones of molecular mass of approximately 115 and 90 kDa. Importantly, fractions both before and after the V5 positive peak were free from gelatinolytic activity indicating that the clear zones are generated by ADAMTS-1. Interestingly, ADAMTS-1 containing fractions eluting later in the imidazole gradient showed further gelatinolytic activity corresponding to a smaller sized protein (~50 kDa) compared to the fractions earlier in the gradient.

Interaction of ADAMTS-1 with MMP2 and MMP13

It has been shown earlier that ADAMTS-1 is a substrate for a number of MMPs, including MMP2 (gelatinase A) [20]. Here we wanted to test if gelatinase (MMP2) or collagenase (MMP13) exposure of ADAMTS-1 influences its gelatinolytic activity. Approximately, 0.2 μg of MMP2 or MMP13 was mixed with similar amounts of purified ADAMTS-1 and incubated at 37 °C for 30 min followed by gelatin zymography. In Fig. 4 the results from this type of experiment show that incubation of ADAMTS-1 with MMP2 removes the ADAMTS-1 specific lytic zones present on the gelatin zymogram of ADAMTS-1 alone. However, if ADAMTS-1 processing by MMP2 produces a ~65 kDa fragment, as suggested by Rodriguez-Manzaneque *et al.* [20], this lytic zone could be masked by MMP2 lytic zones. A similar experiment with collagenase-3 (MMP13) did not remove the ADAMTS-1 specific 90 kDa lytic zone. Interestingly, incubation of the ADAMTS-1 alone revealed the presence of a low molecular weight gelatin degrading activity (~27 kDa) which was not

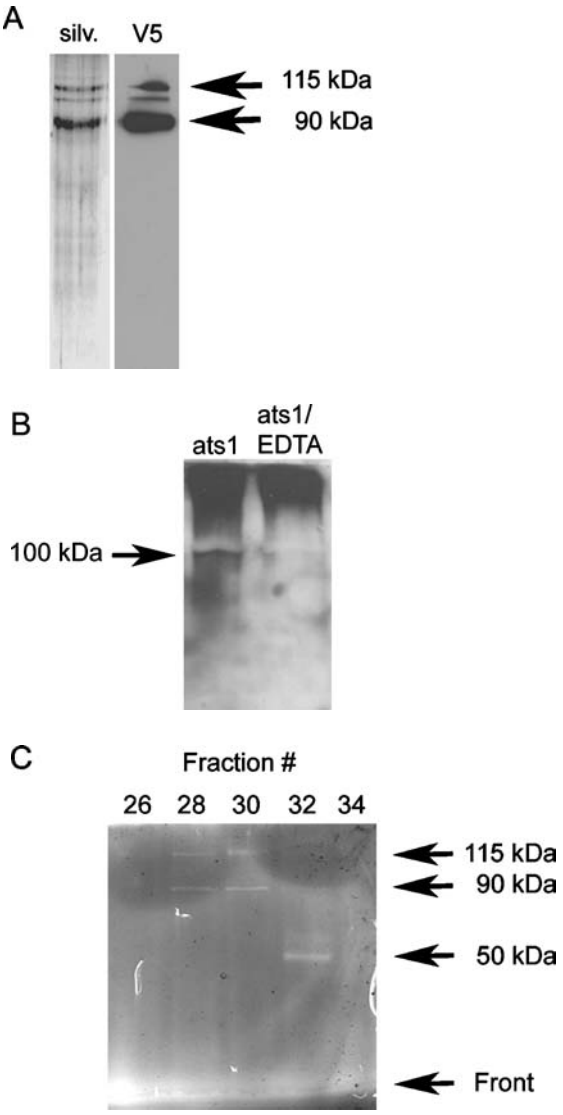

Fig. 3. Characterisation of ADAMTS-1 apperance and activity. SDS-PAGE separation of purified and concentrated recombinant ADAMTS-1 show upon silver staining three bands, the major species of 90 kDa being the pro-domain off mature furin processed version whereas the 115 and 110 kDa versions are likely the full length species still containing the pro-domain, probably produced from alternate usage of the two initiation codons present in the sequence (A, lane silv.). All the three species are positive for the V5 epitope added to the recombinant ADAMTS-1, in Western blotting (A, lane V5). (B) Bovine aggrecan was incubated for 16 h with purified recombinant ADAMTS-1 with (lane ats1/EDTA) or without (lane ats1) 10 mM EDTA. ADAMTS-1 incubation without EDTA generated a 100 kDa degradation product from aggrecan, detected using Western blot with an anti-chondroitin sulphate stub antibody after aggrecan was deglycosylated with chondrotinase ABC. This product was not seen when EDTA was included in the incubation (ats1/EDTA lane). (C) Fractions analysed in Figs. 2B and C where treated with non-reducing sample buffer and separated on SDS-PAGE containing gelatin (type I collagen). After separation the gel was washed in a neutral detergent to remove SDS followed by incubation 18 h at 37 °C. The reaction was stopped by immersing the gel in Coomassie blue staining solution and clear zones were subsequently revealed by washing in destain solution. Molecular weights of proteins in the clear zones are indicated.

present in samples containing either MMP2, MMP13 or if the 37 °C incubation of ADAMTS-1 was omitted. If EDTA was present in the zymographic incubation, the ADAMTS-1 specific zone was removed showing that the zone was generated by a metalloproteinase.

Discussion

The ADAMTS family of proteases have a variety of substrates which in large part are structural components of the ECM. In particular, the ADAMTS-1, -4, -5, and -9 proteins have been shown to degrade high molecular weight proteoglycans such as versican, brevican and aggrecan [3–7, 11]. In this regard, they are considered as important mediators of turnover in cartilage tissue where the loss of proteoglycan is widely considered to be an early step in the degradation of the cartilage matrix where suitable intervention may be targeted to ameliorate pathology [25, 26]. They are however highly expressed in other tissues including the brain and retina where their roles are presently less well defined [6, 11, 27, 28]. A novel finding from the present study demonstrates that in addition to the proteoglycan substrates already known, ADAMTS-1 can also degrade denatured type I collagen as previously described for many MMP enzymes such as MMP2, MMP3 and MMP9 [29–32]. ADAMTS-1 is the first member of the aggrecanse family that has been shown to be able to do this and it may thus be possible that a coupled degradation of proteoglycan and denatured collagen could be mediated by ADAMTS-1, activities already known to be performed by several MMPs.

Interestingly, the molecular weights of the gelatinolytic species detected in this study varied between that expected for full length ADAMTS-1 (115 kDa) down to a 27 kDa species. It is possible that, in line with data for ADAMTS-4, activity of the ADAMTS-1 enzyme on aggrecan requires the full length enzyme [33]. It could therefore be envisaged that subsequent cleavage of the full length enzyme as mediated by proteolysis or autolysis, could be a trigger for a 'substrate switch'. In a tissue, such as cartilage and bone where the turnover of proteoglycan and collagen are intimately related with respect to matrix homeostasis, an enzyme such as ADAMTS-1 with its ability to degrade both substrates could be pivotal to tissue turnover.

Here we used an insect cell expression system free from endogenous gelatinolytic activity [22], together with inhibitors to cysteine, serine and aspartic proteases for production of recombinant human ADAMTS-1. The molecular weights of the ADAMTS-1 species that retain gelatinolytic activity are 115, 90, 50, and 27 kDa. This implies that it is being C-terminally processed in our system, since the metalloproteinase domain is close to the N-terminus (Fig. 1A). C-terminal processing of ADAMTS-1 by MMPs has already been demonstrated

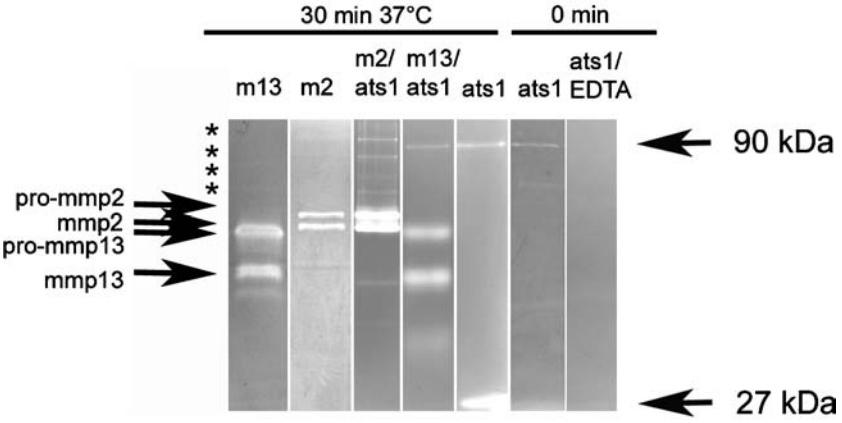

Fig. 4. MMP/ADAMTS-1 interaction zymogram. About 200 ng of partially activated recombinant MMP2 or MMP13 was incubated with similar amounts of purified ADAMTS-1 for 30 min at 37 °C followed by gelatin zymogram analysis in a non-reducing 8% SDS-PAGE. As a control, MMP2 alone and MMP13 alone was incubated. Asterix (*) indicate clear zones from the recombinant MMP2 preparation. ADAMTS-1 alone shows , in addition to the 90 kDa clear zone a 27 kDa zone corresponding to a molecular weight of the catalytic domain alone. Incubation of ADAMTS-1 with MMP13 does not affect the 90 kDa clear zone but the 27 kDa zone disappears. This is in contrast to incubation with MMP2 which remove both the 90 and 27 kDa clear zones generated by ADAMTS-1. Zymographic analysis of ADAMTS-1 alone without a 30 min pre-incubation at 37 °C are shown with and without EDTA present at the in-gel incubation step.

in mammalian cells [20], and autolysis of ADAMTS-1 has already clearly been demonstrated by expression of a catalytically inactive mutant [21]. The autolytic property of ADAMTS-1 complicates its analysis since enrichment of the protein leads to further breakdown. This is illustrated in the zymogram of eluted fractions from the nickel column, where the zymogram show clear zones of 90 and 115 kDa in early ADAMTS-1 fractions, whereas, a late fraction show a 50 kDa clear zone. Furthermore, incubation of purified ADAMTS-1 produces a 27 kDa gelatinolytic fragment, a fragment not present before incubation (Fig. 4). The absence of a 65 kDa form, such as that demonstrated to be formed in mammalian cells underscores that this second ADAMTS-1 processing event probably is mediated by MMPs. This processing may be intimately involved in the change of substrate from proteoglycan to denatured collagen in line with the above hypothesis and may be mediated by a decrease in matrix binding ability due to loss of TSP1 type repeats.

In this context, it is interesting to note that inhibitor studies have been carried out by others on ADAMTS-1 and demonstrated that it is sensitive to both TIMP2 and TIMP3, and insensitive to TIMP1 [34]. ADAMTS-1 therefore could continue to be active in the presence of TIMP1 which is highly expressed in bone cells and in cartilage [35, 36].

This work thus describes purification of recombinant ADAMTS-1 which has facilitated characterisation of ADAMTS-1 proteinase activities. Here, we show in addition to the proteoglycan substrates so far documented, ADAMTS-1 can also degrade preparations of denatured type I collagen. Furthermore, ADAMTS-1 undergoes autolysis upon purification which yields active species with a variety of molecular weights. The finding that ADAMTS-1 possesses additional proteolytic activities beyond cutting large proteoglycans could explain some of its involvement in the multitude of events already described in the literature. It will be important to clarify if other ADAMTS family members also possess the ability to degrade gelatin, or if, this is a unique property of ADAMTS-1.

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