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Proteolytic fragmentation and sugar chains of plasma ADAMTS13 purified by a conformation-dependent monoclonal antibody

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ADAMTS13 is a metalloproteinase that specifically cleaves unusually large von Willbrand factor multimers under high-shear stress. Deficiency of ADAMTS13 activity induces a life-threatening generalized disease, thrombotic thrombocytopenic purpura. We established a simple and efficient method to purify plasma ADAMTS13 (pADAMTS13) from cryosupernatant using an anti-ADAMTS13 monoclonal antibody (A10) that recognizes a conformational epitope within the disintegrin-like domain. Using the purified pADAMTS13, the amino acid residues involved in cleavage by thrombin, plasmin and leucocyte elastase were determined, and the carbohydrate moieties of this enzyme was analysed by lectin blots. Purified pADAMTS13 had a specific activity of 300 U/mg (25,057-fold purification) and the pI was 5.1–5.5. Cleavage sites of the purified pADAMTS13 by three proteases were identified; thrombin cleaved the four peptidyl bonds between Arg257–Ala258, Arg459–Ser460, Arg888–Thr889 and Arg1176–Arg1177, plasmin cleaved the three peptidyl bonds between Arg257–Ala258, Arg888–Thr889 and Arg1176–Arg1177, and elastase cleaved the two peptidyl bonds between Ile380–Ala381 and Thr874–Ser875. Lectin blot analysis indicated the presence of non-reducing terminal α2–6 and α2–3-linked sialic acid residues with penultimate β-galactose residues on the N- and O-linked sugar chains of pADAMTS13, suggesting that pADAMTS13 is cleared from the circulation via the hepatic asialoglycoprotein receptor like other plasma glycoproteins.

Keywords: enzyme digestion/lectin blotting/plasma ADAMTS13/purification.

Abbereviations: ADAMTS13, a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13; IEF, Isoelectric focusing; mAb: monoclonal antibody; PVDF, polyvinylidene difluoride, RT, room temperature, TBS, Tris-buffered saline; UL-VWFM, unusually large von Willbrand factor multimers, VWF, von Willbrand factor.

ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type-1 motifs 13) is a metalloproteinase that specifically cleaves von Willbrand factor (VWF)-A2 domain at the peptidyl bond between Tyr1605 and Met1606 (*1–3*). VWF is synthesized exclusively in vascular endothelial cells, and is then either constitutively secreted into subendothelial matrices or released into the circulation as unusually large VWF multimer (UL-VWFM), which is the most biologically active form of the protein and results in excessive platelet aggregation/thrombus formation under high-shear stress generated in the microvasculature (*4–6*). Failure to control VWF activity due to a lack of plasma (p) ADAMTS13 activity typically results in thrombotic thrombocytopenic purpura (TTP), a life-threatening disease characterized by generalized microvascular platelet thrombi (*7–10*). Thus, ADAMTS13 may play a pivotal role in maintaining normal circulation, by controlling the molecular size of UL-VWFM.

In addition to TTP, recent studies indicate that an extremely low ratio of ADAMTS13 to UL-VWFM may cause a variety of clinical issues complicated by platelet thrombi in the microvasculature (*11*), including hepatic veno-occlusive disease (*12*), liver transplantation (*13*), liver cirrhosis (*14*), sepsis-induced disseminated intravascular coagulation (DIC) (*15*) and severe acute pancreatitis (*16*). However, it is poorly understood how ADAMTS13 activity is regulated in these circumstances.

In this regard, Crawley *et al.* (*17*) reported in 2006 that three serine proteases, thrombin, plasmin and factor Xa each cleaves ADAMTS13 to effect a reduction of ADAMTS13 activity *in vitro*. Using purified recombinant ADAMTS13 (rADAMTS13), Lam *et al.* (*18*) subsequently reported that thrombin cleaves the peptide bonds C-terminal to amino acid residues Arg257 and Arg1176. However, human ADAMTS13, as deduced from the cDNA sequence, has 10 potential N-linked and 6 O-linked sugar chains and the structure of the sugar chain moieties depends on the producing cells. This suggests that rADAMTS13 expressed in other types of mammalian cells may have different

carbohydrate moieties than ADAMTS13 expressed in humans, which may result in a distinct proteolytic signature.

In 1996, Furlan *et al.* (*1*) and Tsai *et al.* (*2*) independently reported the partially purified pADAMTS13 with a feature of metalloproteinase and a molecular mass of 200–300 kDa. Using immunoadsorbent columns coupled to anti-ADAMTS13 polyclonal IgG that had been obtained from plasma of TTP patients, Gerritsen *et al.* (*19*) succeeded in purifying pADAMTS13 with heterogeneous molecular sizes (110–150 kDa) by SDS–polyacrylamide gel electrophoretic analysis, but each had the same N-terminal amino acid sequence (AAGGIL–). Almost simultaneously, Fujikawa *et al.* (*20*) reported the purification of an electrophoretically homogeneous pADAMTS13, with a molecular weight of 150 kDa before and 190 kDa after reduction on SDS–polyacrylamide gel, from a commercial factor VIII/VWF concentrate by a series of conventional chromatographic steps. This purification method was artistic, but the starting material was first treated with thrombin to remove fibrinogen, followed by thrombin inactivation by diisopropyl fluorophosphate. It is now known that pADAMTS13 is proteolyzed by thrombin, and a small portion of ADAMTS13 (<10% of the total in the plasma milieu) can co-sediment as a cryoprecipitate, whereas the majority (>90% of the total) remains in the cryosupernatant. Thus, it is important to establish a high-yield purification method for pADAMTS13, and to characterize this enzyme in comparison with rADAMTS13, as both enzymes appear to be under development for therapeutic use.

In this article, we describe a simple and efficient purification method for human pADAMTS13 from cryosupernatant, utilizing an anti-ADAMTS13 mouse monoclonal antibody (mAb). Using the purified pADAMTS13, we determine the peptide bonds cleaved by thrombin, plasmin and leucocyte elastase. Further, the carbohydrate moieties of this purified enzyme identified by lectin blot suggest that ADAMTS13 can be cleared from the circulation by the hepatic asialoglycoprotein receptor, like many other plasma glycoproteins.

Materials and Methods

Plasma and monoclonal antibodies

Outdated fresh frozen plasmas (FFP), anti-coagulated with acid-citrate-dextrose, were kindly provided by the Japan Red Cross Nara Blood Center as the starting materials for purification of ADAMTS13. The anti-ADAMTS13 mouse mAb (A10, IgG1-κ), which recognizes an epitope residing on the disintegrin-like (Dis) domain of ADAMTS13, was previously described (*21*). Most recently, its conformation-dependent epitope was precisely determined to reside on 72 amino acid residues (Tyr305-Glu376) within the Dis domain (*22*). A10-IgG was purified by protein A-Sepharose CL-4B column, and the purified IgG completely inhibited ADAMTS13 activity at a final concentration of 20 μg/ml in the static assay (*21*). The A10-IgG was conjugated to CNBr-activated Sepharose 4B according to the manufacturer's instructions. Another anti-ADAMTS13 mouse mAb (WH2-11-1, IgG1-κ) was kindly supplied by Dr Kenji Soejima of the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) (*23*).

Protein A-Sepharose CL-4B, CNBr-activated Sepharose 4B, HiTrap DEAE-FF and Superdex 200 HR10/30 were purchased from GE Healthcare Bio-Sciences AB (Piscataway, NJ, USA), and all reagents were analytical grade purchased from Wako Pure Chemicals (Osaka, Japan).

Assay of ADAMTS13 activity and protein concentration

ADAMTS13 activity was measured using a commercial chromogenic ADAMTS13-act-ELISA kit (Kainos, Tokyo) (*24*). In this article, an N-terminal glutathione-*S*-transferase (GST) and C-terminal histidine (His)-tagged fusion protein containing 73 amino acid residues (D1596 to R1668) of human VWF, termed GST-VWF73-His, are used as a substrate. After it is cleaved by ADAMTS13, Tyr-1605 is exposed and is detected by peroxidase-conjugated mAb anti-N10 (IgG) (*24*). Both 1 U (U) and 100% of ADAMTS13 activity were defined as the amount contained in 1 ml of pooled normal plasma. For the standard curve, pooled normal plasma serially diluted with heat-inactivated normal plasma, prepared by incubation at 56°C for 1 h followed by centrifugation, was used.

Protein concentration was estimated by absorption at 280 nm, using an extinction coefficient (E1%) of 10, or by micro BCA protein assay reagent (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Purification of pADAMTS13

One liter of FFP was thawed overnight at 4°C, and was then gently mixed with a cocktail of protease inhibitors [5 mM benzamidine–HCl, 1 mM phenylmethanesulphonyl fluoride and 20 mM ε-amino-n-caproic acid (EACA), each final concentration] followed by centrifugation at 7,000 rpm for 30 min at 4°C. After centrifugation, the cryoprecipitate was discarded and the cryosupernatant (~825 ml) was saved. The cryosupernatant was applied to an A10-Sepharose 4B column ($V_t = 50$ ml) at a flow rate of 50 ml/h at 4°C. The column was then washed with five-bed volumes of 20 mM Tris-buffered saline (TBS, pH7.4), five-bed volumes of high-salt TBS (20 mM Tris–HCl, 1M NaCl, pH 7.4), and five-bed volumes of high-salt TBS containing 10% dimethylsulphoxide (DMSO). Major fractions of ADAMTS13 activity were then eluted with high-salt TBS containing 40% DMSO, pooled and dialyzed overnight against 21 of 20 mM Tris–HCl buffer (pH 7.4) at 4°C. The dialysate was applied to a HiTrap DEAE-FF column ($V_t = 1$ ml) equilibrated with 20 mM Tris–HCl buffer (pH 7.4) at a flow rate of 30 ml/h at room temperature (RT) followed by extensive washing with the same buffer. Bound protein was eluted with 20 mM Tris–HCl buffer (pH 7.4) containing 1 M NaCl, pooled and concentrated using aquacide II (Calbiochem, La Jolla, CA, USA). The protein was then dialyzed against 20 mM imidazole–HCl buffer (pH 6.5) containing 20 mM EACA, 10 mM Na_3citrate, 1 M NaCl and 0.02% NaN_3. The dialysate (~400 μl) was then separated on a Superdex 200 HR10/30 column equilibrated with the same buffer at a flow rate of 0.5 ml/min at RT, and 0.5 ml fractions were collected. The purified protein was exclusively dialyzed against TBS, and kept frozen at −80°C until use in aliquot.

Enzyme stability

pH stability. The purified pADAMTS13 was dialyzed against TBS containing 0.05% Tween-20 (TBST, pH 7.4). The pH of each aliquot of the purified pADAMTS13 was adjusted with 0.1 N HCl or 0.1 N NaOH, and then incubated for 18 h at 4°C. After incubation, 1/22 volume of 1 M Tris–HCl (pH 7.4) was added to each sample to neutralize the pH. Samples were then diluted 11-fold with the reaction buffer (5 mM Na-acetate buffer containing 5 mM $MgCl_2$, pH 5.5) and the residual ADAMTS13 activity was assayed.

Effects of heat and metal ions. The purified pADAMTS13 was dissolved in TBST (pH 7.4) or TBST containing 10 mM $CaCl_2$, $BaCl_2$, $MgCl_2$, $MnCl_2$, $NiSO_4$ or $ZnCl_2$, and incubated for 20 min at various temperatures (from 25 to 65°C). After incubation, samples were chilled on ice, diluted 11-fold in the reaction buffer (pH 5.5) and the residual ADAMTS13 activity was determined. As a control experiment, ADAMTS13 activity was measured at 25°C in the reaction buffer containing 0.9 mM $CaCl_2$, $BaCl_2$, $MgCl_2$, $MnCl_2$, $NiSO_4$ or $ZnCl_2$ without incubation.

Electrophoresis and immunoblotting

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electroblotting onto nitrocellulose or polyvinylidene difluoride (PVDF)

membrane after SDS–PAGE were described previously (*25*). Protein bands in the gels were stained with Coomassie brilliant blue (CBB).

For the immunoblotting studies, blots were incubated with anti-ADAMTS13 mAb A10 (IgG), and the bound antibody was detected using Super Signal Western Blotting Kits (Pierce, Rockford, IL, USA). For the detection of ABO blood group antigens on ADAMTS13, blots were incubated in TBST containing either mAb against blood group A or B (Ortho Clinical Diagnostic Laboratory, Japan) at 1 : 10 dilution for 90 min at RT. HRP-conjugated anti-mouse IgM (Zymed Laboratories, San Francisco, CA, USA) was used as a secondary antibody.

Isoelectric focusing (IEF) was performed according to the manufacturer's instructions using agarose IEF, Pharmalyte TM 3-10 and an IEF calibration kit (Broad pI. pH 3–10) (products of GE Healthcare Bio-Science AB, Sweden). In some experiments, immunoblotting studies were performed after IEF using PVDF membranes, and stained with anti-ADAMTS13 mAb (WH2-11-1).

Lectin blotting

For lectin blotting, pADAMTS13 (0.4 μg/lane) was subjected to SDS–5%PAGE under reducing conditions followed by electro-transfer to PVDF membranes. Each membrane was incubated for 90 min at RT with TBST containing 1–3 μg/ml of biotin- or HRP-conjugated lectin (Seikagaku, Tokyo, EY laboratories, San Mateo, CA, USA), i.e. Concanavalin A (Con A), *Lens culinaris* agglutinin (LCA), *Datura stramonium* agglutinin (DSA), *Sambucus sieboldiamna* agglutinin (SSA), *Agaricus bisporus* agglutinin (ABA), *Maackia amurensis* agglutinin (MAM), *Arachis hypogaea* (peanut) agglutinin (PNA), *Ulex europaeus* agglutinin I (UEA-I) and *Ricinus communis* agglutinin 120 (RCA_{120}). Membranes were washed with TBST and incubated with TBST containing 1 : 1000 diluted HRP-conjugated Streptavidin (Vector Laboratories, Burlingame, CA, USA) where biotinylated lectins were used. After washing with TBST, HRP reaction was performed in a solution containing 50 mM Tris–HCl buffer (pH 7.5) containing 200 mM NaCl, 5 mg/ml DAB and 0.005% H_2O_2 for 2–7 min.

In a separate experiment, the blot was incubated in 100 mM MOPS (3-morpholinopropanesulphonic acid) buffer, pH 6.8, containing 10 mM $CaCl_2$ and 20 mU/ml of *Streptococcus* neuraminidase (Seikagaku) for 1 h at 37°C. The neuraminidase-digested membrane was used for some lectin blot analysis.

Protease digestion and N-terminal amino acid sequencing

The purified pADAMST13 was concentrated by a Microcon-YM10 (Millipore, MA, USA) and aliquots (18 μg ADAMTS13/50–60 μl of TBS) were incubated with 4 U of human thrombin (Calbiochem, CA, USA), 30 mU of human plasmin (Calbiochem, CA, USA) or 1 mU of human leucocyte elastase (Elastin Products Co., MI) at 37°C for appropriate times. Aliquots of the digest were taken at intervals, and kept frozen at −80°C until use. The frozen samples were thawed, diluted 11-fold in reaction buffer and used to assay ADAMTS13 activity. ADAMTS13 activity of pre-incubation samples was defined as 100%. Ten microlitres of each digest were mixed with 2.5 μl of sample buffer (2% SDS, 0.5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris–HCl, pH 6.8) and heated for 3 min at 95°C. The digests were analysed by SDS–5–20% gradient PAGE under reducing conditions. Proteins were electro-transferred onto PVDF membrane as described (*24*), and the protein bands were stained with Coomassie blue. The protein bands on the membrane were carefully cut out and directly subjected to N-terminal amino acid sequence analysis using an Applied Biosystems Procise protein sequencing system (Model 494 protein sequencer connected to a phenylthiohydantoin analyzer).

Results

Purification of pADAMST13

Using immunoadsorbent chromatography on an A10-Sepharose 4B column, bound ADAMTS13 was eluted with 40% DMSO with a high yield (41.7%) of activity (Table I). After dialysis of pooled fractions, the sample was concentrated using a HiTrap DEAE-FF column ($V_t = 1$ ml) followed by elution with 1M NaCl (see 'Materials and Methods' section). The eluates were concentrated and further separated by size exclusion chromatography on a Superdex 200 HR10/30 column (Fig. 1). A single major protein band with a molecular mass of 170 kDa before and 190 kDa after reduction was detected (inset of Fig. 1, left). Immunoblotting using anti-ADAMTS13 mAb (A10) and N-terminal amino acid sequence analysis confirmed that the major band was pADAMTS13. Some minor bands were identified as immunoglobulin heavy chain by both methods (data not shown). Agarose IEF of the purified pADAMTS13, followed by immunoblotting and staining with anti-ADAMTS13 mAb (WH2-11-1), is shown in the inset of Fig. 1, right. The purified pADAMTS13 had a pI of 5.3 (5.1–5.5), equivalent to ADAMTS13 in the plasma milieu.

From this system, a total of 200 μg of purified pADAMTS13 with a specific activity of 300 U/mg protein (25,057-fold activity purification) was obtained from 825 ml of starting material (cryosupernatant), with an activity yield of 8.5% (Table I). This column had been used >7 times without appreciable reduction of the yield of ADAMTS13 activity.

Effect of pH and metal ions on the heat stability of purified pADAMTS13

pH stability. The activity of purified pADAMTS13 was stable and relatively constant at pH ranges between 6.1 and 8.8 for 18 h storage at 4°C. However, ADAMTS13 activity was almost totally lost below pH 3.1 (Fig. 2A).

Divalent cations and heat stability. As shown in Fig. 2B (upper panel), ADAMTS13 activity in the reaction buffer containing 0.9 mM Ca^{2+} without incubation was enhanced 2.7 times compared to the value determined in the absence of 0.9 mM Ca^{2+}. In contrast, ADAMTS13 activity was markedly decreased in the reaction buffer containing 0.9 mM Zn^{2+} or Ni^{2+}.

Table I. Purification of ADAMTS13 from human plasma.

	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (fold)	Yield (%)
Cryosupernatant	825	701	58,575	0.01	1	100
A10-agarose	88	292	6.1	47.8	3,992	41.7
HiTrap DEAE	12	147	1.4	105.0	8,769	20.9
Superdex 200 HR10/30	2	60	0.2	300.0	25,057	8.5

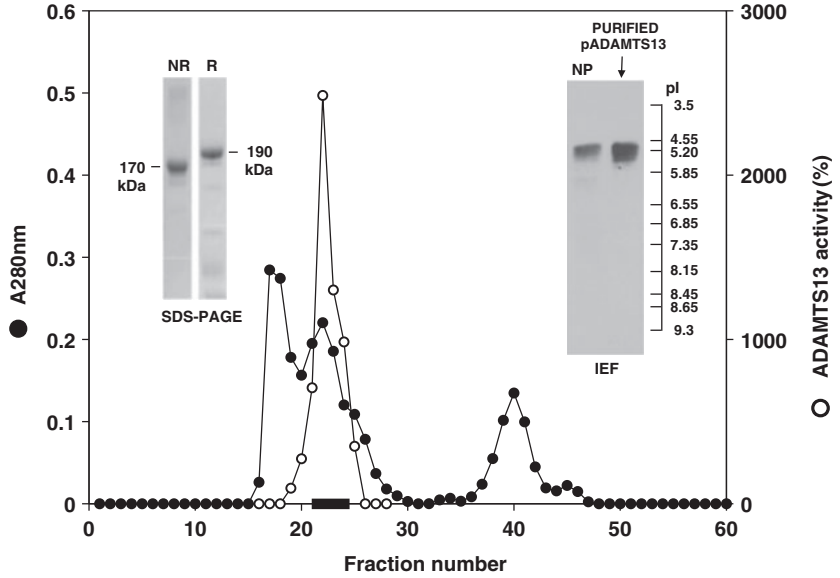

Fig. 1 Gel chromatogram of Superdex 200 HR10/30 as a final step for plasma (p) ADAMTS13 purification. Partially purified pADAMTS13, prepared by immunoadsorbent chromatography and concentrated by HiTrap DEAE gel, was further separated by Superdex 200 HR10/30 column at a flow rate of 0.5 ml/min at RT (see 'Materials and Methods' section for detail). Fractions shown by the black bar were pooled and used as the purified pADAMTS13. The inset (left) shows SDS–5% PAGE of the purified pADAMTS13 under reducing (R) and non-reducing (NR) conditions (stained with CBB). The inset (right) indicates immunoblotting analysis of the purified pADAMTS13 and normal plasma (control), after IEF. ADAMTS13 antigen was detected by anti-ADAMTS13 mAb (WH2-11-1).

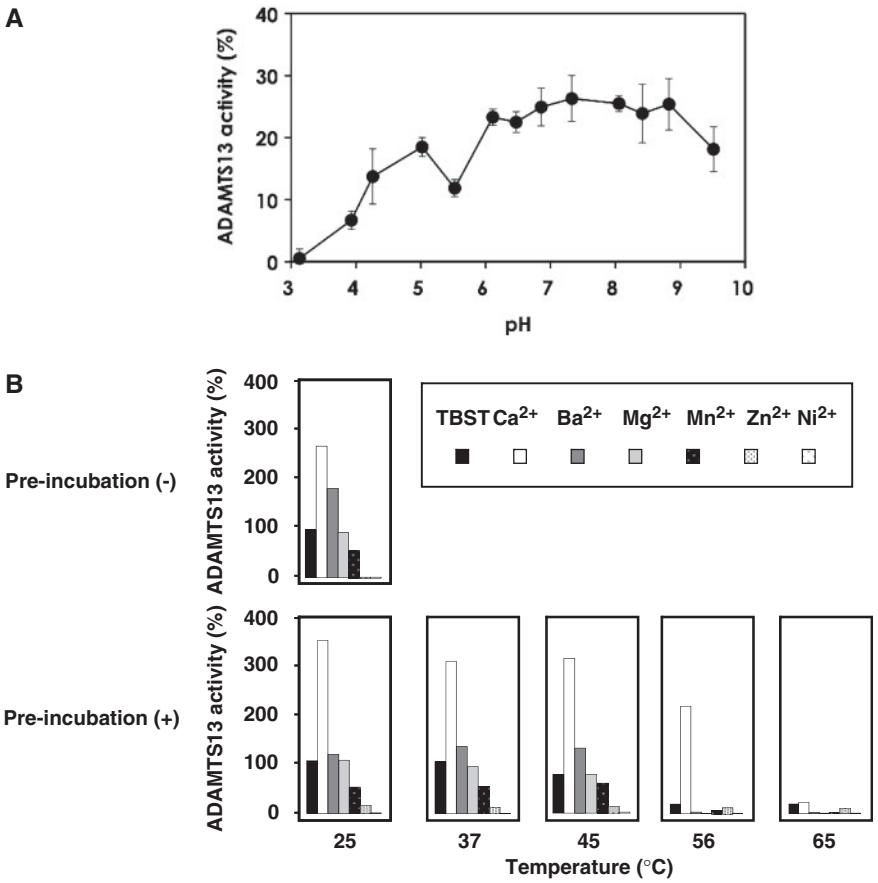

Fig. 2 pH stability, and effects of temperature and divalent metal ions on purified pADAMTS13 activity. (A) The purified pADAMTS13 was dialyzed against 20 mM TBS containing 0.05% Tween-20 (TBST, pH 7.4), and the pH of each aliquot was adjusted from 3.1 to 9.5 with 0.1 N HCl or NaOH, followed by incubation for 18 h at 4°C. After incubation, each sample was neutralized with 1 M Tris–HCl buffer (pH 7.4), diluted and the residual ADAMTS13 activity was determined. (B) The purified pADAMTS13 dissolved in TBST (pH 7.4) or TBST containing 10 mM $CaCl_2$, $BaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$ or $NiSO_4$, was incubated for 20 min at various temperatures from 25 to 65°C. In either before incubation (upper panel) or after incubation (lower panel), samples were diluted, and the residual ADAMTS13 activity was determined. ADAMTS13 activity in TBST incubated at 25°C was arbitrarily defined as 100%.

Next, ADAMTS13 activity was measured after 20-min incubation of purified protein under various temperatures and in the presence of six different divalent cations. The activity of pADAMTS13 in the presence of 10 mM Ca^{2+} was enhanced 3–5-fold as compared with control experiments in TBST alone at temperatures between 25 and 56°C. However, the activity in the presence of 10 mM Ca^{2+} was decreased from 364% at 25°C to 23% at 65°C. In the presence of 10 mM Ba^{2}, the ADAMTS13 activity was enhanced 1.1- to 1.4-fold as compared with control experiments in TBST alone at temperatures between 25 and 56°C. Mn^{2+} had an inhibitory effect on the enzyme activity, but Mg^{2+} was equivalent to the enzyme activity in TBST alone. Both Ni^{2+} and Zn^{2+} had a strong inhibitory effect on the ADAMTS13 activity. Perhaps the most striking result was obtained in the experiment at 56°C for 20 min, where the purified pADAMTS13 containing 10 mM Ca^{2+} maintained 60% of its original activity, whereas enzyme containing other divalent cations almost completely lost activity (Fig. 2B, lower panel), indicating that Ca^{2+} provided thermal resistance to this enzyme. Further, no significant loss of enzyme activity was observed during storage in the presence of 10 mM Ca^{2+} for 2 weeks at 37°C (data not shown).

Cleavage sites of thrombin, plasmin and leucocyte elastase

As shown in Fig. 3, the purified 190-kDa pADAMTS13 was digested with thrombin, plasmin and leucocyte elastase at 37°C for various time intervals. The residual ADAMTS13 activity was measured in each sample, and the degradation of ADAMTS13 antigen was monitored by SDS–5–20% gradient PAGE under reducing conditions. In each instance, the major 190-kDa band was gradually degraded into several fragments of smaller molecular mass. Thrombin digestion resulted in fragments with molecular mass of 37, 40, 48, 70, 100 and 170 kDa (Fig. 3A, left). Plasmin digestion produced 36, 40, 70, 100, 140 and 180 kDa bands (Fig. 3B, centre). Digestion with leucocyte elastase gave 38, 41, 63, 114, 130 and 160 kDa bands (Fig. 3A, right).

In terms of the residual ADAMTS13 activity, 20% of the original ADAMTS13 activity remained at 5 h after thrombin digestion, but the activity was completely lost at 20 h, with a concomitant disappearance of the 190-kDa band (Fig. 3B, left). Similarly, 30–50% of the original activity was maintained at 30 min after plasmin digestion (Fig. 3B, centre) and at 120 min after elastase digestion (Fig. 3B, right) with a loss of the 190-kDa band.

The aforementioned peptides were sequenced, and the N-terminal amino acid sequences are shown in Table II. In summary, thrombin cleaves at least four peptidyl bonds, between Arg257–Ala258, Arg459–Ser460, Arg888–Thr889 and Arg1176–Arg1177, and plasmin cleaves three peptidyl bonds between Arg257–Ala258, Arg888–Thr889 and Arg1176–Arg1177. Interestingly, thrombin and

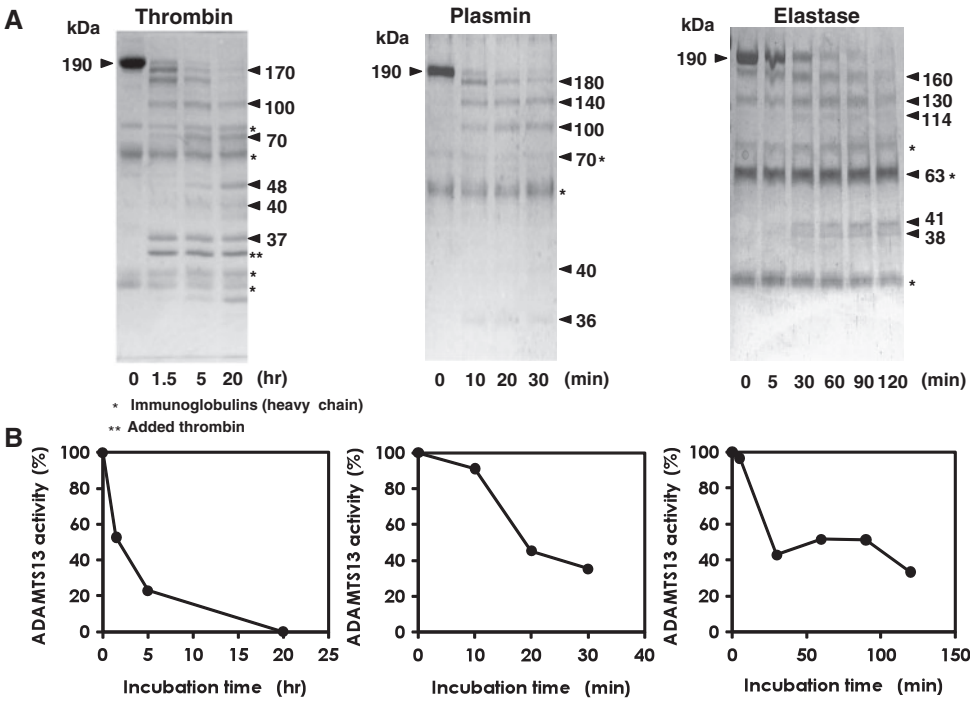

Fig. 3 Proteolytic fragmentation of the purified pADAMTS13 by thrombin, plasmin or leucocyte elastase, and residual activity. (A) The purified pADAMST13 was dissolved in TBS (pH 7.4), and incubated at 37°C for various time intervals with human thrombin (left), plasmin (middle) or leucocyte elastase (right), as described in 'Materials and Methods' section. Aliquots of each mixture were taken at intervals, and kept frozen at –80°C until use. The frozen samples were thawed, and the residual ADAMTS13 activity was determined by simultaneous analysis on SDS–5–20% gradient PAGE under reducing conditions. The indicated arrows were subjected to N-terminal sequencing after electro-blotting onto PVDF membranes (see the results in Table I). (B) Residual ADAMTS13 activity of each digest at various time intervals is shown. The ADAMTS13 activity determined by prior enzyme digestion was arbitrarily defined as 100%.

plasmin both cleaved three peptidyl bonds, just after the Arg residues at positions 257, 888 and 1176. In contrast, leucocyte elastase cleaved two peptidyl bonds between Ile380–Ala381 and Thr874–Ser875 (Table II). Fig. 4 depicts the putative cleavage sites on ADAMTS13 by thrombin, plasmin and leucocyte elastase.

Lectin blotting analysis of purified pADAMTS13 and detection of ABO blood group antigens on ADAMTS13

Carbohydrates on the purified pADAMTS13 were surveyed by lectin blotting analysis (Fig. 5A). Major 190 kDa and minor 170-kDa bands positively reacted to Con A, LCA and DSA. Since Con A and LCA show specific affinity to mannosyl residues and DSA to tri- or tetra-blanched poly-*N*-acetyllactosamine repeats, the presence of high-mannose or complex-type Asn-linked sugar chains is likely. PNA, which has specific affinity to Galβ1-3GalNAc structures, did not bind to purified pADAMTS13 before neuraminidase treatment, but did bind following this treatment. Since NeuNAc-Galβ1-3GalNAc structures are often found in Ser/Thr-linked sugar chains, it is likely that pADAMTS13 contains this type of Ser/Thr-linked sugar chain. SSA, which specifically binds to α2–6-linked sialic acid but not to α2–3-linked sialic acid, bound to pADAMTS13, whereas MAM, which is specific to α2–3-linked sialic acid in Asn-linked sugar chains, reacted only very weakly with pADAMTS13. These results indicate the presence of α2–6 (and partially α2–3-linked) sialic acid residues at the non-reducing terminus. The reactivity of RCA_{120} was also strongly enhanced after neuraminidase digestion, indicating the presence of β1–4-linked Gal residues penultimate to the sialic acid.

Further, the purified pADAMTS13 neither reacted to anti-blood groups A and B antibodies (Fig. 5B) nor UEA-I (anti-H) lectin (data not shown), indicating the absence of ABO-blood group antigens in this enzyme; in contrast, these antigens were found in human VWF.

Table II. The N-terminal amino acid sequence of ADAMTS13 peptides generated by the cleavage of thrombin, plasmin or elastase.

Protease	Fragment (kDa)	N-terminal sequence	Amino acid residue number
Thrombin	170	AAGGILHLE	N-terminal sequence
	100	AGLAxSP	R257-A258
	70	AGLAxSP	R257-A258
	48	SSPGGASF	R459-S460
	40	TGAQAAH	R888-T889
	37	RLLPGPQENS	R1176-R1177
Plasmin	180	AAGGILxL	N-terminal sequence
	140	AAGGILxL	N-terminal sequence
	100	AAGGILxL	N-terminal sequence
	70	AGLAxSP	R257-A258
	40	TGAQAA	R888-T889
	36	RLLPGPQE	R1176-R1177
Elastase	160	AAGGILxL	N-terminal sequence
	130	AAGGILxL	N-terminal sequence
	114	AAGGILxL	N-terminal sequence
	63	AAVHGR	I380-A381
	41	AAGGILxL	N-terminal sequence
	38	SAGEKAP	T874-S875

x, means unknown.

Discussion

Here, we have established a purification method for pADAMTS13 using an immunoadsorbent column containing an anti-ADAMTS13 mAb (A10-IgG). This method is unique, because A10 has recently been shown to bind to a conformational epitope consisting of 72 amino acid residues (Tyr305-Glu376) within the Dis domain of ADAMTS13 (*21*, *22*). The bound protein was eluted with 40% DMSO dissolved in a neutral buffer (pH 7.4) with a high yield (41.7%) of enzyme activity. In the two subsequent chromatographic steps (anion exchange and size exclusion columns), however, the activity yield was reduced by approximately half each, and the final activity yield was ~8.5%. The purified pADAMTS13 was confirmed by SDS–PAGE analysis as a homogeneous band before and after reduction. In addition, the protein had a single N-terminal amino acid sequence of AAGGIL–. The pI of the purified enzyme was 5.3 (5.1–5.5). These data indicated that the purified pADAMTS13 was comparable to ADAMTS13 in the plasma milieu.

The purified pADAMTS13 was stable in buffers with pH ranging from 6.1 to 8.8, but the activity decreased under acidic pH, suggesting that pADAMTS13 bound to the A10-column was not efficiently eluted under acidic conditions. At temperature of 25–45°C, the activity of purified pADAMTS13 was

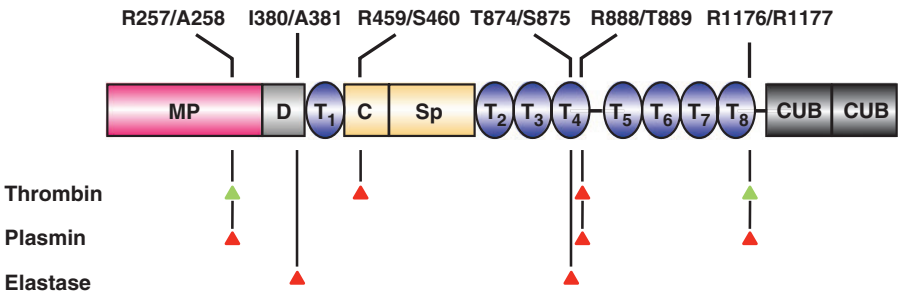

Fig. 4 Sites of pADAMTS13 cleavage by thrombin, plasmin and leucocyte elastase. A Schematic diagram of the identified pADAMTS13 cleavage sites for thrombin, plasmin and leucocyte elastase is shown. Note that thee of the four thrombin cleavage sites are identical to those of plasmin, but are different from of the two leucocyte elastase sites. MP, metalloproteinase; D, disintegrin-like; T, TSP type1; C, cystein-rich; Sp, spacer; CUB, complement C1r/C1s sea urchin epidermal growth and bone morphogenic protein 1.

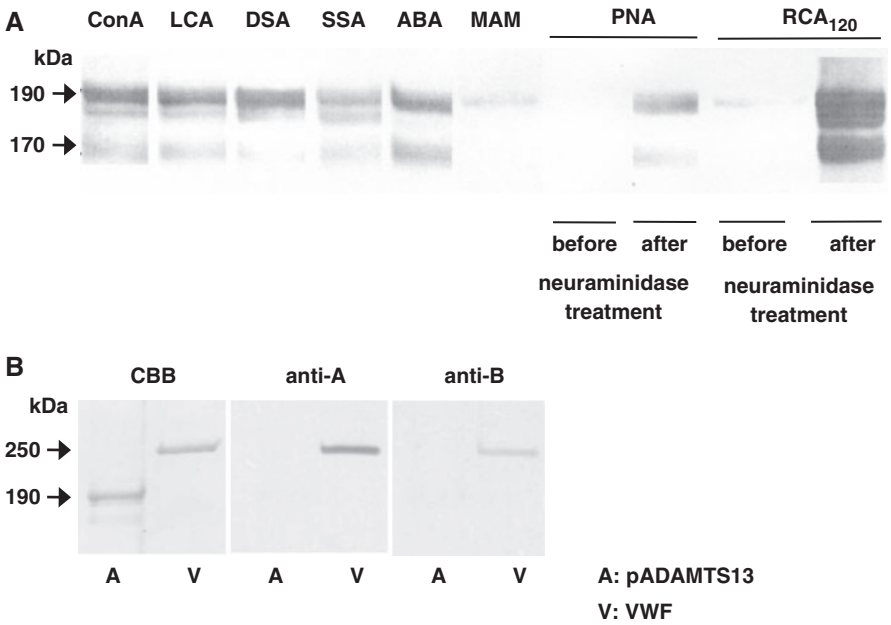

Fig. 5 Lectin blotting analysis of purified pADAMTS13 and detection of ABO blood group antigens on ADAMTS13. (A) Purified pADAMTS13 (0.4 μg) was subjected to SDS–5%PAGE under reducing conditions and transferred to a PVDF membrane. In some experiments, the blot proteins were treated with neuraminidase and then reacted with various lectins as described in 'Materials and Methods' section. Two protein bands, a major band of 190 kDa and a minor band of 170 kDa, are observed. Both bands positively reacted to Con A, LCA and DSA. PNA did not bind to purified pADAMTS13 before neuraminidase treatment, but did following neuraminidase treatment. (B) VWF (0.3 μg/lane) or pADAMTS13 (0.3 μg/lane) purified from pooled plasmas was subjected to SDS–5%PAGE under reducing conditions and transferred to a PVDF membrane as describe above. Protein bands on the membrane were stained with CBB. Reactivity to blood groups A or B antigen was clearly shown in VWF, but not in pADAMTS13.

enhanced by ~3-fold in the presence of 10 mM Ca^{2+}, but strongly inhibited in the presence of 10 mM Zn^{2+} and Ni^{2+}. This result appears to be in good agreement with that of Anderson *et al.* (*26*), who showed that Zn^{2+} at final concentrations of 1–3 mM enhanced the enzymatic activity of rADAMTS13 by the classic VWF multimer assay, but Zn^{2+} at higher concentrations (4–5 mM) inhibited activity. Gardner *et al.* (*27*) recently reported that rADAMTS13, extensively dialyzed against 0.15 M NaCl, 20 mM Tris–HCl (pH 7.8), requires pre-incubation with Ca^{2+} for 40–50 min to restore full enzyme activity. Furthermore, the activity of EDTA-treated enzyme, extensively dialyzed against 0.15 M NaCl, 20 mM Tris–HCl (pH 7.8) containing 5 mM Ca^{2+}, could be fully restored in a Zn^{2+}-dependent manner. These results indicated that dialysis can remove all functional Ca^{2+}, but does not remove the active site-bound Zn^{2+}. In addition, we have demonstrated here that 10 mM Ca^{2+} provides thermal resistance to pADAMTS13 activity, but it remains unaddressed how this happens in relation to the recently identified putative Ca^{2+} binding sites (Glu184 and Asp 187 of ADAMTS13).

Crawley *et al.* (*17*) showed that the three serine proteases, thrombin, plasmin and coagulation factor Xa, down-regulate ADAMTS13 activity by proteolysis. Lam *et al.* (*18*) identified the peptidyl bonds between Arg257–Ala258 and Arg1176–Arg1177 within ADAMTS13 as targets of thrombin. They predicted additional possible thrombin cleavable sites at Arg287, Arg393, Arg415, Arg910 and Arg968 of ADAMTS13 based on primary sequence analysis. In addition to the two thrombin cleavage sites reported by Lam *et al.* (*18*), we report two novel thrombin targets using the purified pADAMTS13: the peptidyl bonds between Arg459–Ser460 and Arg888–Thr889, neither of which was predicted. Of particular interest was the observation that plasmin cleaved the purified pADAMTS13 at the Arg257–Ala258, Arg888–Thr889 and Arg1174–Arg1177 sites, the same as thrombin.

Ono *et al.* (*15*) reported that ADAMTS13 antigen bands with lower molecular weights were found in plasmas of patients with sepsis-induced DIC that might have derived from digestion by bacterial proteases or leucocyte elastase. In this study, we therefore subjected the purified pADAMTS13 to leucocyte elastase digestion, and observed that the two peptidyl bonds between Ileu380–Ala381 and Tyr874–Ser875 were cleaved by this enzyme. These cleavage sites were different from those of thrombin or plasmin, and therefore also exclude the possibility that the three common cleavage sites of thrombin and plasmin were artificially generated during the purification process. According to the partial crystal structure of ADAMTS13 (*28*), Ileu380–Ala381 and Arg459–Ser460 were located on the surface exposure part between the Dis and thrombospondin1-1 domains and of the loop of the cystein-rich domain, respectively (Fig. 6).

Lam *et al.* (*18*) further reported that thrombin-cleaved rADAMTS13 remained partially active against intact VWF, and fully active against a recombinant VWF-A2 fragment (termed VWF115). However, the binding affinity of thrombin-treated rADAMTS13 for intact VWFM was significantly reduced compared

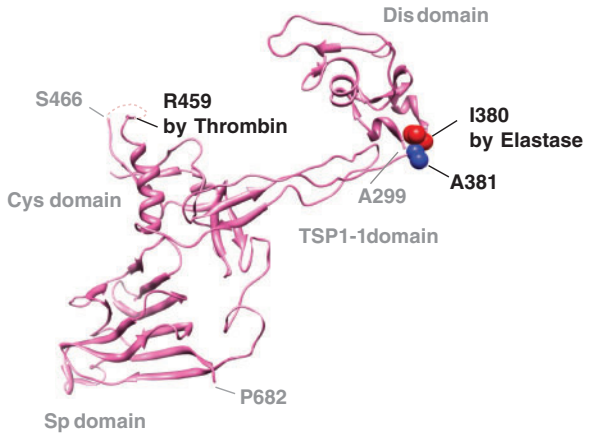

Fig. 6 Partial crystal structure of ADAMTS13 and location of the Ileu380–Ala381 and Arg459–Ser460 peptidyl bonds on ADAMTS13. Ribbon structure of ADAMTS13 (residues 299–682) was drawn with the UCSF Chimera package (*34*) using Protein Data Bank (PDB) entry as 3GHM. I380 and A381 are shown by space-filling model. Cleavage sites of I380–A381 by elastase and R459–S460 by thrombin (existing within the unsolved region) were located on the surface exposure part between the disintegrin-like and thrombospondin1-1 domains and of the loop of the cystein-rich domain, respectively. Dotted line indicates the unsolved region. Dis, disintegrin-like; TSP, thrombospondin1; Cys, cystein-rich; Sp, spacer domains.

to VWF115. As part of this study, we therefore also determined that >30% of the original pADAMTS13 activity against the GST-VWF73-His substrate remained during the incubation period (0.5–5.0 h) in each enzyme digestion.

ADAMTS13 has 10 putative *N*-glycosylation sites and *O*-fucosylated sugar chains in at least six thrombospondin type 1 repeats. Both the *N*- and *O*-glycans were reported to be prerequisite for enzyme secretion using rADAMTS13 (*29*, *30*). We here demonstrated by lectin blotting analysis that the purified pADAMTS13 possesses α2–6 (and partially α2–3-linked) sialic acid residues at the non-reducing terminus, and the presence of β1–4- and β1–3-linked galactose residues penultimate to sialic acid, through the observation that the reactivities of RCA_{120} and PNA were strongly enhanced after neuraminidase digestion. Since almost all sugar chains on pADAMTS13 are capped by sialic acid with no exposure of galactose residues, a clearance mechanism via hepatic asialogrycoprotein receptor might be involved, as is the case for other plasma glycoproteins (*31*, *32*). These sialo-sugar chains may also protect ADAMTS13 from proteolytic cleavage. In addition, neither blood group A nor B antigens were detected on the purified pADAMTS13, as previously reported by other investigators (*33*).

This study may in part contribute to understanding how ADAMTS13 activity is modulated by proteases generated in various clinical settings, and also to preparing the heat-treated pADAMTS13 concentrates.

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Conflict of interest

None declared.

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