The cleaved and latent forms of antithrombin are normal constituents of blood plasma: A quantitative method to measure cleaved antithrombin.

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Summary

Antithrombin (AT), a member of the serine protease inhibitor family, is the key regulator of thrombin activity *in vivo*. Thrombin inhibition is accomplished by the formation of a covalent thrombin-antithrombin (TAT) complex. The rate of inhibition is accelerated by heparin, which also leads to formation of a substantial amount of cleaved AT. We have made a murine monoclonal antibody (M9) that is specific for the two forms of AT, in which the reactive center loop is inserted into β-sheet A, i.e. cleaved and latent AT. The antibody has no measurable affinity for native AT. Using M9 as a catcher antibody in conjunction with a monoclonal antibody (M27), that does not bind latent AT, we have developed a sandwich assay that measures cleaved AT without interference from latent and native AT. The concentration in healthy subjects was determined to 1.3 (1.0-1.9) mg/L, which is about 100-fold lower than the plasma concentration of native AT and 1000-fold higher than the concentration of the TAT complex. The cleaved AT concentration is higher than what would be expected from the rate of formation of cleaved AT *in vitro* in conjunction with TAT complex formation in the presence of heparin. The concentration of cleaved AT did not correlate with the TAT concentration in plasma from patients with venous thrombosis.

Keywords: antithrombin, cleaved antithrombin, DELFIA-assay, latent antithrombin, monoclonal antibody, thrombin-antithrombin complex

Introduction

Antithrombin (AT)[1] belongs to the serine protease inhibitor family (serpin) and is by way of inhibition of especially thrombin (T) and activated factor X an important regulator of blood coagulation [1]. A characteristic structural feature of AT and of other members of the serpin family is the five stranded β-sheet A, and the mobile reactive center loop (RCL), which contains the reactive P_1–P'_1 Arg-Ser bond in AT [2].

Complex formation between a serpin and a protease induces profound structural changes in the inhibitor. An initially formed Michaelis complex is transformed to a covalent acyl ester intermediate between the P_1 residue in the inhibitor and the protease active site serine residue by cleavage of the RCL. The cleaved RCL, with attached protease, is inserted as strand four in β-sheet A with translocation of the protease to the opposite pole of the inhibitor [3]. The inhibitory reaction follows a branched pathway with the final formation of a stable protease-inhibitor complex as well as a small amount of cleaved inhibitor [4]. The crystal structure of the trypsin-antitrypsin complex reveals that the active site of the protease is distorted, thus precluding cleavage of the acyl bond between the inhibitor and the protease [5].

In contrast to most other serpins, native AT has the residues at positions P15 and P14 in the so-called hinge region of the RCL inserted into β-sheet A [6]. By heparin binding this region is expelled from sheet A, contributing to an increased flexibility of the exposed RCL [7]. This not only accelerates the rate of protease inhibition but also promotes the pathway leading to cleavage of the acyl intermediate between AT and T. In the presence of heparin about 30 % of the reaction products are cleaved AT as compared to 7 % in the absence of heparin [8,9].

A common feature for inhibitory serpins is the stability of the cleaved, loop-inserted form in even 6 M Guanidine-HCl, conditions under which the native serpin is denatured. An alternative stable structure of AT is the so-called latent form (L-AT), in which the RCL has been inserted into β-sheet A without previous cleavage of the reactive P_1-P'_1 bond [10]. A similar conformational variant has been reported for plasminogen activator inhibitor 1, α_1-antitrypsin and α_1-antichymotrypsin [11-13]. Formation of L-AT is induced by incubation of purified native AT at 50-60°C or of plasma at 37°C, which may explain the contamination of commercial AT preparations by L-AT [14,15]. Both cleaved and latent AT inhibit tumor angiogenesis [16,17].

As a result of the drastic conformational changes in the serpin structure, following loop insertion into β-sheet A, new epitopes appear. Monoclonal antibodies (mabs) have been described reacting with such neoepitopes in cleaved free and protease complexed plasminogen activator inhibitor type-1 and typ-2, in C1-inhibitor, and in protein C inhibitor [18-22]. Protein C inhibitor in complex with activated protein C has been found to be a marker for venous thrombosis [23,24].

In the present study we describe a high affinity murine mab, M9, selected to be specific for loop inserted cleaved and latent AT. No binding was observed to native AT. Using M9 together with a second monoclonal antibody against AT, which does not recognize L-AT, we have devised a sandwich assay to measure the plasma concentration of cleaved AT.

Materials and Methods

Proteins and peptides

Native AT was purified from human plasma as described [25]. Cleaved AT was produced by the incubation of native AT with porcine elastase (Sigma-Aldrich, Stockholm, Sweden) at a 100:1 molar ratio [26]. After 2 h at 37°C, α_1-antitrypsin was added to inhibit the elastase. The cleaved AT was purified on a 5 ml HiTrap Heparin HP Column (Amersham Biosciences, Uppsala, Sweden), equlibrated with 50 mM Tris, 0.1 M NaCl pH 7.5. A NaCl-gradient of 50 ml (0.1-1 M) eluted cleaved AT at 0.4 M NaCl. L-AT was produced by incubating native AT (10 mg) at 50°C for 72 h in 50 mM Tris, 0.1 M NaCl pH 7.4, containing 20% glycerol [17]. The L-AT was purified using the same method as for cleaved AT. TAT complexes were formed in 50 mM Tris, 0.1 M NaCl pH 7.4. Native AT (52 nmol) was mixed with 30 nmol T, incubated at room temperature for 4 min after which 20 nmol PPACK (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) was added. TAT was purified on a Heparin HiTrap column as described above. SDS-PAGE showed one major band corresponding to TAT and two minor bands corresponding to cleaved AT and T. Protein concentrations were determined by amino acid analysis after acid hydrolysis. Prothrombin was purified using a standard procedure [27]. The protein was activated as described [28].

The RCL-peptide was synthesized on a Milligen 9050 Plus instrument (Perkin-Elmer, Stockholm, Sweden), and purified by reverse-phase HPLC. Insertion of the RCL into native AT was performed, by incubating 6.9 nmol native AT with 690 nmol of a synthetic RCL-peptide at 37°C for 20 h [29]. The RCL-complexed AT (RCL-AT) was then purified on a Heparin HiTrap Sepharose Column. The RCL insertion was confirmed by protein sequencing.

Monoclonal antibodies

Murine mabs were produced as described [22]. Mice were immunized with TAT. To find mabs specific for cleaved AT, an ELISA screening method was used, based upon the formation of complexes between biotinylated bovine activated factor X and AT (BXa-AT) in human heparin plasma. The BXa-AT containing plasma was diluted 1/50 with plasma diluted 1/5 in 50 mM Tris, 0.1 M NaCl, 0.1 % (w/v) bovine serum albumin (BSA), 0.05 % (v/v) TWEEN 20, pH 7.4 (ELISA buffer). The final concentration of BXa-AT was 0.6 µg/ml. Cell supernatants were added to microtiterplates (Costar®, Corning Incorporated, Corning, NY) coated with 50 µl Rabbit Anti Mouse Immunoglobulins (10 µg/ml) (DAKO, A/S Denmark). After 30 min incubation at room temperature followed by washing, 50 µl BXa-AT was added.

The plate was incubated for 30 min at room temperature and washed, followed by the addition of 50 µl horseradish peroxidase-conjugated streptavidin (DAKO) diluted 1/50. After another incubation of 30 min and washing, 100 µl 1.2-phenylenediamine dihydrochloride (OPD) (DAKO) was added and the absorbance at 490 nm was measured. The mabs recognizing BXa-AT were selected. Positive clones were recloned twice. Mab-containing media from cells, cultured in an INTEGRA CELLine CL1000 flask (INTEGRA Biosciences AG, Wallisellen, Switzerland), were purified by Protein A-Sepharose chromatography. One of the mabs, M9-4 (M9), was investigated for its specificity to cleaved and latent AT.
Mab M27, specific for native and cleaved AT, has been described earlier and mab M38 recognizes all forms of AT [30].

Affinity chromatography

Mixtures of equal amounts (50 µg) of purified native and latent as well as native and cleaved AT were applied at a flow rate of 0.5 ml/min to a 1 ml HiTrap Heparin HP Column, equlibrated with 50 mM Tris-HCl, 0.1 M NaCl, pH 7.4 (TBS). The proteins were eluted with a NaCl-gradient (0.1-1.5 M, 25 ml). The absorbance of the effluent was monitored at 280 nm. For measurements of inhibitory activity 100 µl of the fractions, diluted 1/25 in TBS containing 0.1 % BSA (w/v) and heparin, 4 U/ml, (Lövens Kemiske Fabrik, Ballerup, Denmark), were transferred to microtiter plates and supplemented with 100 µl T (0.2 µg/ml) diluted in the same buffer. After 15 min incubation at room temperature, 50 µl T substrate S-2238 (Chromogenix, Gothenburg, Sweden) was added, and the absorbance at 405 nm was recorded for 10 minutes. Binding to M9 was analyzed in an ELISA as follows: In non-adsorptive microtiter plates (Bibby Sterilin Ltd., Staffs, UK) 50 µl of the fractions, diluted 1/80 in ELISA buffer, were mixed with 50 µl biotinylated M9 (4 µg/ml). After incubation for 15 min at room temperature the samples were transferred to Streptavidin coated plates (Wallac, Turku, Finland) and incubated for 1 h, followed by washing with ELISA buffer and subsequent incubation with 100 µl of a rabbit polyclonal immunoglobulin fraction against human AT (PabAT) for 1 h. After washing, 100 µl of a horseradish peroxidase conjugated goat-anti-rabbit antibody (DAKO), diluted 1/1500 in ELISA buffer without BSA was added to each well and the plate was incubated for 1 h followed by washing. OPD substrate was added (100µl) and absorbance recorded at 490 nm.

A 1 ml HiTrap NHS-activated HP Column was coupled with 10 mg M9 following the instructions of the manufacturer. After equilibration with TBS, 5 ml human blood plasma was applied. The column was washed with 50 mM Tris-HCl, 0.5 M NaCl, pH 7.4 and bound

proteins were eluted with 0.1 M glycine, 0.5 M NaCl pH 3.0. The eluate was analyzed employing PabAT or M9 as catcher antibodies and Eu^{3+}-labeled M38 as reporter mab. Microtiterplates were coated with 100 µl PabAT (20 µg/ml in 0.15 M NaCl), incubated over night at +4°C and washed with Wash solution (Wallac). After blocking with 200 µl 1 % BSA in Assay Buffer (Wallac) for 1 h followed by washing, 100 µl of fractions diluted 1/200 in Assay Buffer were added. The plates were incubated shaking for 1 h. After washing, 100 µl of Eu^{3+}-labeled mab M38 (0.2 µg/ml) was added and the plate was again incubated shaking for 1 h, followed by washing, subsequent addition of 200 µl Enhancement Solution (Wallac) and measurement in a DELFIA plate fluorometer (Wallac). The eluted fractions (50 µl) diluted 1/200 in Assay Buffer were mixed with 50 µl biotinylated M9 in a non-adsorptive microtiter plate. After 15 min the solution from each well was transferred to a streptavidin coated plate and incubated for 1 h followed by washing. Then 100 µl of a Eu^{3+}-labeled mab M38 (0.2 µg/ml) was added. The plate was incubated for 1 h, followed by washing, addition of Enhancement Solution and fluorescence measurement. Pooled eluted fractions were run on SDS-PAGE (12 %).

To estimate the amounts of latent and cleaved AT in plasma and to confirm that no protein cleavage occured after blood collection, 50 ml blood from two persons was drawn in tubes containing 50 mM benzamidin (ACROS, New Jersey, US), 41 µM Leupeptin (Sigma Aldrich Chemie GmbH, Germany), 0.42 µM DEGR (Calbiochem), 4.3 µM PPACK (Calbiocem), 1 mM AEBSF (Boehringer Mannheim GmbH, Germany) and 50 µM Chymostatin (Peptide Institute Inc.). The plasma was applied to the M9 Sepharose column as described above. The eluted proteins were sequenced on a Perkin-Elmer ABI Procise 494-pulsed liquid-phase sequencer.

Competitive binding

Different formations of AT competed with Eu^{3+}-labeled cleaved AT for the binding to M9 or M27. Native, latent, and cleaved AT, TAT, incubated native AT and RCL-AT in 25 µl Assay Buffer (0-400 µg/ml) were added to 25 µl Eu^{3+}-labeled cleaved AT (10 ng/ml) in a non-adsorptive microtiterplate. The solutions were supplemented with 50 µl biotinylated M9 or M27 (0.5 µg/ml). After 15 min of incubation the samples were transferred to a streptavidin plate and incubated for 1 h. The plate was washed with Wash solution. Enhancement Solution was added and the fluorescence measured.

Biacore studies

The affinity of M9 for different forms of AT was determined by surface plasmon resonance (BIACORE 2000, Biacore AB, Uppsala, Sweden). M9 was diluted in 10 mM Na-acetate pH 4.8 and coupled to a CM5 sensor chip to about 4000 response units (RU). Latent, cleaved and thrombin complexed AT were diluted to 3.38, 6.75, 13.5, 27, 54 nM, 3.75, 7.5, 15, 30, 60 nM, and 4.5, 9, 18, 36, 74 nM respectively in 10 mM Hepes, 0.15 M NaCl, 3 mM EDTA, 0.005 % (v/v) TWEEN 20 pH 7.4 and injected at 30 µl/min with an association time of 180 s and dissociation time of 600 s. The sensor chip was regenerated with 2 x 5µl 0.1 M glycine-HCl, 0.5 M NaCl pH 3.0 at a flow rate 5 µl/min. The results were evaluated by the use of Biaevaluation 3.0 software program.

Determination of cleaved AT and TAT in human plasma

Samples from 75 female and 35 male healthy individuals were collected in 5 ml tubes containing 0.5 ml 0.129 M Na-citrate. Cleaved AT was analyzed in a DELFIA by the use of biotinylated M9 as catcher antibody and Eu^{3+}-labeled M27 as reporter antibody. Cleaved AT standard solutions of 50 µl (0-40 ng/ml), diluted in Assay Buffer or in latent AT/cleaved AT deficient plasma, and 50 µl blood plasma diluted 1/200 in Assay Buffer were mixed with 50 µl biotinylated M9 (2 µg/ml) in non-adsorptive microtiter plates. After 15 min at room temperature the samples were transferred to streptavidin coated plates and incubated for 1 h on a plate shaker and washed four times with Wash solution. Eu^{3+}-labeled M27, (20 µg/ml in 100 µl Assay Buffer), was added followed by shaking for 1 h and washing, after which 200 µl Enhancement Solution was added and fluorescence measured as described above.

Cleaved AT and TAT were measured in plasma from 38 patients with DVT diagnosed by phlebography. Blood was collected before pharmaceutical treatment and phlebography. Control samples were from 51 patients with negative phlebography. The measurement of TAT was performed by the use of Enzygnost® TAT micro (Dade Behring Marburg GmbH, Germany). Cleaved AT was measured in plasma samples from 20 patients treated with Warfarin and controlled by a functional Prothrombin assay, PK(INR= International Normalized Ratio). Patients with PK(INR) values between 3.5 and 6.4 were chosen.

Blood samples were drawn from 9 patients before and 15 h after administration of a single dose of 40 mg low molecular weight heparin (Klexane®) prior to hip surgery.

Stability of cleaved AT and TAT in citrated and heparinised plasma

Blood from a healthy person was drawn in tubes containing either 0.5 ml 0.129 M Na-citrate or heparin. The tubes were immediately centrifuged at 1500 g for 10 min, after which the plasma was incubated at room temperature. Aliquots were taken after 0, 1, 2, 4, 6, 24, and 52 h and frozen at –70°C. Cleaved AT and TAT were measured as described above.

Other methods

Eu^{3+}-DTTA chelate labeling (Wallac) and biotinylation with NHS-LC-biotin (Pierce, Rockford, IL) were performed as described by the manufacturers. SDS-PAGE was carried out using a standard method.

Results

Properties of mab M9 specific for the cleaved and latent forms of AT

The monoclonal antibody, M9, was obtained by immunizing mice with the TAT complex. The screening procedure was designed to ensure that only mabs specific for the loop-inserted form of the inhibitor were identified. Thus, biotinylated bovine factor Xa was added to human plasma to obtain BXa-AT complexes. The substitution of thrombin for factor Xa in complex with AT ensured that mabs reacting with the protease were not detected. Moreover, due to the large molar excess of free, uncleaved AT in plasma over the forms with the RCL inserted in β-sheet A, only mabs with the desired specificity were identified. Two mabs were selected and one of them, M9, was characterized in detail.

In a first test of the specificity of M9, mixtures of native and cleaved and of native and latent AT were chromatographed on a HiTrap heparin HP column that was eluted with a NaCl gradient (Fig. 1). The eluate was analyzed for its ability to inhibit thrombin in an amidolytic assay and for its ability to bind to M9 in a sandwich assay as described in Materials and Methods. Cleaved and latent AT, which have lower affinity for heparin than native AT, eluted in the first peak at about 0.4 M NaCl. They had no thrombin inhibitory activity but both were recognized by M9. Native AT eluted in the second peak at about 0.9 M NaCl. It inhibited thrombin but did not bind M9.

The three forms of AT were also analyzed in competitive binding experiments. Competition with Eu^{3+}-labeled cleaved AT for binding to biotinylated M9 was measured. Cleaved and latent AT and the TAT complex competed equally well for binding to the mab (Fig. 2A). In contrast, native AT had no discernible affinity for the mab. There appeared to be a weak competition with RCL-AT. This type of loop-inserted AT is obtained by incubation with a large molar excess of the synthetic loop at 37°C. However control AT, incubated without RCL, displayed the same degree of competition. We therefore infer that the displacement was due to a conversion of about 1% of the native AT to the latent conformation during the incubation rather than competition by the form with inserted synthetic peptide.

The affinity of different forms of AT for M9 was also studied by surface plasmon resonance using a Biacore 2000 instrument. Binding of different concentrations of AT to the immobilized mab was analyzed using the Biaevaluation 3.0 software package. The K_Ds of cleaved and latent AT and of the TAT complex were from 0.96×10^{-9} M to 3.07×10^{-9} M with the complex having the highest affinity (Table 1). The differences were accounted for by

differences in the rate of dissociation. The affinity of the native/stressed form of AT could not be measured as it was too low to give a signal.

Isolation of cleaved/latent AT from plasma using M9

As cleaved/latent AT has been found to contaminate commercial AT concentrates used in clinical medicine we investigated whether the mab could be used to isolate the two species of AT by immunoaffinity chromatography. To that end 5 ml of undiluted citrated plasma was applied to a M9-conjugated Sepharose column and after washing, the bound AT was eluted at a low pH (Fig. 3). With PabAT as catcher and Eu^{3+}-labeled mab M38 as reporter mab, AT in the flow through was identified as well as a small amount of immunoreactive material that had been bound to the column. With biotinylated M9 as a catcher no signal was observed from native/stressed AT in the flow through, whereas the material eluted at low pH contained immunoreactive material, i.e. cleaved and/or latent AT. SDS-PAGE and sequence analysis showed that the eluted material contained both latent and cleaved AT. To estimate the relative amounts of cleaved and latent AT in the eluate, 25 ml plasma from two persons was chromatographed and sequenced. In addition to the N-terminal His-Gly-Ser-Pro-Val the sequence Ser-Leu-Asn-Pro-Asn was obtained, consistent with cleavage of AT at the thrombin cleavage site. The latent AT accounted for ~75% of the loop-inserted forms in both plasmas.

Properties of mab M27, specific for the cleaved and native forms of AT

An assay that measures cleaved AT and utilizes a catcher mab specific for the loop-inserted forms of AT requires that the reporter mab binds to cleaved AT and has no affinity for latent AT. We have previously characterized a monoclonal antibody, mab M27, with these properties [30]. The epitope of this mab involves the sequence Phe-Ile-Arg-Glu-Val-Pro (residues 411-416). The affinity of the mab for latent AT is more than 200-fold lower than it is for cleaved AT making it useful as a reporter mab in a sandwich assay (Fig. 2B).

A sandwich assay for measurements of cleaved AT in plasma

A sandwich assay for cleaved AT was devised using M9 as catcher mab and Eu^{3+}-labeled M27 as reporter mab. Due to the high affinity of the mabs the assay gives linear dose-response curves (Fig. 4). The assay measures cleaved AT in the physiological range (see below) as well as at subnormal levels. The standard curves in buffer and in plasma deficient in cleaved AT are identical within experimental error. The standard curve with plasma

supplemented with purified cleaved AT has the same slope as the curve in buffer and has an intercept corresponding to the plasma concentration of cleaved AT.

The intra (10 measurements) and inter (14 measurements) assay variations of a sample within the normal range of cleaved AT (1.7 mg/L) were 4.7 and 6.2%, respectively (CV). The lowest detection limit of the assay was defined by five measurements of cleaved AT performed on a plasma sample depleted of this analyte and diluted 1/200. The mean value + 3 SD was 0.05 mg/L. The recovery obtained from 5 determinations of two normal plasma samples supplemented with 1 and 2 mg/L cleaved AT was found to be 94% (±3) and 95% (±1), respectively.

The frequency distribution curve of cleaved AT measured in 75 female and 35 male healthy adults was positively skewed (Fig. 5). The median was calculated to 1.3 mg/L (range 1.0-1.9). There was no difference in the concentration of cleaved AT between males and females.

The medians of cleaved AT of 38 patients with DVT and 51 control patients were 1.3 mg/L (range 0.9-2.2) and 1.3 mg/L (range 0.9-2.5), respectively. TAT, measured in the same samples gave median values of 12.5 µg/L (range 0.1-61.0) and 3.0 µg/L (range 0.1-53.0), respectively. No correlation was found between cleaved AT and TAT, as tested by the calculation of Pearson correlation coefficient (r) (Fig. 6). We also measured the concentration of cleaved AT in patients treated with Warfarin. There was no correlation with the PK(INR) (not shown). The concentration of cleaved AT was not influenced by the administration of low molecular weight heparin (not shown).

Generation of cleaved AT in citrated and heparinised plasma

Normal heparinised and citrated plasmas were incubated at room temperature for 52 h and the formation of cleaved AT and TAT was examined. There was no change in the concentration of either cleaved AT or TAT in the citrated plasma within the incubation time. In the heparinised plasma both TAT and cleaved AT increased with time, resulting in an approximal duplication of the concentrations in 52 h (Fig. 7).

Discussion

Unlike the native form of AT both the cleaved and latent forms have antiangiogenic properties |16,17]. Recently, it was shown by densitometric scanning that both these forms of AT are trace components in human plasma [31]. The latent form has also been found to constitute a significant part of AT preparations that are used therapeutically [14]. Latent AT does not inhibit T or active factor X but forms a non-covalent, inactive dimer with the native form of AT [15]. For these reasons high concentrations of latent AT are linked to an increased risk to develope venous thrombosis [32].

It is of potential clinical interest to be able to measure both cleaved and latent AT in plasma, for instance, in patients with malignancies and/or with pathologic proteolysis. It is also important to be able to measure these forms of AT in concentrates intended for clinical use. Two methods have been devised to measure cleaved/latent AT. They depend on preanalytic removal of native AT either by thermal or SDS-mediated denaturation, which is followed by ELISA. Both methods presumably measure the sum of the cleaved and latent forms of AT [33,34]. Therefore, we have developed a sandwich assay that specifically measures cleaved AT without interference from the native or latent forms. The method depends on a very high affinity catcher mab that binds AT with the RCL inserted into β-sheet A, i.e. AT in complex with T/factor Xa as well as the cleaved and latent forms, but has no measurable affinity for the native form. The reporter mab binds cleaved AT but has a ~200-fold lower affinity for the latent form.

The location of the epitope of mab M9 has not been defined. A mab (M36) against protein C inhibitor has properties similar to those of mab M9, i.e. it is specific for the loop-inserted form of the inhibitor [22]. This mab has the epitope in the vicinity of residue P13 of the loop inserted into β-sheet A. It could be induced by insertion of the RCL into β-sheet A of the protein C inhibitor. However, insertion of the corresponding synthetic reactive site loop into β-sheet A of AT did not induce the epitope.

We found the median concentration of cleaved AT in plasma to be 1.3 mg/L. The TAT complex is codetermined, but since its concentration is ~10^3-fold lower (1.0-4.1 μg/L in adults), it does not pose a problem even in pathological conditions.

The pathway for inhibition of T by AT is branched, leading to complex formation and inhibition of T as well as to cleavage of AT and liberation of active T due to cleavage of the acyl-enzyme intermediate. It has been observed *in vitro* that TAT formation in the presence of heparin is accompanied by the formation of about 30% of cleaved AT relative to TAT, and

that the amount of cleaved AT produced under physiological conditions is independent of the AT/T ratio in the reaction [8,9]. *In vivo*, the complex between T and AT is formed under heparinised conditions due to heparin-like substances, which are bound to the surface of capillary endothelial cells [35]. This poses the question whether the cleaved AT in plasma can be accounted for solely by the cleavage of AT that is coupled to complex formation with T, or if there are other sources, for instance AT cleavage mediated by enzyme(s) other than thrombin [33,36]. In humans the half-life of native AT is ~64 h, whereas the half-lives of cleaved and latent AT have not been determined, nor has that of the TAT complex [37]. In rabbits the half-life of native as well as cleaved and latent AT is 20 h and that of the TAT complex is 18 min [31]. We assume that the three-fold longer half-life of native AT in humans than in rabbits and presumably also of cleaved and latent AT is accompanied by a similar elongation of the half-life of the TAT complex, i.e. to ~54 min, even if there is no experimental evidence to support this notion. Under those premises and assuming a first order rate of elimination, steady state conditions and equal distribution volumes of 3 L (plasma volume) for the TAT complex and cleaved AT, 0.6 pM/min of TAT and 12.1 pM/min of cleaved AT would be formed. More cleaved AT is thus generated than would be expected from the *in vitro* measurements performed in buffers of different ionic strengths. At physiological ionic strength about 30 % cleaved AT was generated relative to TAT (9). This conclusion is supported by the fact that there was no correlation either between cleaved AT and TAT in DVT patients or between cleaved AT and PK(INR) in patients treated with Warfarin.

Elastase might be involved in the production of cleaved AT, as it binds to glycosaminoglycans like AT and has been reported to cleave AT effectively *in vitro* in the presence of heparin [38]. However, the elastase cleavage of AT is at the Ile390–Ala391 peptide bond, whereas we only have observed the thrombin Arg393-Ser394 cleavage in AT purified from plasma by affinity chromatography utilizing mab M9. The production of cleaved AT in the microvasculature thus exceeds that observed *in vitro* under heparinised condition and the mechanism is unclear.

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[1]Abbreviations: AT, antithrombin; BXa-AT, biotinylated bovine activated factor X-AT complex; BSA, bovine serum albumin; DELFIA, dissociation enhanced lanthanide fluorescence immunoassay; ELISA, enzyme linked immunosorbent assay; L-AT, latent AT; mabs, monoclonal antibodies; OPD, 1,2-phenylenediamine dihydrochloride; PabAT, rabbit polyclonal immunoglobulin fraction against human AT; RCL, reactive center loop; RCL-AT, RCL-complexed AT; TAT, thrombin-antithrombin complex; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide electrophoresis; TBS, 50 mM Tris, 0.1 M NaCl pH 7.4.

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Table 1. Binding constants of M9 for the interaction with L-AT, cleaved AT and TAT, measured on a Biacore 2000.

Analyte	k_a ($M^{-1}.s^{-1}$)	k_d (s^{-1})	k_D (M)
latent AT	3.13×10^{4} (±0.04)	9.61×10^{-5} (±0.27)	3.07×10^{-9} (±0.11)
cleaved AT	2.13×10^{4} (±0.14)	5.94×10^{-5} (±0.26)	2.79×10^{-9} (±0.07)
TAT	2.94×10^{4} (±0.54)	2.76×10^{-5} (±0.33)	0.96×10^{-9} (±0.23)

Values represent the mean value (±SD).

Legends to figures

Fig. 1. Affinity chromatography of mixtures of purified latent, cleaved and native AT on a HiTrap Heparin HP Column. A. A mixture of equal amounts of native and cleaved AT was applied on a HiTrap Heparin HP Column of 1 ml and eluted with a NaCl-gradient (0.15-1.5 M, 25 ml). The effluent was monitored at 280 nm (—). The eluate was analyzed with an ELISA by the use of biotinylated M9 as a catcher and PabAT as reporter antibody, followed by horseradish peroxidase conjugated goat-anti-rabbit antibody as detector (O). An amidolytic assay was used to measure the ability of the eluate to inhibit T. The results are presented as the relative inhibition, where the inhibition by an equimolar amount of native AT towards thrombin was set to 100 % (Δ). **B.** The same as in A performed with a mixture of latent and native AT.

Fig. 2. Competition of different forms of AT with Eu^{3+}-labeled cleaved AT for binding to M9 and M27. A. Competition of native AT (●), native AT incubated for 20 h at 37°C (■), cleaved AT (▲), RCL-AT, attained by incubating native AT with 100-fold molar excess of a synthetic RCL for 20 h at 37°C (Δ), latent AT (♦) and TAT (O) for binding to M9 as described in Materials and Methods. **B.** Competition of native AT (Δ), latent AT (●), and cleaved AT (O) for binding to M27. The y-axis shows the binding of Eu^{3+}-labeled cleaved AT to M9 or M27 relative to its binding with no competitor present.

Fig. 3. Affinity chromatography on a M9 coupled Hi Trap column. Human normal plasma (5 ml) was applied to a M9 conjugated Sepharose. The effluent was monitored at 280 nm (). Elution of the bound material is indicated with an arrow. The eluate was analyzed for binding to PabAT (❑) in **A** and to M9 (O) in **B**. Eu^{3+}-labeled M38 was used as reporter mab in both assays. **C**. SDS-PAGE (12%) of eluate. Lane 1: molecular markers 94, 67 and 43 kDa, lane 2: cleaved AT, lane 3: native AT, lane 4: latent AT, and lane 5: eluted bound material.

Fig. 4. Dose response curves for cleaved AT. Increasing concentrations of cleaved AT were diluted in Assay buffer (O), human blood plasma (●), latent and cleaved AT-deficient human blood plasma (Δ). Latent and cleaved AT-deficient human blood plasma was supplemented with 6 mg/L latent AT (❑). The assay was performed with biotinylated M9 as a catcher and Eu^{3+}-labeled M27 as a reporter mab.

Fig. 5. Frequency histogram of cleaved AT in healthy adults (n=110).

Fig. 6. Plot diagram of the concentrations of TAT and cleaved AT measured in 17 patients with DVT and positive in phlebography (O) and in 13 control patients with negative phlebography (■). A correlation (Pearsons r) is shown as the coefficient of determination, R^2 = 0.0689.

Fig. 7. Comparison of the generation of cleaved AT and TAT in citrated plasma and in heparinised plasma incubated at room temperature. (O) cleaved AT and (◊) TAT in citrated plasma, (▲) cleaved AT and (●) TAT in heparin plasma.

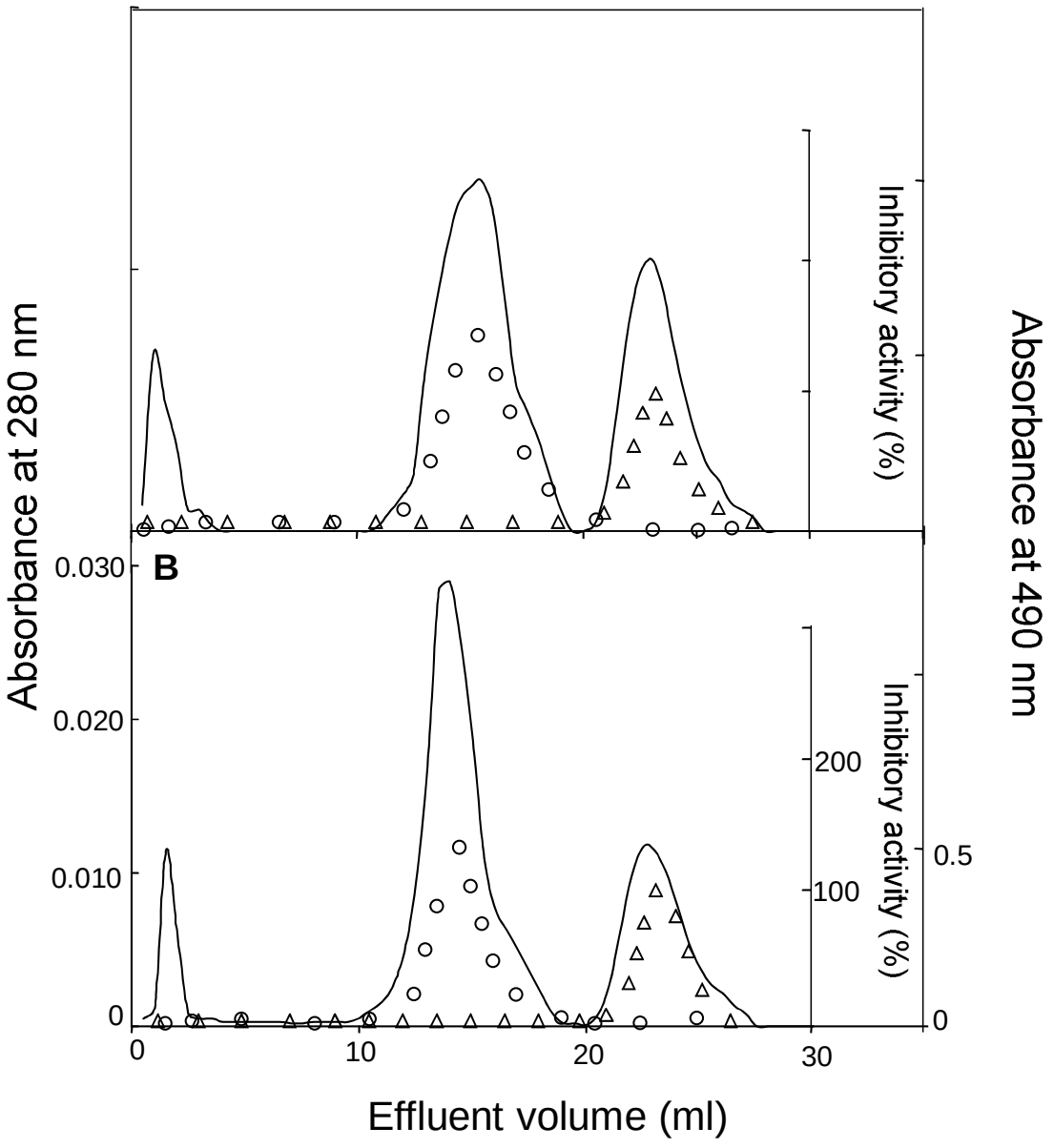
Absorbance at 280 nm
Absorbance at 490 nm
Inhibitory activity (%)
Inhibitory activity (%)
B
0.030
0.020
0.010
0
200
100
0.5
0
0
10
20
30
Effluent volume (ml)

Fig. 1

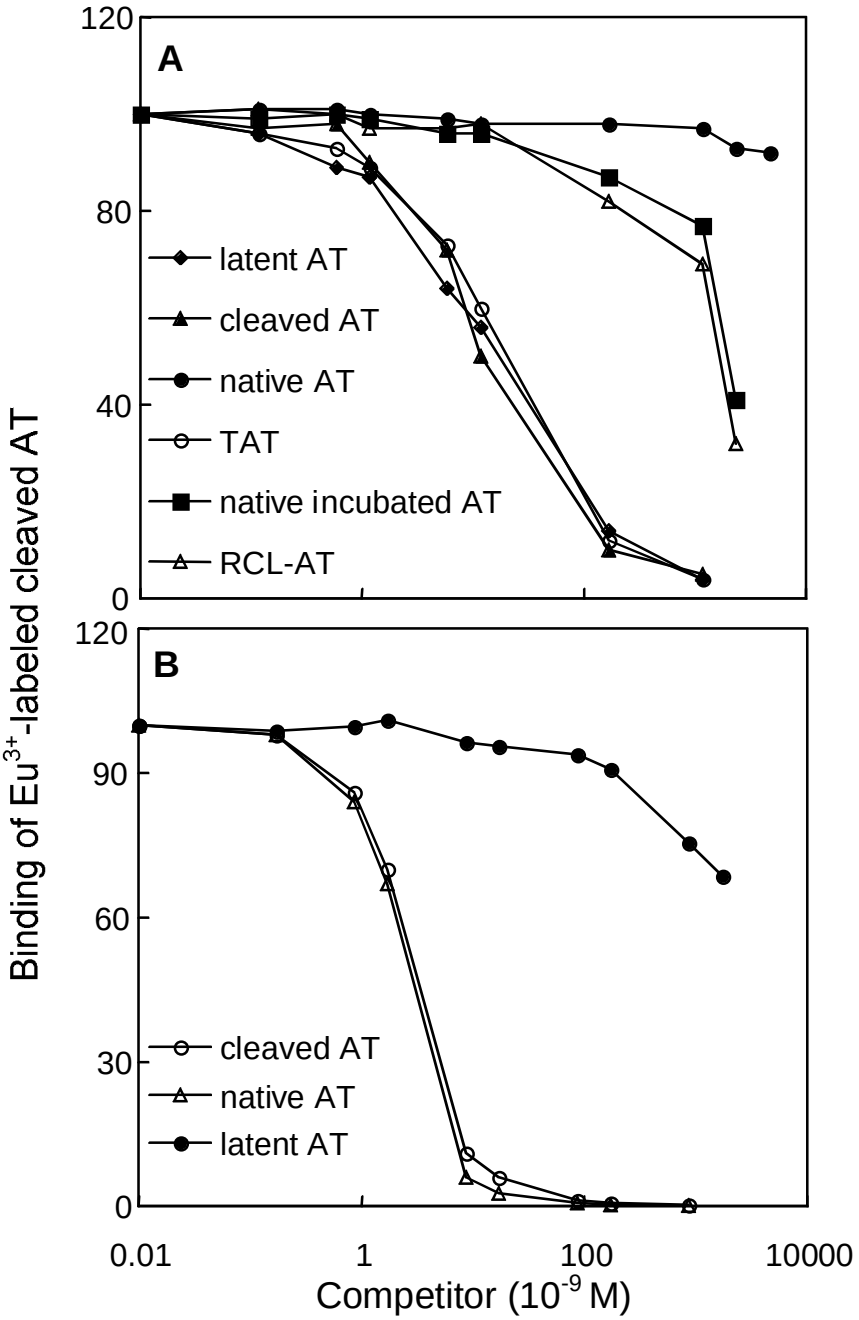
A
B
latent AT
cleaved AT
native AT
TAT
native incubated AT
RCL-AT
Binding of Eu3+-labeled cleaved AT
Competitor (10-9 M)
0
40
80
120
30
60
90
0.01
1
100
10000

Fig. 2

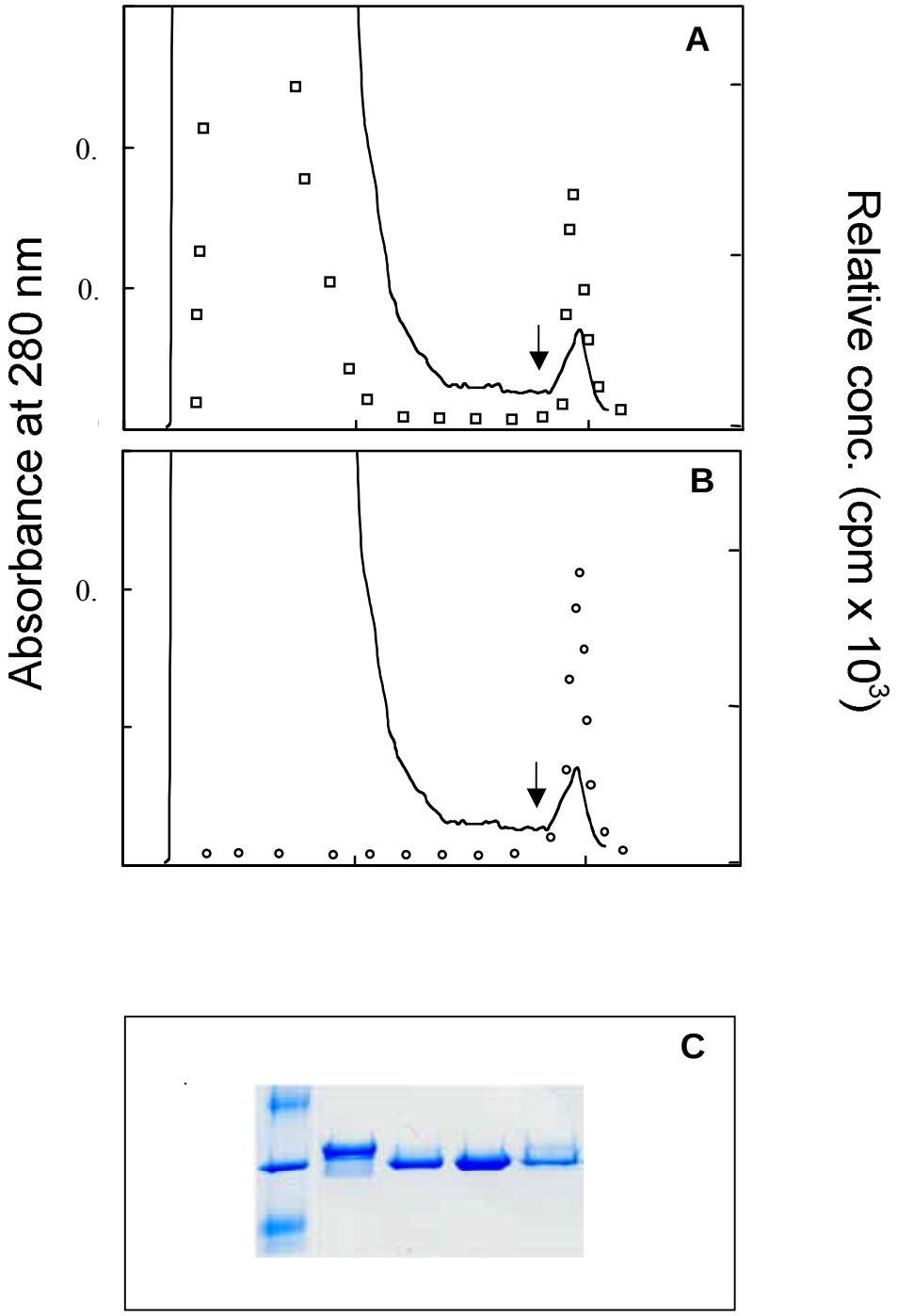

Fig.3

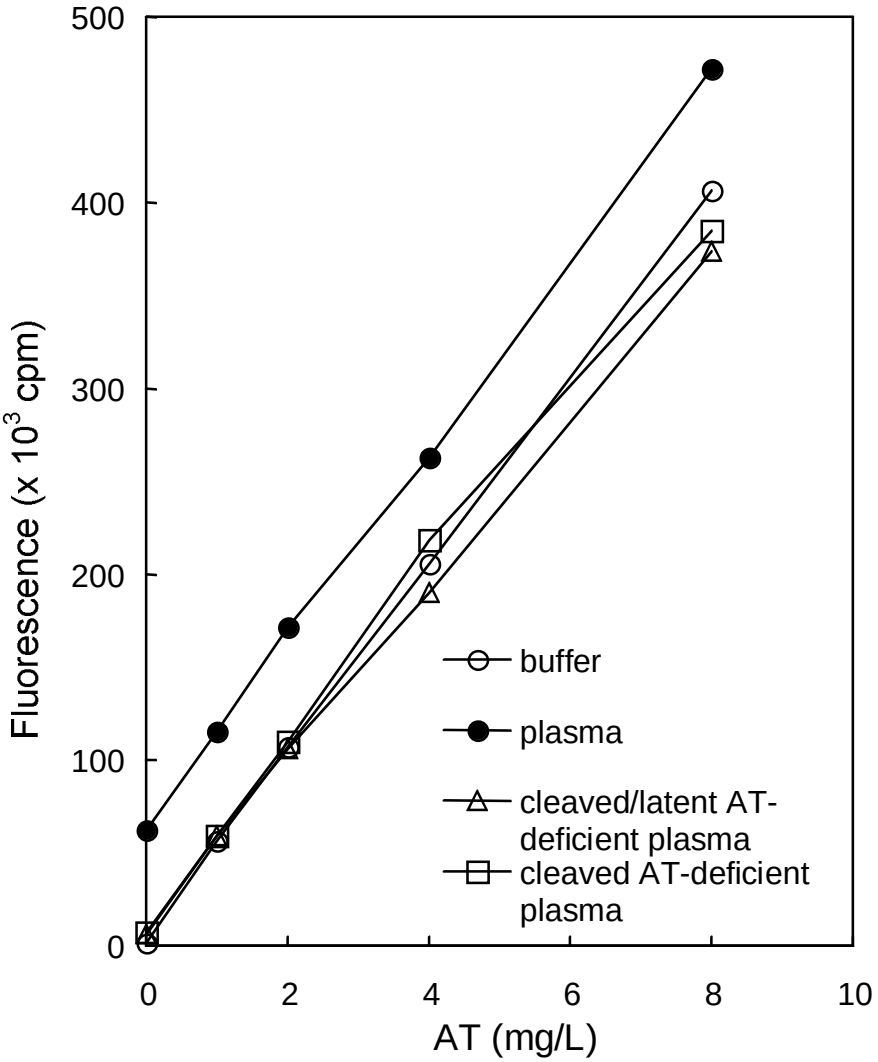
500
400
300
200
100
0
Fluorescence (x 10^3 cpm)
0
2
4
6
8
10
AT (mg/L)
buffer
plasma
cleaved/latent AT-deficient plasma
cleaved AT-deficient plasma

Fig. 4

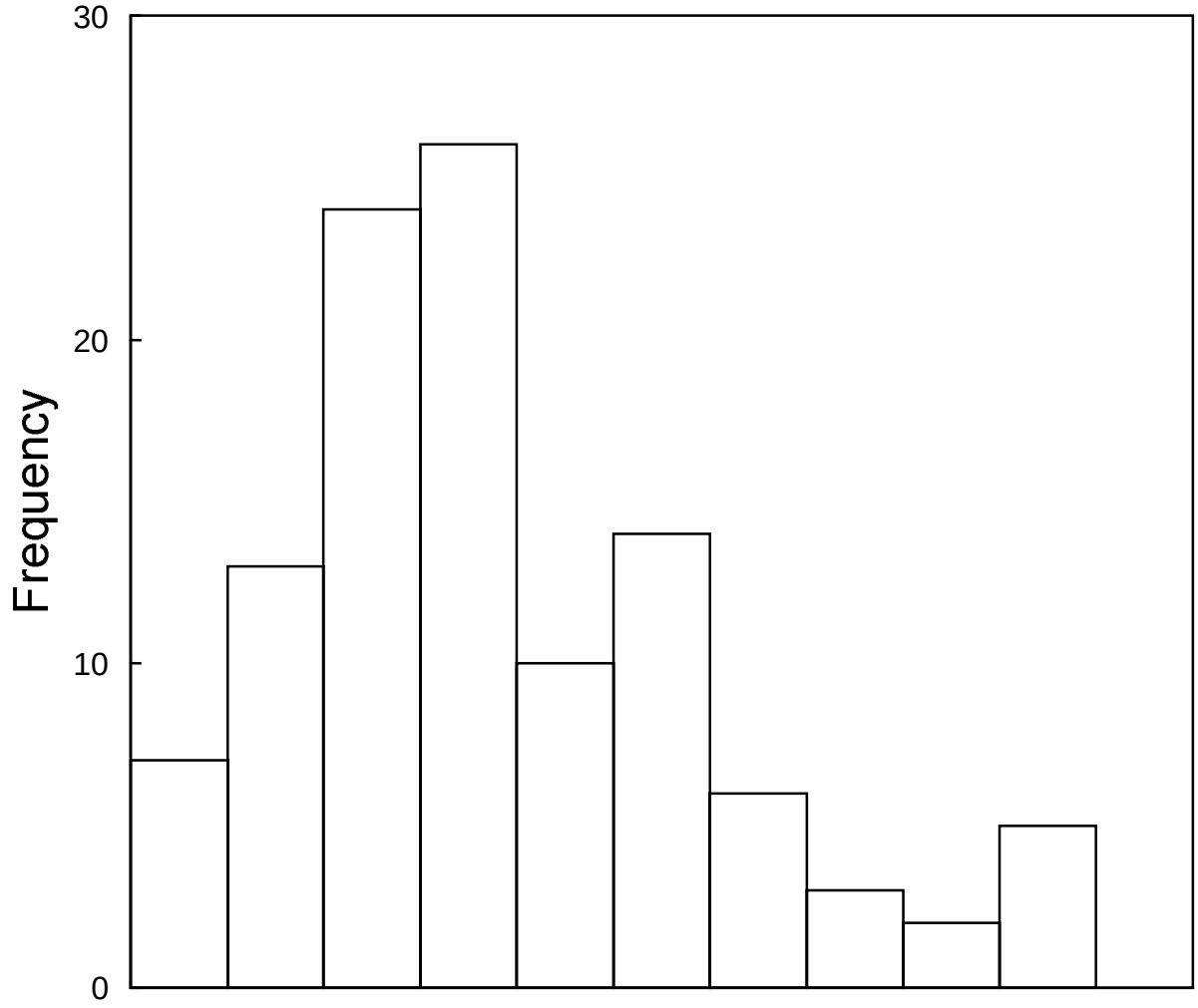

Fig. 5

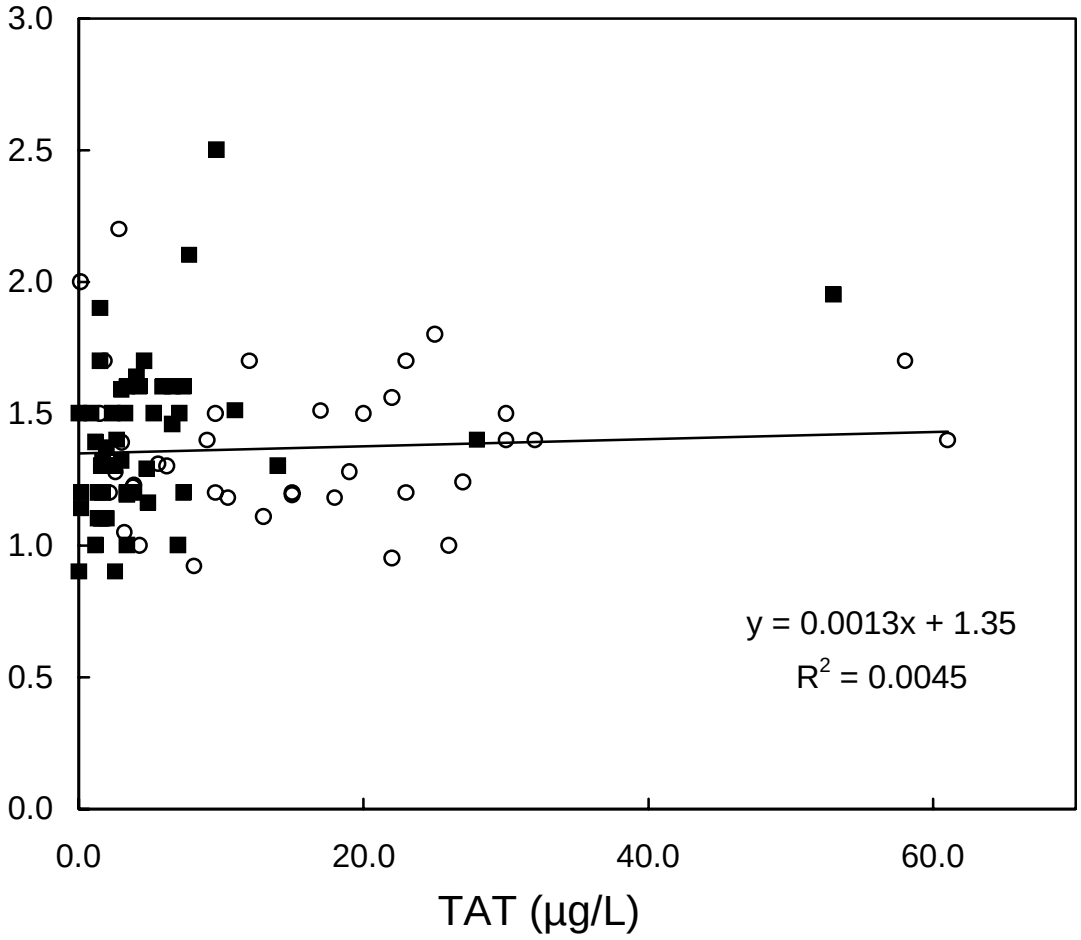

Fig. 6

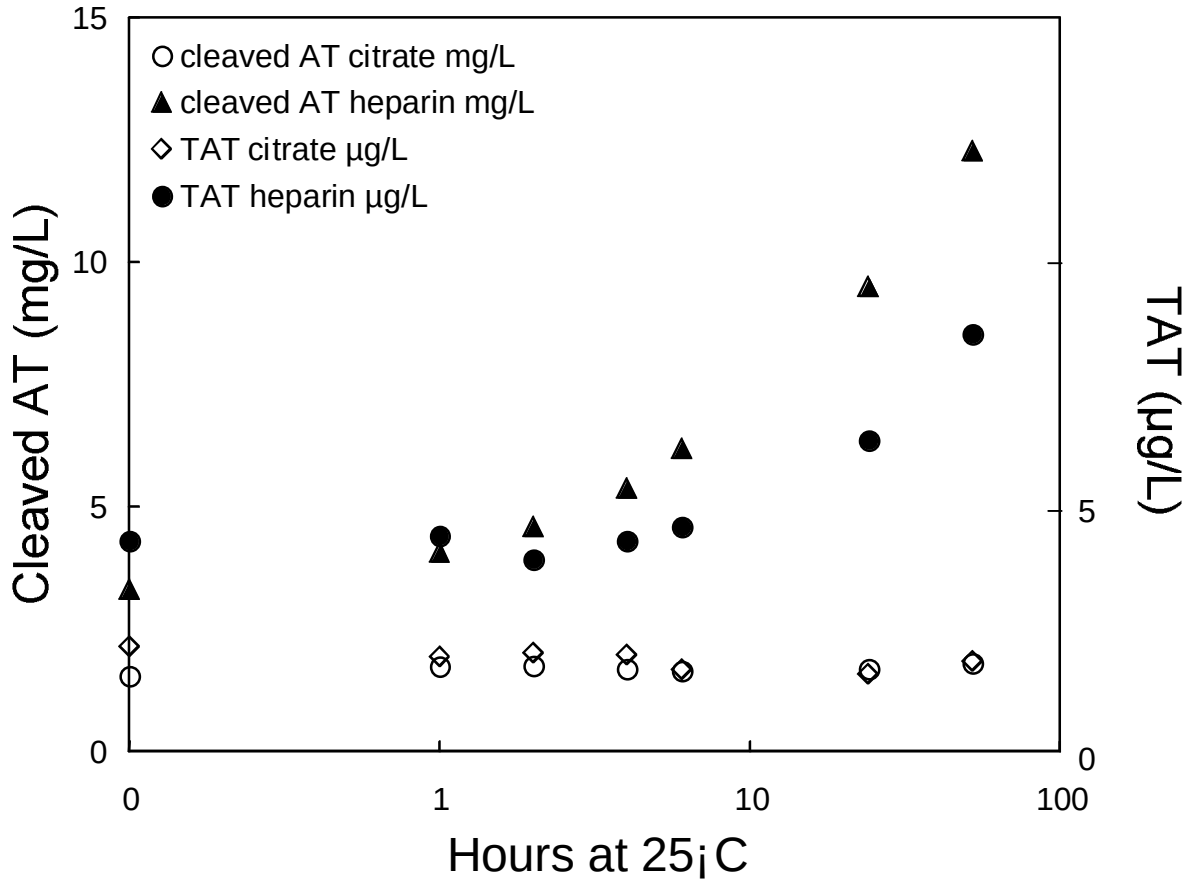
cleaved AT citrate mg/L
cleaved AT heparin mg/L
TAT citrate µg/L
TAT heparin µg/L
Cleaved AT (mg/L)
TAT (µg/L)
Hours at 25¡C
0
5
10
15
0
1
10
100

Fig. 7