Subcellular Localization and Purification of Platelet $\alpha_1$-Antitrypsin

By Andranik Bagdasarian and Robert W. Colman

A protease inhibitor has been purified from platelets by ammonium sulfate fractionation, QAE Sephadex chromatography, Sephadex G-200 gel filtration, and affinity chromatography on concanavalin A-Sepharose to apparent homogeneity on disc gel electrophoresis. The following properties of the inhibitor suggest its relationship to plasma $\alpha_1$-antitrypsin: antigenic identity with $\alpha_1$-antitrypsin, molecular weight of 60,000 estimated from gel filtration, $\alpha$-globulin mobility on electrophoresis, and adsorption to concanavalin A-Sepharose with elution by $\alpha$-methyl-D-glucoside. The protein inhibits the proteolytic and amidoletic activities of trypsin, the esterolytic activity of chymotrypsin, and the coagulation activity of factor Xla. Exposure of platelets to aggregating agents such as thrombin, epinephrine, and ADP causes the release of protease-inhibitory activity in parallel with the release of serotonin, suggesting that the inhibitors may be released from the dense granules. Thrombin also causes the release of 37% of $\alpha_1$-antitrypsin antigen from platelets. Subcellular fractionation confirms the localization in platelet granules but also reveals considerable membrane-bound $\alpha_1$-antitrypsin.

Platelets have been studied from two $\alpha_1$-antitrypsin deficient patients: A with 10% and B with 0% plasma $\alpha_1$-antitrypsin. In patient A, normal concentrations of $\alpha_1$-antitrypsin antigen and trypsin-inhibitory activity were found in gel-filtered platelets as well as in the granular and soluble subcellular fractions, further suggesting that $\alpha_1$-antitrypsin is an intrinsic platelet protein. However, unlike normal individuals, platelets from patient A contained no membrane-bound $\alpha_1$-antitrypsin, indicating that its origin may be from plasma. In contrast, the platelets from patient B, like the plasma, contained no demonstrable $\alpha_1$-antitrypsin antigen in any subcellular fraction. However, the trypsin-inhibitory activity was 50% of normal, corresponding to the occurrence in normal platelets of inhibitory activity distinct from $\alpha_1$-antitrypsin.

Naturally occurring protease inhibitors play an important role in the control and regulation of the action of proteolytic enzymes. Plasma contains several inhibitors effectively limiting the extent of the proteolysis when proteolytic zymogens are activated in the circulation. These plasma inhibitors are known to play a major regulatory role in coagulation, fibrinolysis, and kinin formation. At least four plasma inhibitors, $\alpha_1$-antitrypsin, $\alpha_2$-macroglobulin, Cl inhibitor, and antithrombin III, are involved in the regulation of the above pathways. Each of these plasma inhibitors is known to demonstrate specificity toward several enzymes, although considerable overlap exists.

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Platelets play an important part in the formation of arterial thrombi by forming aggregates, and in the process release subcellular components from discrete organelles. Platelets have also been shown to contain protease inhibitors such as antiuropinase,1 antiplasmin,2,3 and α2-macroglobulin.4 We have recently identified in washed human platelets an inhibitor of trypsin that reacts with an antibody to plasma α1-antitrypsin.5 This observation has subsequently been confirmed and extended by Nachman and Harpel,4 who have shown that the inhibitor has a similar subunit structure to its plasma counterpart. Nalli et al.,6 using an immunofluorescent method, have also demonstrated the presence of α1-antitrypsin in platelets.

In this paper, we describe the purification and characterization of a functionally active platelet α1-antitrypsin that inhibits trypsin, chymotrypsin, and factor Xla and is released from human platelets by ADP, epinephrine, and thrombin. In addition, we have studied two patients with 0% and 10% plasma α1-antitrypsin. The latter had normal levels of this inhibitor in her platelets, and the former had no detectable platelet α1-antitrypsin.

MATERIALS AND METHODS

Substrates

N'-Benzoyl-phenylalanyl-valyl-arginine-p-nitroanilide (BPVA-pNA) was purchased from AB Bofors Nobel Division, Peptide Research, Molndal, Sweden, and dissolved in distilled water, either by stirring for 1 hr or by sonication for four periods of 15 sec each to yield a 1 mM solution. N'-Carbobenzoxy-tyrosine-p-nitrophenyl ester (CBZ-T-pNP) was purchased from Sigma Chemical, St. Louis, Mo., and dissolved in acetone to yield a 2.5 mM solution. P-nitrophenyl p'-guanidinobenzoate HCl was purchased from Vega-Fox Biochemicals, Tucson, Ariz. Casein was obtained from Fisher Scientific, Fairlawn, N.J.

Synthetic Inhibitors of Proteases

Benzamidine hydrochloride was purchased from Aldrich Chemical, Milwaukee, Wis.

Enzymes

Bovine pancreatic trypsin (crystallized three times) treated with tosylphenylalanyl chloromethyl ketone to render it chymotrypsin free was obtained from Worthington Biochemical, Freehold, N.J. The concentration of active trypsin in solution was determined by the active site titration method of Chase and Shaw.7 Bovine pancreatic α-chymotrypsin (six times crystallized, type II) was purchased from Sigma. Plasminogen was prepared by affinity chromatography by the procedure of Chibber et al.8 or was purchased from Kabi, Stockholm, Sweden. Both preparations of plasminogen gave similar results. Varidase (Lederle Laboratories, Pearl River, N.Y.) was the source of streptokinase. Human plasma kallikrein was prepared in our laboratory by alcohol and salt fractionation and ion exchange chromatography9 and was further purified by gel filtration.10 Bovine thrombin was obtained from Lederle and was further purified by chromatography on DEAE-cellulose and cellulose phosphate columns followed by gel filtration.11

Human thrombin was a gift of Dr. John Fenton, Division of Laboratories, New York State Department of Health, Albany, N.Y.;12 and by active site titration it was 98%, active enzyme.

Two preparations of factor Xla were used. One was a highly purified preparation, which was a gift from Dr. Allen P. Kaplan, National Institutes of Health, Bethesda, Md., prepared as previously described.13 The second, human factor Xla, was a byproduct of the kallikrein purification. Human normal plasma was subjected to alcohol and salt precipitation, and the "ceruloplamin precipitate" was chromatographed on CM-Sephadex, DEAE-cellulose and a second CM-Sephadex.9 After elution of kallikrein, factor Xla activity was eluted with 0.016 M sodium
PLATELET α 1 -ANTITRYPSIN

phosphate, pH 8.0, containing 0.5 M NaCl. On disc gel, factor XIa migrated as a single band and contained no detectable quantities of kallikrein, thrombin, or factor XIIa by functional assays. Furthermore, it did not contain kallikrein or γ-globulin as assayed by double immunodiffusion.

Deficient Plasmas

Fresh-frozen plasmas congenitally deficient in either factor XI or XII were purchased from George King, Salem, N.H.

Molecular Weight Standards

Twice crystallized bovine serum albumin (BSA), rabbit muscle lactate dehydrogenase, type II crystalline suspension in ammonium sulfate, α-chymotrypsinogen (bovine pancreas, type II, six times crystallized), ovalbumin (crystallized and salt free) and human γ-globulin were obtained from Sigma.

Other Chemicals

Acrylamide, N,N-methylene bisacrylamide (Bis) and Coomassie brilliant blue were obtained from BioRad Laboratories, Richmond, Calif. N,N,N,N-tetramethyl ethylenediamine was obtained from Eastman, Rochester, N.Y., and ammonium persulfate was obtained from Fisher. Dithiothreitol and dextran blue were purchased from Sigma. Sodium dodecyl sulfate was obtained from Schwartz/Mann, Orangeburg, N.Y. QAE-Sephadex, Sephadex G-200, Sepharose-4B, Sepharose-2B, concanavalin A (Con A)-Sepharose and dextran blue 2000 were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. DEAE-cellulose (DE-52), preswollen, was obtained from Whatman, Clifton, N.J.

Antisera

Antibodies prepared in rabbits against human plasma proteins, anti-α1-antitrypsin, anti-inter-α1-trypsin inhibitor, anti-α2-macroglobulin, anti-C1 inhibitor, and anti-thrombin III were obtained from Behring Diagnostics, Somerville, N.J. Anti-human whole serum prepared in goat was obtained from Miles Laboratories, Elkhart, Ind.

Column Chromatography and Gel Filtration

QAE- and G-200 Sephadex and preswollen DE-52 were equilibrated with the starting buffer according to the directions of the manufacturer, and were packed into columns by gravity. A standard curve was constructed to estimate molecular weights by gel filtration. The following proteins were used: IgG, BSA, ovalbumin, and α-chymotrypsinogen. Sephadex G-200 filtration was carried out using a downward flow column (1.6 x 86.5 cm) at a constant operating pressure of 40 cm. Samples of up to 3 ml were applied. The flow rate was 8 ml/hr.

Affinity Chromatography

To remove albumin from purified inhibitor (after gel filtration) either of the following two methods was employed:

Con A-Sepharose column. A small column (2-ml volume) was packed and equilibrated with 0.05 M Tris-HCl buffer at pH 8.0. Inhibitor samples of up to 2 ml were passed through the resin at 4°C. After the removal of the unabsorbed proteins by the starting buffer, the inhibitor was eluted from the Con A-Sepharose with a 0.1 M solution of α-methyl-D-glucoside in the 0.05 M Tris buffer. One-ml fractions were collected and assayed for trypsin inhibition. For functional studies, fractions containing the inhibitor were pooled and stabilized with human serum albumin 100 μg/ml prior to concentration. The recovery of inhibitor was about 70% based on the total trypsin inhibitory capacity of the material applied to the column. Con A Sepharose was routinely used as the final step in the purification of the protease inhibitor.

Sepharose-dextran blue column. Occasionally a Sepharose-dextran blue column was used to remove albumin contamination from inhibitor. Sepharose-4B was activated by cyanogen bromide and dextran blue was linked onto activated Sepharose-4B. The partially purified inhibitor was
passed through a Sepharose-dextran blue affinity column and eluted with 0.05 \( M \) Tris-HCl buffer, pH 8.0.

**Gel Electrophoresis**

Analytical polyacrylamide disc gel electrophoresis was performed according to Davis.\(^{16}\) Samples in 4\% sucrose were applied to 7\% polyacrylamide gels, and electrophoresis was carried out for 2 hr at 4\(^\circ\)C, 2.5 mA per gel. To locate protein bands, gels were stained for 3 hr with 0.1\% Coomassie blue. To locate activity, duplicate gels were sliced immediately after the run. Corresponding slices (4.7 mm) were pooled and eluted with 0.01 \( M \) Tris-HCl, pH 8.0, in saline containing 200 \( \mu \)g/ml human serum albumin and 0.01\% sodium azide for 48 hr.

**Preparation of Platelets**

For the platelet aggregation and release studies, 9 volumes of freshly drawn blood was mixed with 1 volume of 3.8\% sodium citrate (final concentration 0.013 \( M \)). Platelet-rich plasma (PRP) was obtained by centrifuging blood in plastic tubes at 80 \( g \) for 10 min at 23\(^\circ\)C. To separate platelets from plasma, PRP was gel filtered through Sepharose-2B according to a modification\(^{17}\) of the original procedure of Tangen et al.\(^{18}\)

Fresh blood was obtained by venipuncture with informed consent from normal donors, from an \( \alpha_1 \)-antitrypsin-deficient donor (PiZZ) with 10\% plasma \( \alpha_1 \)-antitrypsin, and from a unique patient with 0\% \( \alpha_1 \)-antitrypsin (null gene). The blood was collected into acid citrate dextrose in standard blood bank packs and centrifuged at 80 \( g \) for 15 min to obtain the PRP. For purification procedures, however, platelet concentrates (3–5 days old, obtained from American Red Cross, Philadelphia, Pa.) were used. No consistent difference in inhibitor recovery or functional activity was found between the Red Cross platelets and the fresh platelets. Each concentrate was diluted with equal volumes of Tyrode’s buffer (containing 1 mM EDTA) and 0.01 \( M \) benzamidine. To separate platelets from plasma the diluted platelet concentrate, or PRP, was centrifuged at 700 \( g \) for 15 min. The platelet buttons were resuspended gently with 40 volumes of the same buffer and centrifuged as above. This step was repeated three times. In some instances, when platelets were significantly contaminated with red blood cells, the latter were removed by centrifuging suspended cells for 30 sec at 800 \( g \). The supernatant containing the platelets was then centrifuged to sediment platelets. All the operations up to this point were carried out at room temperature and in plastic containers.

**Lysis of Washed Platelets**

*Sonication.* Platelet concentrates were resuspended in 0.01 \( M \) Tris-HCl, pH 8.0, containing 0.13 \( M \) NaCl at 0\(^\circ\)C and sonicated three times, for 15 sec each, at 50 W using a Branson sonifier (Plainview, N.Y.). The sonicate was centrifuged at 100,000 \( g \) for 1 hr at 5\(^\circ\)C to obtain the supernatant.

*Homogenization.* Platelet concentrates were resuspended in 0.44 \( M \) sucrose containing 1 \( mM \) EDTA, homogenized, and centrifuged to obtain the supernatant as described by Marcus et al.\(^{19}\)

**Subcellular Fractionation**

Platelet homogenate (1.0 ml) was layered on top of a 30\% to 60\% discontinuous sucrose density gradient in 5 ml cellulose nitrate tubes and spun at 130,000 \( g \) for 2 hr at 5\(^\circ\)C. The subcellular fractions (supernatant, membrane, and granules) were then removed with a Pasteur pipette.

**Ammonium Sulfate Precipitation**

Solid ammonium sulfate was added to the 100,000 \( g \) supernatant from washed homogenized or sonicated platelets at 0\(^\circ\)C with constant stirring. After 15 min the precipitate was collected by centrifuging at 3000 \( g \) at 4\(^\circ\)C for 15 min and then was redissolved in the 0.01 \( M \) Tris-HCl buffer, pH 8.0. The procedure was repeated until a final saturation of 80\% was reached. The ammonium sulfate fractions were dialyzed against 0.05 \( M \) Tris buffer, pH 8.0, overnight.
Immunodiffusion

Double diffusion was performed by the method of Ouchterlony\textsuperscript{20} with prepared 1\% agarose plates (Hyland Labs, Costa Mesa, Calif.).

Radial Immunodiffusion

The general procedure was that of Mancini et al.,\textsuperscript{21} with a final concentration of 1.2\% agarose in barbital buffer, pH 7.6, 1:8 dilution of antiserum, and 0.01\% merthiolate. The 2.25-mm diameter wells contained 10-15 \mu l of antigen solution. A plot of the square of the diameter versus antigen concentration was linear for both standards and samples.

Platelet Aggregation and Release

The procedures for ADP- and epinephrine-induced aggregation and release were identical to those of Colman and Schreiber.\textsuperscript{17} In the case of thrombin (0.01-1.0 U/ml), stirring was not required and fibrinogen was not required or added. After the aggregation and release, the supernatant free of platelet aggregates was obtained by centrifuging the mixture at 12,000 \textit{g} for 4 min in an Eppendorf microfuge at 23°C.

\textsuperscript{14}C-Serotonin Release

\textsuperscript{14}C-Serotonin release was measured by the method of Jerushalmy and Zucker.\textsuperscript{22}

\(\beta\)-Glucuronidase

\(\beta\)-Glucuronidase was assayed according to the method of Fishman et al.\textsuperscript{23} with phenolphthalein glucuronate as substrate.

Inhibition of Trypsin Amidase Activity

Inhibition by platelet components was determined by measuring the degree of hydrolysis using a tripeptide \(p\)-nitroaniline substrate.\textsuperscript{24} Up to 0.25 ml of platelet sample from different stages of purification was mixed with 0.05 \textit{M} Tris-HCl-imidazole buffer, pH 8.1, containing 0.1 \textit{M} NaCl and 0.5 mg/ml BSA (in a total volume of 0.9 ml), and 0.01 ml of trypsin (0.2 \mu g). The mixture was incubated at 30°C for 5 min, unless otherwise stated. At the end of the incubation period, 0.1 ml of 1 \textit{M} substrate (BPVA-pNA) was added and incubated for another 2 min. The reaction was stopped by the addition of 0.1 ml glacial acetic acid and the color formed measured at 405 nm with a Gilford 240 spectrophotometer. The controls for the platelet release studies consisted either of Tyrode's buffer at pH 7.4 or supernatant obtained from gel-filtered platelets not exposed to aggregating agents, to which the same amounts of thrombin, ADP, or epinephrine were added. Another set of controls was run by incubating a sample alone with substrate in order to correct for possible endogenous amidase activity and absorbance at 405 nm.

Inhibition of Trypsin Protease Activity

The substrate used was casein. A mixture of up to 0.3 ml sample, 0.3 ml 0.1 \textit{M} phosphate buffer at pH 7.65, 0.01 ml trypsin (0.2 \mu g), and BSA at a final concentration of 0.5 mg/ml was incubated for 50 min at 30°C. Casein solution (0.4 ml of 50 mg/ml) was then added to the mixture and incubated for a period of 62 min. A control experiment was carried out in which buffer was substituted for the sample. The degree of casein hydrolysis and the extent of the inhibition were determined by a method similar to the procedure described for plasmin assay.\textsuperscript{25}

Inhibition of Chymotrypsin Esterase Activity

The method employed was that of Kosow et al.\textsuperscript{26} 0.25 ml of platelet sample was mixed with 0.25 ml 0.1 \textit{M} Tris-HCl, pH 7.4, containing 0.5 mg/ml BSA and 10 \mu l chymotrypsin (0.1 \mu g). The mixture was incubated at 30°C and 10 \mu l 2.5 \textit{mM} substrate (CBZ-T-pNP) was added to the cuvette and mixed; adsorption at 300 nm was recorded by a recorder or read at 30-sec intervals for about 2 min.
Inhibitor Units

Hydrolysis of BPVA-\(p\)-NA and CBZ-T-\(p\)-NP was linear for at least 3 min under these conditions. In the trypsin amidase or chymotrypsin esterase assays, when the remaining enzyme activity was plotted on a semilog graph against the concentration of the inhibitor, a linear relationship was obtained. Therefore, we arbitrarily chose 1 unit of inhibitor to be equal to that amount that could inhibit 50% of trypsin or chymotrypsin activity when 0.2 \(\mu\)g of the former and 0.1 \(\mu\)g of the latter were used.

Inhibition of Plasmin

The procedure used to activate plasminogen and to measure plasmin activity was that of Alkjaersig et al.\textsuperscript{25} 0.5 casein units of plasmin was then incubated with platelet samples for an additional 15 min at 30°C in a total volume of 0.25 ml, containing 0.1 \(M\) phosphate, pH 7.6, in 0.13 \(M\) NaCl, and residual plasmin activity was measured.

Inhibition of Human Plasma Kallikrein

A mixture of 20 \(\mu\)l kallikrein (1.5 \(\mu\)moles tosyl arginine methyl ester hydrolyzed per minute), 0.10 ml platelet sample or 0.05 \(M\) Tris- \(H\)-Cl, pH 8.0 (as a control), was incubated for 15 min at 30°C, followed by the addition of 0.05 ml heated plasma and further incubation for 5 min at 30°C. The levels of bradykinin generated from heated plasma were then measured by the bioassay method,\textsuperscript{27} employing guinea pig ileum.

Inhibition of Bovine Thrombin

Thrombin amidase activity was measured by a procedure similar to that described for trypsin. Platelet inhibitor samples of up to 50 \(\mu\)l in 0.01 \(M\) Tris-saline, pH 8.0, were preincubated with 10 \(\mu\)l thrombin (1 unit) for 5 min at 30°C. After preincubation, the sample was transferred to a fibrometer (Baltimore Biological, Baltimore, Md.) maintained at 37°C, and to it was added 0.2 ml fibrinogen (5 mg/ml) in 0.01 \(M\) barbital buffer in 0.15 \(M\) NaCl, and the clotting time was recorded.

Inhibition of Factors Xla and XII

Factor Xla was assayed by the modified partial thromboplastin time,\textsuperscript{26} eliminating the kaolin. The inhibition of factor Xla was measured by preincubating it with the platelet inhibitor for 5 min at 37°C. At the end of the incubation the mixture was assayed for its residual factor Xla activity. The factor XII assay was performed similarly to that for factor Xla using plasma from a patient congenitally deficient in factor XII.

Determination of Protein Concentration

Routinely fractions obtained from chromatography were examined for their adsorption at 280 nm. For a more sensitive estimation of the protein concentration, the micro-Folin-Cioicalteu method was used.\textsuperscript{29} The specific activities of inhibitors were expressed as units per milligram protein determined by Folin's method.

Concentration of Protein Samples

Fractions containing various inhibitory activities were pooled and concentrated in an Amicon ultrafiltration device (Amicon, Waltham, Mass.) using a PM-10 membrane.

RESULTS

Platelet Count and Release of Trypsin Inhibitors

When 1 ml of gel-filtered normal platelet suspensions, each containing 2 \(\times\) \(10^9\) platelets, were disrupted by sonication or with 0.2\% Triton X-100, 2.4 \(\pm\) 0.68 units of trypsin-inhibitory activity was found, while the \(\alpha\)-antitrypsin antigen concentration in the same sample was 1.2 \(\mu\)g/ml. In order to ascertain
Table 1. Release of Protease-inhibitory Activity From Human Platelets by Aggregating Agents

<table>
<thead>
<tr>
<th>Aggregating Agent</th>
<th>Conc.</th>
<th>Platelet Aggregation (%)</th>
<th>Trypsin-inhibitory Activity</th>
<th>α1-Antitrypsin Activity</th>
<th>α1-Antitrypsin Antigen</th>
<th>14C-Serotonin Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>10 μM</td>
<td>68.1 ± 8.43</td>
<td>36.7 ± 9.5</td>
<td>ND*</td>
<td>26.0 ± 5.4</td>
<td></td>
</tr>
<tr>
<td>Epinephrine</td>
<td>10 μM</td>
<td>70.6 ± 5.0</td>
<td>40.7 ± 16</td>
<td>ND</td>
<td>43.6 ± 5.0</td>
<td></td>
</tr>
<tr>
<td>Human thrombin</td>
<td>0.1 U/ml</td>
<td>68.3 ± 3.5</td>
<td>61.7 ± 8.6</td>
<td>37</td>
<td>57.7 ± 12.9</td>
<td></td>
</tr>
</tbody>
</table>

The concentrations of the agents were chosen to give maximum aggregation and serotonin release and were therefore in excess of threshold levels. The percent release of trypsin inhibitor was determined by comparison between the total inhibitory activity in sonicated gel-filtered platelets and concentration of inhibitor in the supernatant of the centrifuged platelets after exposure to the aggregating agents. Total trypsin inhibitory activity in 2 ± 10^6 platelets of sonicated gel-filtered platelets was 2.4 ± 0.68 units. The percent release of α1-antitrypsin antigen was determined by the comparison between the total antigen in Triton X-100-treated gel-filtered platelets (40 x 10^9) and the supernatant of the same number of centrifuged platelets after exposure to thrombin. The platelet aggregation, trypsin-inhibitory activity, and serotonin release are reported as the mean ± SEM of experiments on four separate donors.

*ND: not determined.

Table 2. Purification Data on Platelet Protease Inhibitors

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Volume</th>
<th>α1-Antitrypsin Antigen†</th>
<th>Trypsin-inhibitory Activity (U/ml)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet sonicate 60%-80% saturation (NH4)2SO4 fraction</td>
<td>2</td>
<td>30</td>
<td>(28)</td>
<td>20</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>QAE Pool I</td>
<td>0.235</td>
<td>2.2</td>
<td>(26)</td>
<td>31</td>
<td>132</td>
<td>13.2</td>
</tr>
<tr>
<td>Pool II</td>
<td>0.20</td>
<td>2.25</td>
<td>±</td>
<td>22</td>
<td>110</td>
<td>11.3</td>
</tr>
<tr>
<td>Q-200 Pool I (on QAE pool I)</td>
<td>0.10</td>
<td>2.2</td>
<td>(27)</td>
<td>25</td>
<td>250</td>
<td>25</td>
</tr>
<tr>
<td>Pool III (on QAE pool II)</td>
<td>0.20</td>
<td>1.35</td>
<td>(23)</td>
<td>25</td>
<td>125</td>
<td>12.5</td>
</tr>
<tr>
<td>Con A-Sepharose (on Q-200 pool I, and pool III)</td>
<td>0.032</td>
<td>4.62</td>
<td>+</td>
<td>14</td>
<td>437</td>
<td>43.7</td>
</tr>
</tbody>
</table>

* Determined according to Harriott.†
† Samples (10 μl) were tested in radial immunodiffusion plate containing antibody to plasma α1-antitrypsin. A positive reaction is indicated as +, and the concentrations as determined by reference to a standard curve are in parentheses.
† The yield is based on the combination of both Q-200 pools.
releasing the inhibitory activity from platelets with a fair correlation between inhibitory activity and serotonin release, rather than aggregation. Thrombin released 37% of the \( \alpha_1 \)-antitrypsin antigen from platelets, which corresponded at least to 13% of the total inhibitory activity released. However, this calculation was based on the specific activity of the purified inhibitor (Table 2) and represents a minimum estimate. The relationship between thrombin concentration and inhibitor release is presented in Fig. 1. A maximum of 82.5% of the inhibitor was released from platelets of a normal donor by thrombin (0.03 NIH U/ml). At a concentration of 0.1 NIH U/ml, 75% of the trypsin inhibitory activity was released, which is within the normal range, 61.7% ± 8.6% (Table 1).
Purification of Protease Inhibitors From Normal Platelets

The starting material for the purification procedure was the sonicate of washed platelets. Fifty percent of the inhibitory activity and all of \( \alpha_1 \)-antitrypsin antigen was found in the fraction precipitated between 60\% and 80\% saturation of (NH\(_4\))\(_2\)SO\(_4\); thus this fraction was chosen for further purification.

The results of QAE-Sephadex chromatography on the 60\%–80\% fraction are presented in Fig. 2. Almost all the protein was adsorbed onto the resin. The trypsin proteolytic inhibitory peaks were eluted after the major protein peaks. All inhibitor peaks also demonstrated chymotrypsin (esterolytic) inhibition, which correlated well with inhibitory activity against trypsin. When each peak was concentrated and tested by radial immunodiffusion, only the first major inhibitory peak revealed the presence of \( \alpha_1 \)-antitrypsin antigen.

Each of the major inhibitory peaks (pools I and II) from QAE was then gel-filtered through Sephadex G-200; these results are summarized in Figs. 3 and 4. QAE pool I on G-200 revealed a single corresponding trypsin and chymotrypsin inhibitory peak, which was completely resolved from the major inactive larger proteins (Fig. 3). The molecular weight of the inhibitor was estimated at 60,000 daltons and was identified as \( \alpha_1 \)-antitrypsin immunochromically. The QAE pool II, however, was resolved into three inhibitory peaks, each capable of inhibiting both trypsin (amidase) and chymotrypsin (esterase) activities.
(Fig. 4). When each peak was concentrated and tested by radial immunodiffusion, the major inhibitor (pool III, Fig. 4) revealed the presence of \( \alpha_1 \)-antitrypsin antigen. The molecular weight of the inhibitor was estimated at 60,000 daltons, similar to that from previous gel filtrations (Fig. 3). The larger inhibitor peaks had estimated molecular weights of 165,000 for pool II and greater than 300,000 for pool I (Fig. 4). Protease inhibitors at different stages of purification were tested for immunoreactivity with anti-\( \alpha_1 \)-antitrypsin by radial immunodiffusion. The 60%–80% ammonium sulfate fraction and highly purified 60,000-dalton inhibitors (pool I, Fig. 3; pool III, Fig. 4) gave positive reactions, while the larger inhibitor (pool II, Fig. 4) did not react with the antibody.

Concentrated peaks from gel filtration were tested by double immunodiffusion against antibody to whole human serum. Both major inhibitory peaks (pool I, Fig. 3; pool III, Fig. 4) revealed an additional precipitin line. These fractions formed a precipitin arc with goat anti-human albumin on double immunodiffusion.

The final step of inhibitor purification, Con A Sepharose affinity chromatography of platelet \( \alpha_1 \)-antitrypsin obtained from G-200 gel filtration, is presented in Fig. 5. After the contaminating albumin was removed by the starting buffer, the \( \alpha_1 \)-antitrypsin was eluted by the 0.1 \( M \) solution of \( \alpha \)-methyl-D-glucoside.

**Polyacrylamide Disc Gel Electrophoresis of Purified Platelet \( \alpha_1 \)-Antitrypsin and Elution of Inhibitory Activity**

On disc gel electrophoresis of the \( \alpha_1 \)-antitrypsin obtained from Con A Sepharose (Fig. 6) a single major protein band was found comprising over 80% of the total protein. Analysis of the eluates of the gel slices revealed the trypsin inhibitory activity corresponding to the protein band (segment 7). Factor X1a inhibitory activity was also found in the eluate of segment 7 corresponding to the protein. Since the starting material for the Con A Sepharose step was a
combined preparation of α1-antitrypsin (pool I, Fig. 3; pool III, Fig. 4), the results from the disc gel suggest that both inhibitors have similar electrophoretic mobility in addition to identical molecular weight and antigenic identity with α1-antitrypsin. The resolution of disc gel electrophoresis, however, was not sufficient for the detection of the genetic variants of the α1-antitrypsin.

Table 2 summarizes the purification of protease inhibitors from platelets. At the final stage of Con A-Sepharose affinity chromatography, a 43-fold purification was achieved with a specific activity of about 430 U/mg protein with a yield of 11%.

**Inhibition of Factor XIa by Platelet α1-Antitrypsin**

The effect of the inhibitor on plasma factor XIa is presented in Table 3. Purified α1-antitrypsin obtained from Con A-Sepharose and eluted from disc gels demonstrated dose-dependent inhibition of highly purified factor XIa.13 Similar results were obtained with a second preparation of factor XIa (described in Materials and Methods) that was interacted with platelet α1-antitrypsin containing albumin.

<table>
<thead>
<tr>
<th>Platelet α1-Antitrypsin (μg/ml)</th>
<th>Mean Clotting Time (sec)</th>
<th>Factor XIa (U/ml)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>150.5</td>
<td>0.18</td>
<td>33</td>
</tr>
<tr>
<td>0</td>
<td>142.5</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>168.5</td>
<td>0.10</td>
<td>58</td>
</tr>
<tr>
<td>0</td>
<td>145.0</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>16.0</td>
<td>210.0</td>
<td>0.03</td>
<td>85</td>
</tr>
<tr>
<td>0</td>
<td>150.5</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

Human factor XIa, 5 μl, was preincubated for 5 min at 37°C in a total volume of 0.1 ml containing varying volumes of either buffer or platelet α1-antitrypsin. The inhibitor protein (32 μg/ml) was obtained by elution from disc gels and was stabilized by addition of 10 μg human serum albumin/ml eluate. Residual factor XIa activity was assayed as in Materials and Methods. A separate control was run for each concentration of inhibitor, and the percent inhibition was calculated. The actual clotting times are recorded and represent the mean of duplicate determinations.
Effect of Platelet α1-Antitrypsin on Other Plasma Proteases

Concentrations of platelet α1-antitrypsin similar to those that inhibited trypsin, chymotrypsin, and factor Xla failed to inhibit the caseinolytic activity of human plasmin, the esterolytic and kinin-forming ability of human kallikrein, and the clotting activity of thrombin.

Kinetic Studies

The inhibition of trypsin by platelet α1-antitrypsin and 165,000-dalton inhibitor at various substrate (BPVA-pNA) concentrations is presented by the Lineweaver-Burk plot in Fig. 7. Both inhibitors demonstrated competitive inhibition of trypsin. $K_i$ for the larger inhibitor (165,000 daltons) was calculated at 0.67 U/ml and for platelet α1-antitrypsin it was 0.54 U/ml.

Comparison of the Levels of the Platelet Protease Inhibitor From Normal and α1-Antitrypsin-Deficient Individuals

The concentration of α1-antitrypsin antigen in 1 ml of gel-filtered platelets containing $2 \times 10^8$ platelets was 1.2 μg, or about 1/2000 that of platelet-free plasma. The α1-antitrypsin concentrations in platelet subcellular fractions from a normal donor and two patients with plasma α1-antitrypsin deficiency are shown in Table 4. Although patient A had 10% of normal plasma α1-antitrypsin antigen, the content of antigen and trypsin-inhibitory activity in the granule and soluble subcellular fractions was normal. Moreover, on exposure to thrombin, patient A’s platelets released a comparable percentage of trypsin-inhibitory activity and $^{14}$C-serotonin. However, the platelet membrane fraction of the patient’s platelets did not have demonstrable α1-antitrypsin antigen, in contrast to the normal donor. The platelets of patient B, with no demonstrable plasma α1-antitrypsin, were shown to contain no α1-antitrypsin antigen in any of the subcellular fractions. However, these fractions contained about 50% of trypsin-inhibitory activity. Chromatography of the platelet-soluble fraction from patient B on QAE-Sephadex showed a similar pattern to normal platelets (Fig. 2). The trypsin-inhibitory activity, however, was about one-third that of normal, and no α1-antitrypsin antigen was detected.
Table 4. \(\alpha_1\)-Antitrypsin Levels of Human Gel-filtered Platelets, Platelet Subcellular Fractions, and Plasma

<table>
<thead>
<tr>
<th>Donor</th>
<th>Gel-filtered Platelets</th>
<th>Platelet (\alpha_1)-Antitrypsin</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antigen (µg/ml)</td>
<td>TIA (U/µg)</td>
<td>Antigen (µg/ml)</td>
</tr>
<tr>
<td>Normal</td>
<td>1.2 ± 0.68*</td>
<td>3.8 ± 2.4</td>
<td>5.2</td>
</tr>
<tr>
<td>Patient A</td>
<td>0.72</td>
<td>3.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Patient B</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
</tr>
</tbody>
</table>

Platelets obtained from normal individuals and two individuals with plasma \(\alpha_1\)-antitrypsin deficiency were fractionated into subcellular fractions. The concentration (µg/ml) of \(\alpha_1\)-antitrypsin (antigen) was determined by radial immunodiffusion. Trypsin-inhibitory activity (TIA) and protein concentration were determined as in Materials and Methods. The levels of \(\alpha_1\)-antitrypsin were measured using platelet buttons obtained from 7-10 ml of gel-filtered platelets, resuspended, and lysed with Triton X-100.

*Value determined as in Table 1.
†ND: not done.

DISCUSSION

When platelets were subjected to sonication or were solubilized with nonionic detergent, inhibitory activity against bovine trypsin was demonstrated, in agreement with the findings of Wakabayashi et al.\(^3\). That this activity might be physiologically available is suggested by the release of 62% of the antitrypsin activity by thrombin, which includes 37% of the total platelet \(\alpha_1\)-antitrypsin. Thus, during blood coagulation, trypsin inhibitors would be liberated into the plasma. This release is independent of platelet aggregation since it occurs after thrombin stimulation, even when platelets are not stirred and, therefore, do not aggregate. Thrombin is known\(^3\) to mediate release from both dense granules and lysosomal granules. However, the liberation of up to 40% of the trypsin-inhibitory activity by ADP and epinephrine suggests that the inhibitors may be present in the dense granules since these aggregating agents do not promote lysosomal release. One cannot rule out the possibility, however, that this inhibitor, like platelet factor 4,\(^3\) may, despite release in parallel with serotonin, be present in a granule component other than dense or lysosomal granules.

During homogenization, much of the activity is apparently released from the granules into the soluble fraction. Using the same homogenization procedure,\(^9\) 53.6% of a lysosomal protein—\(\beta\)-glucuronidase—is released into the soluble fraction. Assuming a subcellular protein distribution similar to that reported by Marcus et al.,\(^9\) it can be calculated that 70% of the total \(\alpha_1\)-antitrypsin antigen is in the soluble fraction and 24% remains in the granules. Therefore, we chose platelet sonicate as a starting material to purify these inhibitors, although we have also purified them from granules obtained by sucrose density gradient ultracentrifugation of the platelet homogenate. Although purifying the inhibitors from granules allows prior elimination of soluble proteins such as albumin, the yield of the inhibitors was substantially increased from the sonicate.

It was obvious even from fractionation on one ion-exchange column that the platelet antitrypsin activity was heterogeneous, and that a major component showed antigenic identity with plasma inhibitor \(\alpha_1\)-antitrypsin. Two sites of ori-
gin may be postulated for platelet α₁-antitrypsin. The presence of this inhibitor in thoroughly washed platelets as well as gel-filtered platelets, the localization of a substantial portion of the inhibitor to platelet granules, and its release by thrombin, all suggest that platelet granular α₁-antitrypsin is an intrinsic platelet component. Although small molecules such as serotonin can be transported into the granules from plasma, no such mechanism has been described for proteins. The intrinsic nature of this inhibitor is further substantiated by the finding of α₁-antitrypsin in normal concentrations in the granules of a patient with 10% plasma α₁-antitrypsin. In contrast, the portion of α₁-antitrypsin bound to the membrane may be acquired from the plasma since membrane-bound α₁-antitrypsin was missing from this patient.

Our results agree with those of Nachman and Harpel,⁴ who found α₁-antitrypsin in both membrane and granule fractions. The absence of α₁-antitrypsin from the platelet-soluble fraction found by these authors may be due to differences in the homogenization process. Normal plasma contains 2000 times the amount of α₁-antitrypsin found in platelets. Our results were fivefold greater than the amount of α₁-antitrypsin in platelets, 0.2 µg/ml whole blood, estimated by Nachman and Harpel.⁴ This difference may arise in part from different methods of measuring protein or from the different ways of measuring antigen (radial immunodiffusion versus electroimmunoassay).

The trypsin inhibitors from sonicated platelets were isolated by ion-exchange chromatography and gel filtration. Gel filtration of each of two peaks resolved on QAE-Sephadex indicated that both contained inhibitors that displayed antigenic identity to plasma α₁-antitrypsin and had an estimated molecular weight of 60,000 daltons on a calibrated Sephadex G-200 column. This value is in reasonable agreement with that of 52,000 calculated from sedimentation equilibrium.³² Nachman and Harpel also found that platelet α₁-antitrypsin displayed antigenic identity as well as a similar molecular weight to plasma α₁-antitrypsin. However, they did not purify the inhibitor in a functional form and did not measure its release from platelets by aggregating agents. The two forms of α₁-antitrypsin probably represent microheterogeneity, which, like fibrinogen, is more easily appreciated on ion-exchange chromatography than on gel electrophoresis.³³ The platelet inhibitor adsorbed and eluted from Con A-Sepharose under similar conditions to those described for plasma α₁-antitrypsin inhibitor. This property allowed further purification and removal of albumin, which is not a glycoprotein. However, the inhibitor eluted from Con A-Sepharose, although fully active immediately after elution, was rather unstable, probably due to its low protein concentration, and lost a significant portion of its activity following concentration. For this reason, the functional studies that were carried out on inhibitors eluted from Con A-Sepharose were performed using a preparation to which crystalline human serum albumin was added as a stabilizing agent.

Inhibition of trypsin by highly purified platelet α₁-antitrypsin is competitive in nature, as evidenced by the kinetic data (Fig. 7). The inhibition of renin by plasma α₁-antitrypsin similarly follows the kinetics of competitive inhibition.³⁴ By cleaving a single peptide bond in α₁-antitrypsin, the inhibitor is converted to a reactive molecule that then complexes with trypsin to form a covalent com-
plex joined by an ester bond. Since the initial phases of the interaction are proteolytic, α1-antitrypsin functions as a substrate for trypsin. Competitive inhibition therefore is expected at short time intervals. Inhibition of thrombin apparently proceeds by a similar mechanism.

The role of plasma α1-antitrypsin in controlling proteolysis in human disease is not clear. The inhibitor has a wide range of specificity and has been claimed to inhibit trypsin, elastase, collagenase, chymotrypsin, coagulation factor Xla, plasmin, and thrombin. However, the effect of α1-antitrypsin on the last two enzymes is controversial since other investigators have found no inhibition of plasmin or thrombin. These discrepancies might be due to the contamination of α1-antitrypsin with antithrombin III, a known inhibitor of both of these plasma proteases. Using α1-antitrypsin free of antithrombin III, Burrowes and Movat could detect no inhibition of thrombin. Collen has recently described a new fast-reacting plasmin inhibitor that, together with the more slowly reacting α2-macroglobulin, accounts for virtually all of the plasmin-inhibitory activity of plasma. Specific removal of α1-antitrypsin did not alter the inactivation rate of plasmin by plasma. α1-Antitrypsin does not appear to be a major inhibitor of kallikrein. Platelet α1-antitrypsin has been shown to resemble the plasma inhibitor in its inhibition of trypsin, chymotrypsin, and factor Xla. No inhibition of human plasmin, kallikrein, or thrombin was detected at concentrations that inhibited these enzymes.

Our studies revealed a normal level of the inhibitor in patient A’s platelets, suggesting that α1-antitrypsin synthesis in megakaryocytes may be under different genetic regulation than α1-antitrypsin synthesis in the liver, the presumed source of plasma α1-antitrypsin. Alternatively, the presence of α1-antitrypsin in the platelets of patient A may be coupled with its defective release such as that proposed to explain the finding of α1-antitrypsin inclusion bodies in the liver of patients with homozygous (PiZZ) deficiency. Against the hypothesis of defective release is our finding that thrombin released trypsin-inhibitory activity from patient A’s platelets to the same extent as serotonin. However, the secretory mechanism in hepatic cells may be different from that in platelets. Talamo et al. described a patient who totally lacked functional and antigenic α1-antitrypsin in his plasma. We have further demonstrated a total lack of platelet α1-antitrypsin antigen in the same patient (patient B). In this case, the gene controlling synthesis of α1-antitrypsin may be missing rather than altered and therefore may not be expressed in the megakaryocytes. Compared to normal individuals, there was 50% platelet trypsin-inhibitory activity, which therefore differed for α1-antitrypsin.

Because of the high levels of α1-antitrypsin in normal plasma, the significance of the low concentrations in the platelets of normal individuals is questionable. However, since the inhibitor can be readily released from platelets into the immediate environment of the platelet aggregate, it may make some contribution to the α1-antitrypsin available in areas where platelets are concentrated, as in the hemostatic plug. The local concentration of platelet α1-antitrypsin in such aggregates may be sufficiently high to exert a limiting effect on coagulation by its action on plasma coagulation factor Xla. Whether this action is important in physiologic or pathologic processes is unknown. Studies by Walsh have
suggested that platelet-associated factor XI may be activated by collagen and bypass factor XII. If this alternative pathway is important, platelet α1-antitrypsin may mediate its regulation. Finally, the α1-antitrypsin and other protease inhibitors present in platelets may have a role in the control and regulation of proteolysis, which might be initiated by platelets. The occurrence in platelets of proteolytic enzymes such as collagenase, elastase, and kinin-forming enzymes indicates that inhibitors such as α1-antitrypsin, α2-macroglobulin, or the two other distinct inhibitors purified from platelets may play a role in controlling the activity of these proteases.

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Subcellular localization and purification of platelet alpha1-antitrypsin

A Bagdasarian and RW Colman