

Human Mast Cell Tryptase Fibrinogenolysis: Kinetics, Anticoagulation Mechanism, and Cell Adhesion Disruption[†]

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ABSTRACT: Tryptase is a 31 kDa, glycosylated, trypsin-like enzyme stored in and released from mast cell granules. Human tryptase exists as a tetramer, binds heparin, and has a limited substrate specificity, yet it displays remarkable resistance to inhibition by blood plasma proteinase inhibitors. In this study we have examined the cleavage of human fibrinogen by tryptase. α chain cleavage was shown to occur in the carboxyl terminal region at Arg⁵⁷² and β chain cleavage was found to occur at Lys²¹. Kinetic analyses of these reactions yielded K_m values of 0.2 μ M for α chain cleavage and 0.26 μ M for β chain cleavage, as well as k_{cat}/K_M values of 7×10^5 and 4.6×10^5 M⁻¹ s⁻¹ for α and β chain reactions, respectively. Proteolysis at Arg⁵⁷² destroyed the Arg-Gly-Asp (RGD) sequence motif recognized by cell surface $\alpha_v\beta_3$ integrins, and endothelial cell binding to tryptase-modified fibrinogen was significantly reduced, consistent with loss of the RGD motif. Tryptase competed with thrombin in clotting assays using pure fibrinogen with heparin or blood plasma in the absence of heparin. Thrombin failed to initiate the clotting of fibrinogen following modification by tryptase, and fibrin clotting initiated with Ancrod was stopped and partially reversed by tryptase. These data provide insight concerning the mechanism by which tryptase renders fibrinogen unclottable by thrombin and suggests a novel role for tryptase in the modulation of cellular interactions with fibrin(ogen).

Tryptase, a trypsin-like serine proteinase (EC: 3.4.21.59), is stored fully active in the cytoplasmic granules of all human mast cells (1). The enzyme exists as a tetramer composed of 31 kDa glycoprotein subunits and accounts for more than 20% of the total cellular protein (2). Heparin stabilizes tryptase activity (3) and is critical to the maintenance of active tetramers (4–6). One of the most unusual properties of tryptase is its remarkable resistance to inhibition by blood plasma proteinase inhibitors, such as anti-thrombin III and α_1 -proteinase inhibitor (7). Tryptase contains 21 additional amino acids relative to trypsin, and these additional residues are thought to form two peptide loops positioned on either side of the active site, which serve to protect the enzyme from inhibitors and restrict its activity to a limited number of proteins (8). Identified substrates include fibrinogen (9), high molecular weight kininogen (10, 11) and vasoactive intestinal peptide (12). Tryptase also activates matrix metalloproteinase 3 (pro-stromelysin) (13) and pro-urokinase (14).

Human fibrinogen is a 340 kDa dimeric glycoprotein, the two subunits of which are linked via amino terminal disulfides. Each subunit is composed of three different polypeptide chains, designated α (66.1 kDa), β (52.3 kDa),

and γ (46.4 kDa) (15). Thrombin converts fibrinogen to fibrin via cleavages at α -Arg¹⁶ and β -Arg¹⁴, resulting in the aggregation critical to the formation of blood clots (16). Fibrin(ogen) also interacts with cells and contains a single RGD motif near the carboxyl terminus of the α chains that binds to integrin $\alpha_v\beta_3$ on endothelial cells (17, 18). It has previously been reported that tryptase cleavage of fibrinogen prevents thrombin-initiated clotting (9). To address the mechanism of fibrinogen inactivation, we have performed detailed analysis of fibrinogen cleavage by human tryptase. Our data indicate that tryptase specifically cleaves both the α and β chains of fibrinogen. As a consequence of this cleavage, physiologic functions of fibrinogen, including clot formation and modulation of endothelial cell adhesion, were significantly disrupted. These data support the role for tryptase as an anticoagulant and suggest a new function for this enzyme in modulating cellular interactions.

EXPERIMENTAL PROCEDURES

Materials. Human fibrinogen (plasminogen free) was purchased from CalBiochem and Enzyme Research Laboratory (South Bend, IN), and fibrinogen concentrations were measured using the $A^{1\%}_{280}$ value of 15.5 (19). Human lung tissue was obtained from the National Disease Research Interchange (Philadelphia). Blood from a volunteer was collected in Vacutainer tubes (Becton Dickinson) containing buffered sodium citrate as the anticoagulant, and plasma was obtained by centrifugation at 2000g for 10 min, followed by centrifugation at 14000g for 15 min. Tryptase was

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isolated as previously described (7, 11), except that affinity chromatography on *p*-aminobenzamidine agarose (Sigma), which had been used to purify rat skin tryptase (20), was substituted for cellulose-phosphate chromatography. Enzyme obtained from hydrophobic interaction chromatography was made 0.1 M in Tris-HCl¹ pH 8 and loaded onto the affinity column in 0.1 M Tris-HCl, 0.5 M NaCl, 10% glycerol, pH 8.0, washed with loading buffer, and eluted with 20 mM sodium acetate, 1 M NaCl, 0.5 M KSCN, pH 5.0. The enzyme was immediately transferred to 10 mM MES, 2 M NaCl, 10% glycerol, 0.01% NaN₃, pH 6.1, and stored at 4 °C. Affinity-purified tryptase corresponded to the "high-HLT" form that accounts for 80% of the total tryptase activity in lung tissue (11), and this material was consistent with our previously published preparation (7). Tryptase protein concentrations were based on $E^{1\%}_{280}$ of 28 (7). Tryptase monomer (31 kDa) concentrations were determined by active site titration with 4-methylumbelliferyl *p*-guanidinobenzoate (MUGB) (21), and enzyme preparations were at least 95% active. Tryptase preparations were also assayed using the CBZ-Lys-thiolbenzyl ester substrate, as previously described (6). Human α thrombin (3800 units/mg; $M_r = 36\,500$), Ancrod (a snake venom thrombin-like protease), 4-(2-aminooethyl)benzenesulfonyl fluoride (AEBSF), poly(ethylene glycol) 8000 (PEG), trichloroacetic acid 100% solution (TCA), MUGB, heparin (porcine intestinal mucosa sodium salt; product no. H-3393), and the Arg-Gly-Asp-Ser peptide were purchased from Sigma Chemical Co. Thrombin was stored at 0.1 mg/mL in 0.15 M NaCl, 0.05 M sodium citrate, pH 6.5, at -20 °C. Bovine aortic endothelial cells were a generous gift of Dr. Gerald Soff (Northwestern University, Department of Hematology). Acrylamide/bis(acrylamide) solution, SDS, MES, CAPS, and dithiothreitol (DTT) were Fisher Biotech products.

Cleavage of Fibrinogen by Tryptase and Thrombin. Cleavage reactions were performed in 0.1 M Hepes, 0.02% NaN₃, pH 7.5, containing 0.1 mg/mL heparin at room temperature (22 ± 2 °C). Molar ratios of fibrinogen to tryptase were based on the moles of fibrinogen α (or β) chains, a molecular weight of 170 000, and the moles of tryptase active sites. Human fibrinogen (1 nmol) was reacted with tryptase (4 pmol; 1.5 h) or thrombin (4.2 pmol; 1 h). Reactions were stopped by TCA precipitation (20%), and precipitated proteins were washed with 10% TCA, followed by acetone. Dried samples were dissolved in SDS-PAGE sample buffer (22) containing 10% SDS and 50 mM DTT. SDS-PAGE gel electrophoresis was performed on 9% minigels (Bio-Rad) at 120 V for 2.6 h, which placed the bands of interest near the bottom of the gel to better resolve the fragments and native chains. Proteins were electroblotted onto PVDF (Bio-Rad; 0.2 μ m) membrane, using 20 mM CAPS buffer, pH 11, containing 10% methanol with a semidry apparatus (Bio-Rad) at 200 mA for 35 min. Coomassie stained fragments were excised and sequenced

at the St. Jude Children's Research Hospital Center for Biotechnology (Memphis, TN).

α Chain Cleavage Site Identification. The carboxyl terminal peptide cleaved from the α chain by tryptase was isolated after reacting 40 mg of fibrinogen with 0.8 nmol of tryptase (300:1 molar ratio) in 0.1 M Hepes, pH 7.5, 0.1 mg/mL heparin, 0.02% NaN₃ at room temperature (22 ± 2 °C) for 30 min. After protein precipitation with 0.1 M acetic acid and centrifugation, the supernatant fraction was passed through a 50 000 molecular weight cutoff membrane (Filtron) and peptides were concentrated on a 3000 molecular weight membrane (Amicon). The peptide was isolated by HPLC chromatography on a C₁₈ column (4 × 250 mm; Scientific Glass Engineering) using gradient elution from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 80% acetonitrile with a flow rate of 0.5 mL/min over 40 min. The major A₂₂₅ peak was collected, dried, and sequenced (St. Jude Children's Research Hospital Center for Biotechnology, Memphis, TN), with yields of 11 and 4.4 pmol for cycles 1 and 10, respectively, and with a combined repetitive yield of 96%.

β Chain Cleavage Site Identification. The β chain fragment was isolated by SDS-PAGE and electroblotting onto PVDF membrane (Figure 1) and sequenced with an initial yield of 5.7 pmol and a repetitive yield of 91.3%.

Kinetic Studies. Relative cleavage rates and reaction linearity were investigated by incubating fibrinogen (136 μ g; 800 pmol) with tryptase (4 pmol) in 100 μ L of 0.1 M Hepes, 0.4 mg/mL heparin, 0.02% NaN₃, pH 7.5 (8 μ M fibrinogen), at room temperature (22 ± 2 °C), followed by SDS-PAGE. α and β chain cleavage kinetics were performed by incubating fibrinogen (34 μ g; 200 pmol) with tryptase (1 pmol) in 0.1 M Hepes, pH 7.5, 0.4 mg/mL heparin, 0.02% NaN₃ at room temperature (22 ± 2 °C) for 10 min, with final reaction volumes of 1200–50 mL to give fibrinogen concentrations ranging from 0.17 to 4 μ M (based on individual α or β chains). α and β chain cleavage was measured by densitometry of Coomassie Blue stained gels using Millipore's BioImage software on a SUN Microstation. Band boundaries were set manually while enlarged images were viewed. Potential errors in sample loading were corrected by normalizing the integrated intensity of each product band to the total integrated intensity for all three α or both β bands to yield a percentage cleavage. Molar amounts of cleavage products were calculated from these percentages and the amounts of fibrinogen in the reaction mixtures. For the K_m and V_{max} determinations only data obtained over the range from 0.17 to 2 μ M were used and one data point was omitted from analysis of the β chain cleavage data, because it was an obvious error. K_m and V_{max} values were derived from nonlinear regression curves fit to the Michaelis-Menton plot (Jandel Scientific's SigmaPlot; hyperbolic fit), which had correlation coefficients of 0.99 and 0.96 for α and β chain data, respectively. V_{max} values for each chain were divided by the number of enzyme active sites to give k_{cat} values.

Endothelial Cell Adhesion Assays. Tryptase-modified fibrinogen (54 μ g, 320 pmol) was prepared by incubation with tryptase (3.2 pmol) in 124 μ L of TBS (20 mM Tris, 0.15 M NaCl, 0.02% NaN₃, pH 7.4) containing 0.4 mg/mL heparin at 37 °C for 30 min. Reactions were stopped by adding 4 μ L of 10 mM AEBSF. Native fibrinogen control samples were identical to the cleavage reactions with the

¹ Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DTT, dithiothreitol; ECM, extracellular matrix; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(4-morpholino)ethane sulfonic acid; MMP3, matrix metalloproteinase 3 (stromelysin); MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; PEG, poly(ethylene glycol) 8000; PVDF, poly(vinylidene difluoride); RGD, Arg-Gly-Asp; scu-PA, single chain urinary-type plasminogen activator; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TRIS, tris(hydroxymethyl)aminomethane.

exception that the tryptase was first inhibited with AEBSF prior to the addition of fibrinogen and incubation at 37 °C for 30 min. Samples were frozen and stored at -80 °C. Culture plates (24 well) were coated with native fibrinogen or tryptase-modified fibrinogen (5 µg/mL) in 0.1 M Na₂CO₃, pH 9.5, at 4 °C for 18 h, followed by three washes with 10 mM Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.5. There were no differences in the coating efficiency of native and tryptase-modified fibrinogen, based on Coomassie blue staining of similarly coated 96 well plates, with bound stain measured spectrophotometrically following methanol elution. Wells were blocked with 2% BSA in PBS for 2 h at room temperature. Bovine aortic endothelial cells (2 × 10⁵) were added in a final volume of 500 µL of serum-free media and allowed to attach for 90 min at 37 °C. Nonadherent cells were removed by gentle washing in PBS. Adherent cells were fixed with 3.7% formaldehyde for 10 min, washed with PBS, stained, and enumerated by counting a minimum of six fields (10× magnification), using an ocular micrometer. RGD-dependent binding was determined by adding 20 and 200 µg/mL of the peptide, Arg-Gly-Asp-Ser, to the wells both with and without tryptase treatment. Adhesion data were analyzed by parametric ANOVA using Systat. Similar results were obtained in a duplicate experiment, but data from only one experiment are shown.

Tryptase Competition with Thrombin. Clotting assays using pure human fibrinogen or blood plasma were performed in triplicate. Fibrinogen (0.168 mg) was added to flat-bottomed, microtiter plate wells containing 0.1 M Hepes, 0.2 mg/mL heparin, 0.05% PEG, 0.02% NaN₃, pH 7.5, and thrombin. The total reaction volume was 0.2 mL, resulting in 5 µM fibrinogen and 3.4 nM thrombin. Tryptase was mixed with the thrombin just prior to the addition of fibrinogen to start the reaction. Final concentrations of tryptase were 1.7, 3.4, and 6.8 nM, giving tryptase:thrombin molar ratios of 0.5:1, 1:1, and 2:1, respectively. Increasing turbidity was based on absorbance changes at 405 nm using a Bio-Tek 312e kinetic microtiter plate reader at room temperature (22 ± 2 °C). When blood plasma was used as the source of fibrinogen, the buffer did not contain heparin, 75 µL of plasma was used in each reaction mixture, and the concentrations of thrombin and tryptase were as previously stated. Data presented are the means of triplicate assays.

Modified Fibrin Cleavage. A modified fibrin was produced by treating 50 µg of pure fibrinogen with 3 milliunits (munits) of Ancrod for 30 min at 37 °C. Tryptase was added to the modified fibrin, resulting in a fibrin to tryptase molar ratio of 100:1 and incubated 30 min at 37 °C. Control samples contained fibrinogen, fibrinogen plus Ancrod, and fibrinogen plus tryptase, each incubated at 37 °C for 30 min. All reactions were stopped with 20% TCA, and precipitated proteins were prepared for reduced SDS-PAGE. In addition, Ancrod-mediated clotting and clot dissolution by tryptase were followed kinetically in microtiter plate wells. Fibrinogen (0.516 mg, 15.7 µM) was added to microtiter plate wells containing 6 munits of Ancrod in 0.1 M Hepes, 0.15 M NaCl, 0.5 mg/mL heparin, 0.02% NaN₃, pH 7.5 (0.2 mL total volume), with turbidity monitored as before at room temperature (22 ± 2 °C). Reactions were momentarily interrupted after 2 h for the addition of 27 µL of tryptase (7.5 µg, 1.2 µM) in storage buffer to the top of the undisturbed loose clot, followed by turbidity monitoring for

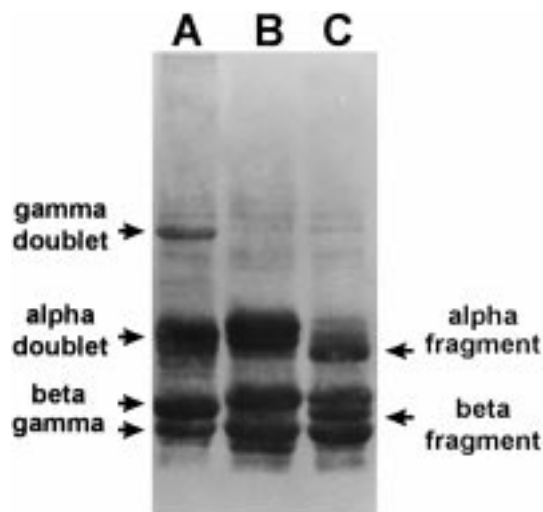


FIGURE 1: Comparison of tryptase and thrombin cleavage products. The Coomassie-stained PVDF blot of 9% SDS-PAGE analysis of fibrinogen shows cleavage differences with thrombin and tryptase. Human fibrinogen was incubated with thrombin for 1 h or tryptase for 1.5 h. Lane A: fibrinogen (12 µg) reacted with thrombin (11 ng). Lane B: fibrinogen control (17 µg). Lane C: fibrinogen (17 µg) after reaction with tryptase (13 ng). α and β fragment bands from lane C were excised for sequencing.

another 2 h. Data presented are the means of triplicate assays.

RESULTS

Tryptase cleavage of fibrinogen was compared with that of thrombin by SDS-PAGE of the reaction products (Figure 1). Whereas thrombin activated factor XIII, tryptase did not, as evidenced by the absence of cross-linked γ doublet bands in lane C in comparison to lane A in Figure 1. α chains are doublets on SDS-PAGE due to the *in vivo* removal of a 27 residue C-terminal peptide from a portion of the circulating α chains (23). Tryptase reduced the α doublet to a single band (α fragment; Figure 1), indicating cleavage at a single site near the carboxyl terminus of both α chains with removal of the ragged ends. Confirmation of this was obtained by amino acid sequence analysis of the α fragment (Figure 1) blotted onto PVDF membrane, which yielded the normal α chain amino terminal residues of Ala-Asp-Ser-Gly-Glu-Gly, proving that the size reduction resulted from cleavage in the carboxyl terminal region. Identification of the tryptase cleavage site in the α chain was accomplished by isolation of the carboxyl terminal peptide by HPLC and amino acid sequencing, yielding the amino terminal sequence of Gly-Asp-Ser-Thr-Phe-Glu-Ser. A review of the sequences of the three fibrinogen chains revealed that this peptide could only have resulted from cleavage of the Arg⁵⁷²-Gly⁵⁷³ peptide bond in the sequence Arg⁵⁷²-Gly⁵⁷³-Asp⁵⁷⁴-Ser⁵⁷⁵.

β chain cleavage by tryptase also resulted in a smaller band (β fragment; Figure 1), relative to the thrombin-modified β chain. This modified β chain was sequenced and found to have a new amino terminal sequence, relative to the native β chain. The new sequence (Lys-Arg-Glu-Glu-Ala-Pro-Ser-Leu) clearly identified the cleavage site to be near the amino terminus between Lys²¹ and Lys²².

Kinetic studies were performed to better characterize tryptase cleavage of fibrinogen. Cleavage of the α and β

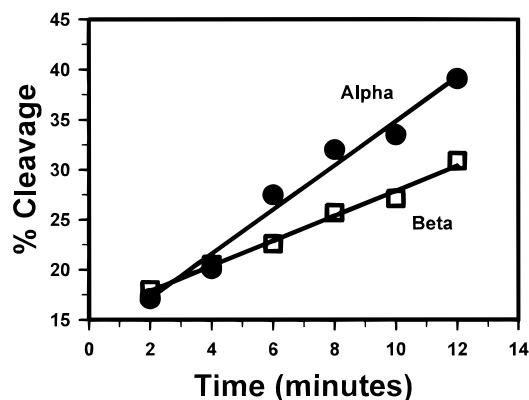


FIGURE 2: Fibrinogen chain cleavage vs time. Fibrinogen (8 μM , 800 pmol) was incubated with trypsin (4 pmol) in 100 μL of 0.1 M Hepes, 0.4 mg/mL heparin, 0.02% NaN_3 , pH 7.5, at room temperature ($22 \pm 2^\circ\text{C}$). Reactions were stopped at 2 min intervals by TCA precipitation and products were analyzed on a 9% SDS-PAGE gel with reduction. Cleavage of α chains (\bullet) and β chains (\square) was quantified by densitometry of the Coomassie-stained bands.

chains was measured by densitometry of stained SDS-PAGE gels, as described in the Experimental Procedures. Comparison of the cleavage rates of the α and β chains showed that the α chain was cleaved more rapidly than was the β chain (Figure 2). Linear regression analysis of the percent cleavage vs time plots showed that the cleavage rates of both chains were linear over 12 min. K_m and V_{\max} values for both α and β chain cleavage reactions were based on the conversion of each chain to a single band on SDS-PAGE (Figure 3A) in reaction mixtures containing increasing concentrations of fibrinogen. Michaelis-Menton kinetics

Table 1: Kinetic Values

enzyme-Fbgn chain	$K_m, \mu\text{M}$	$k_{\text{cat}}, \text{s}^{-1}$	$k_{\text{cat}}/K_m, \text{M}^{-1} \text{s}^{-1}$
trypsin ^a - α	0.20	0.14	7.0×10^5
trypsin ^a - β	0.26	0.12	4.6×10^5
thrombin ^b - α	7.2	84	2.3×10^7
thrombin ^b - β	7.5	46	6.5×10^6

^a Data from the current study at room temperature ($22 \pm 2^\circ\text{C}$). ^b Data from Ng et al. (24) at 37°C .

apply to the cleavage of both α and β chains, since hyperbolic fits to the rate versus substrate concentration curves had correlation coefficients of 0.99 and 0.96 for cleavage of the α and β chains, respectively (Figure 3B). Kinetic data are reported in Table 1 and, for comparison, similar data for thrombin (24) are included. Since trypsin kinetic data were obtained at room temperature ($22 \pm 2^\circ\text{C}$), the k_{cat} data could vary by as much as 0.06, but the effect of temperature variance on K_m cannot be estimated. Although the K_m and k_{cat} of trypsin for cleavage of the fibrinogen α and β chains are similar, comparison of the k_{cat}/K_m ratios indicate that the α chain is preferred by a ratio of 1.5:1, relative to the β chain, which agrees with the data in Figures 1 and 2.

To address the potential functional consequences resulting from trypsin cleavage of fibrinogen α and β chains, experiments were performed to assess the effect of trypsin on integrin-mediated endothelial cell adhesion and on fibrin clot formation.

Since α chain cleavage in sequence Arg⁵⁷²-Gly⁵⁷³-Asp⁵⁷⁴ would destroy an RGD motif, which has been identified as an endothelial cell adhesion site in fibrinogen (18), we sought

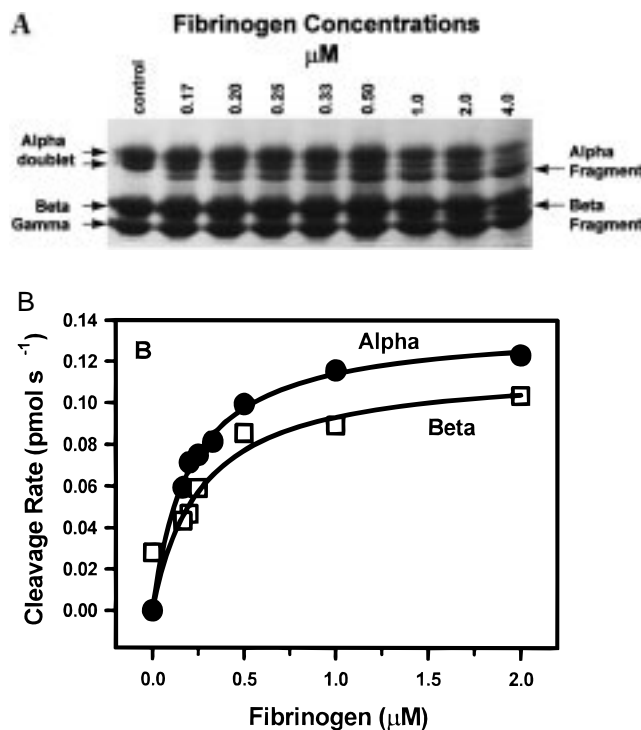


FIGURE 3: Fibrinogenolysis kinetics. (A) Coomassie Blue stained 9% SDS-PAGE of fibrinogen after reaction with trypsin for 10 min at room temperature ($22 \pm 2^\circ\text{C}$). Samples of 34 μg (200 pmol) of fibrinogen and 1 pmol of trypsin were reacted for 10 min before precipitation with 20% TCA and preparation into SDS sample buffer. The sample load was 17 μg /lane, and the fibrinogen concentrations in the reactions are given at the top of each lane. (B) Kinetic data derived from the percent cleavage of α and β chains. K_m and V_{\max} values were calculated from a hyperbolic nonlinear regression fit of the rate curves, which had correlation coefficients of 0.99 for α (\bullet) and 0.96 for β (\square).

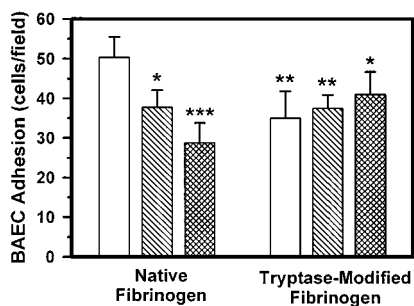


FIGURE 4: Trypsin effects on endothelial cell binding. Fibrinogen was treated with trypsin or catalytically inactivated trypsin and coated by passive adsorption onto 24-well culture plates as described in the Experimental Procedures. Bovine aortic endothelial cells (2×10^5) were added in 500 μ L of serum-free medium and allowed to adhere for 90 min, nonadherent cells were removed by washing in PBS, and bound cells were enumerated. Native fibrinogen wells were coated with unmodified fibrinogen containing inactive trypsin. Trypsin-modified fibrinogen wells were coated with trypsin-treated fibrinogen. Key: (open bars) cells only; (slashed bars) cells + 20 μ g/mL RGDS; (hatched bars) cells + 200 μ g/mL RGDS; (error bars) standard deviations. Statistical analysis of cell binding relative to native fibrinogen with *, **, and *** indicating *p* values of <0.05, 0.001, and 0.0001, respectively.

to test whether fibrinogen cleavage by trypsin would alter cell binding. Bovine aortic endothelial cells were incubated with fibrinogen and trypsin-modified fibrinogen that had been coated on tissue culture wells, followed by washing to remove nonspecifically bound cells. The numbers of adherent cells were determined by fixing, staining, and counting. Cell adhesion to trypsin-modified fibrinogen was significantly ($p = 0.008$) reduced by 30%, relative to adhesion to native fibrinogen (Figure 4). Inclusion of an RGD-containing peptide in the incubations with native fibrinogen resulted in similar inhibition of cell adhesion, indicating that the cleaved RGD site contributed to endothelial cell adhesion. Addition of the RGD peptide to wells containing trypsin-modified fibrinogen did not result in any further reduction in cell adhesion.

A clotting assay was used to examine the effect of trypsin on the conversion of fibrinogen to fibrin by thrombin. In these experiments thrombin and trypsin were mixed just prior to the addition of pure fibrinogen to initiate clotting in reaction mixtures containing 0.5 mg/mL heparin. Clot formation was monitored as the increase in turbidity with time. In Figure 5A it can be seen that the addition of an equal molar amount of trypsin to thrombin resulted in an approximately 50% decrease in both the rate of clot formation and the final amount of the clot. When a 2-fold molar excess of trypsin to thrombin was used, clot formation was totally prevented and clotting was not observed even after an additional hour of incubation (data not shown). To test whether trypsin could also compete with thrombin in the presence of blood plasma proteinase inhibitors and in the absence of heparin to stabilize trypsin activity, similar assays were performed in the absence of heparin, using human blood plasma as the source of fibrinogen. The results, shown in Figure 5B, are essentially the same as in Figure 5A, showing that trypsin cleaved fibrinogen in the presence of blood plasma protease inhibitors and that heparin was not needed to stabilize trypsin.

Because the cleavage sites identified in fibrinogen also exist in fibrin, we next sought to determine whether trypsin

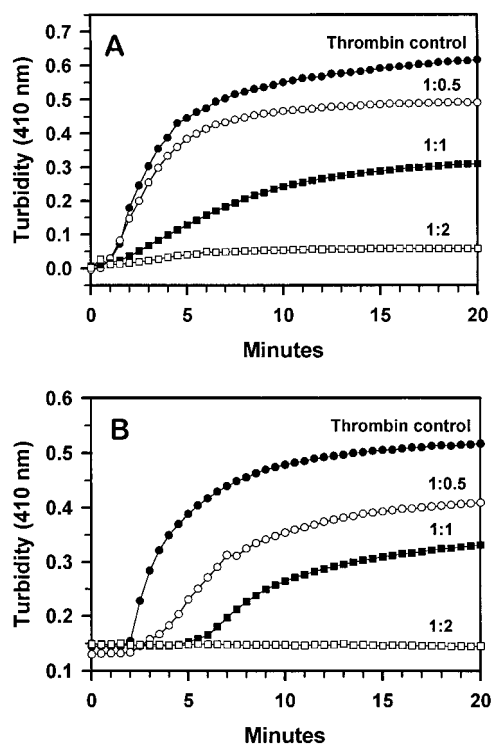


FIGURE 5: Trypsin competition with thrombin in a clotting assay. (A) Clotting was initiated by adding fibrinogen (1 nmol) to thrombin (0.69 pmol) alone or mixed with trypsin (0.35, 0.69, 1.38 pmol) in microtiter plate wells in 0.2 mL buffered with 0.1 M HEPES, 0.2 mg/mL heparin, 0.05% PEG, 0.02% NaN_3 , pH 7.5. Molar ratios of thrombin to trypsin of 1:0.5, 1:1, and 1:2 are indicated in the figure. Turbidity was based on absorbance changes at 405 nm at room temperature (22 ± 2 °C). (B) Clotting was initiated by adding 75 μ L of citrate-buffered blood plasma as the source of fibrinogen. The conditions and amounts of enzymes were the same as in A, but the buffer was 0.1 M HEPES, 0.05% PEG, 0.02% NaN_3 , pH 7.5. Heparin was not present in the buffer or other solutions. Data presented are the means of triplicate assays.

could also cleave fibrin in a clot. Thrombin-initiated clotting produces an insoluble fibrin due to the activation of factor XIII that is present in the purest samples of fibrinogen. To avoid the problems associated with analyzing trypsin cleavage of an insoluble substrate, Ancrod was used to produce a modified fibrin clot as a substrate for trypsin, because resulting cleavage products could be visualized on SDS-PAGE gels. Ancrod, a snake venom protease that does not activate factor XIII, causes fibrinogen clotting by cleaving only the α chains at the normal thrombin cleavage site after Arg^{16} (25). As expected, Ancrod cleaved only the α chain, resulting in small reductions in the sizes of the α chain doublet bands (Figure 6, lane B) as compared to native fibrinogen in lane A. Treatment of Ancrod-modified fibrin with trypsin resulted in the cleavage of α and β chains, as shown in lane D. For comparison, trypsin-cleaved fibrinogen was run in lane C. Trypsin cleavage of Ancrod-generated fibrin resulted in conversion of the α chain doublet into a single band, consistent with C-terminal cleavage at Arg^{572} , as with native fibrinogen. Trypsin also cleaved the β chain of Ancrod-generated fibrin, yielding a β fragment band identical to the β fragment resulting from trypsin cleavage of fibrinogen (lane C). The effect of trypsin on Ancrod-generated fibrin was further examined by following the turbidity resulting from clotting in microtiter wells. Figure 7 shows the increase in turbidity resulting from

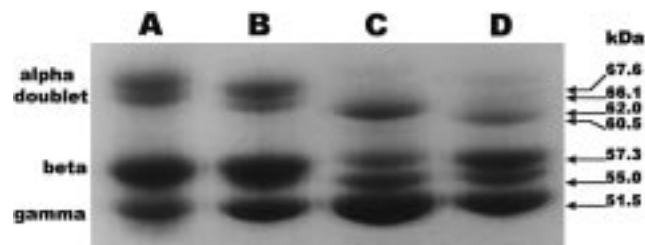


FIGURE 6: Tryptase cleavage of modified fibrin: SDS-PAGE of fibrinogen reacted with Ancrod, tryptase, and Ancrod followed by tryptase. A modified fibrin was produced by treating 50 μg of pure fibrinogen with 3 munits of Ancrod for 30 min at 37 $^{\circ}\text{C}$. Tryptase was added to the modified fibrin, resulting in a fibrin to tryptase molar ratio of 100:1 and incubated 30 min at 37 $^{\circ}\text{C}$. Key: (A) fibrinogen control; (B) Ancrod-modified fibrin; (C) fibrinogen cleaved by tryptase; (D) modified fibrin cleaved by tryptase. Reactions were stopped by adding 2 \times SDS sample buffer and heating.

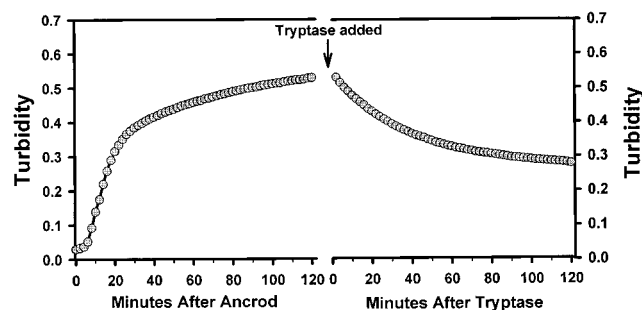


FIGURE 7: Tryptase dissolution of modified fibrin clots: Clotting was initiated by adding fibrinogen (0.516 mg; 3.13 nmol) to 6 munits of Ancrod in 0.2 mL of 0.1 M HEPES, 0.15 M NaCl, 0.5 mg/mL heparin, 0.02% NaN_3 , pH 7.5, and turbidity was measured at 410 nm. Reactions were momentarily interrupted after 120 min and 27 μL of tryptase (7.4 μg , 239 pmol) in 10 mM MES, 2 M NaCl, 10% glycerol, 0.02% NaN_3 , pH 6.1, was pipetted on top of the undisturbed loose clot. Turbidity measurements were then continued for another 120 min. Data presented are the means of triplicate assays.

Ancrod-induced clotting and the decrease in turbidity upon the addition of tryptase on top of the clot.

DISCUSSION

It has previously been reported that mast cell tryptase-catalyzed cleavage of fibrinogen prevents thrombin-mediated conversion to a fibrin clot (9). On the basis of this preliminary observation, the present study was undertaken to elucidate the mechanism underlying the anticoagulant property of tryptase. Consistent with the findings of Schwartz et al. (9), we found that tryptase cleaved both the α and β chains of fibrinogen and that tryptase-modified fibrinogen failed to form thrombin-mediated clots. However, our studies focused on early events and were designed to monitor the initial cleavage rates, thereby enabling determination of the kinetics of tryptase reaction with both α and β fibrinogen chains. Furthermore, we have identified both initial cleavage sites, show that tryptase negatively affects cell binding to fibrinogen and that tryptase cleaves a modified-fibrin.

Sites at which tryptase cleaves human fibrin (ogen) are summarized in Figure 8. Although there are several arginine and lysine residues in fibrinogen where trypsin-like proteases might cleave, tryptase demonstrates considerable specificity by cleaving only one site in each of the α and β chains.

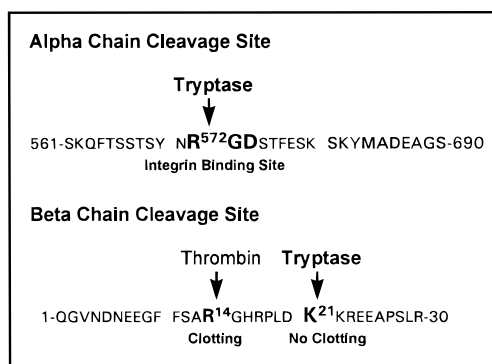


FIGURE 8: Sequences of tryptase cleavage sites in fibrinogen.

Cleavage at α chain Arg⁵⁷² and at β chain Lys²¹ with similar kinetics shows that tryptase does not discriminate between these basic residues. Previously, we have shown that tryptase cleaves human high molecular weight kininogen at an Arg (11) and human single chain plasminogen activator at a Lys (14). Residues P5 to P4' flanking the cleavage sites in these protein substrates and fibrinogen lack sequence homology, suggesting that tryptase specificity depends more on the conformation of the substrate than on specific interactions with residues on either side of the P₁ residue, Arg or Lys. Computer modeling showed that tryptase has two loops positioned on either side of the active site (8) that may be responsible for the resistance of tryptase to inhibition by natural proteinase inhibitors. Similarly, the presence of these loops may also require that potential cleavage sites be positioned in a bend or some other conformation that allows access to the active site. Fibrinogen is obviously an excellent protein substrate for tryptase as the K_m values (Table 1) of tryptase with α and β chains are 36 and 29-fold, respectively, lower than the K_m values of thrombin with these chains (24). The only other protein substrate for which tryptase kinetic data are known is single-chain urinary-type plasminogen activator (scu-PA) (14), for which the K_m is 34 μM and the k_{cat} is 0.08 s^{-1} for a k_{cat}/K_m value of $2.35 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. Interestingly, the k_{cat} values for fibrinogen and scu-PA are similar, indicating a low turnover for tryptase with protein substrates. Comparison of the k_{cat}/K_m values of tryptase and thrombin with fibrinogen (Table 1) shows that thrombin's specificity constants are 32 and 14 times higher than tryptase for the α and β chains, respectively. These constants are probably closer in value than this comparison appears, because the thrombin values were obtained at 37 $^{\circ}\text{C}$, whereas tryptase kinetic data were obtained at room temperature (22 ± 2 $^{\circ}\text{C}$).

The inability of thrombin to clot fibrinogen after reaction with tryptase results from the cleavage at Lys²¹ in the β chain, which removes the thrombin cleavage site at Arg¹⁴ and splits the DKKREE sequence that has recently been shown to be an important polymerization site (26). A similar cleavage at Arg⁴² in the β chain by protease III of *Crotalus atrox* venom has been shown to greatly reduce thrombin-induced clotting (27). A change from a negative to a positive net charge upon removal of the fibrinopeptide B is thought to be important for fibrin polymerization (15), but tryptase removes three acidic and three basic residues, resulting in no change in the net charge at the amino terminus. While cleavage at Arg⁵⁷² of the α chain may have some effect on clotting, the C-terminal regions are not thought to be critical

to polymerization (15). Although the kinetics of β chain cleavage do not at first appear to explain the 1:1 competition results with thrombin obtained in clotting assays (Figure 5), this is due to differences in the two assays. In contrast to cleavage reactions, clot formation requires polymerization of fibrin monomers to build insoluble particles and tryptase-cleaved fibrinogen, which lacks a critical polymerization site, presumably acts as a chain terminator in the polymerization process. The concentration of fibrinogen in human plasma is approximately 18 μ M, indicating that tryptase reaching the blood would be saturated with the fibrinogen substrate, particularly considering that tryptase is fully active when released from the mast cells and that there are no known tryptase inhibitors in blood plasma. It would appear from the clotting assays (Figure 5B) in the absence of heparin that tryptase is stabilized by fibrinogen, which is consistent with our previous finding that tryptase activity binds to cartilage (6).

Human lung mast cells are about 12 μ m in diameter (28), resulting in a calculated tryptase concentration of 11 mg/mL or 355 μ M with regard to active sites, and tryptase concentrations as high as 19 nM have been observed in the serum of patients with systemic anaphylaxis (29). Although blood-clotting abnormalities have not been reported in patients experiencing anaphylaxis or having mastocytosis, mast cell deficient mice were more susceptible to thromboembolism caused by the intravenous injection of india ink (30) and to ADP-induced thrombus formation (31). Extracellular tryptase is also a common feature of advanced atherosclerotic plaques and is often associated with localized edema and stromal matrix disruption (32). Thus, mast cells may contribute to the regulation of coagulation via the release of tryptase, which would remain active in association with heparin (4, 5, 6, 33). The anticoagulant function for tryptase, resulting from fibrinogen cleavage, complements its profibrinolytic function as a activator of single-chain urinary plasminogen activator (14).

Cleavage of the RGD site in human fibrinogen α chain at amino acids 572–574 by tryptase reduced bovine endothelial cell binding by 30%, the same reduction obtained when the synthetic peptide RDGS was added to block integrin binding. Arg-Gly-Asp (RGD) is a consensus cell adhesion sequence recognized by numerous integrins on cell surfaces (34). Human fibrinogen contains RGD sites at residues 95–97 and 572–574 of the α chains. Only the site at 572–574 is likely to be exposed in a β -turn, because the 95–97 site is folded in the triple helical “coiled-coil” domain (35). Additionally, the 572–574 site was reported to be the primary site for endothelial cell adhesion based on studies using mutant recombinant fibrinogens (18). Tryptase cleavage after Arg⁵⁷², but not after Arg⁹⁵, provides additional evidence that the 572–574 RGD site is exposed and further evidence of the limited specificity of tryptase. Cleavage of the 572–574 RGD site in the α chain of fibrinogen and fibrin would prevent the binding of cells via integrins, such as $\alpha_v\beta_3$, that recognize the RGD motif (17, 18). Blocking endothelial cell binding to fibrin with antibodies to $\alpha_v\beta_3$ promotes capillary tube formation which is thought critical to angiogenesis (36), suggesting that proteolytic disruption of the 572–574 RGD site in fibrin may have a similar effect. This is supported by a recent report that tryptase stimulates the organization of endothelial cells into vascular tubes in the

Matrigel angiogenesis model system (37). Interestingly, mast cells also express $\alpha_v\beta_3$ integrins (38, 39) and degranulate in response to several substances other than allergens (28). Protease expression was a noted feature of a recently identified bone marrow-derived precursor of murine mast cells (40), and these authors suggested that mast cell proteases may play a role in tissue invasion from the blood. Therefore, it is interesting to speculate that tryptase may serve to free mast cells from fibrin and extracellular matrix proteins with cleavable RGD sites. Such a function would complement tryptase activation of the matrix metalloproteinase (13) and plasminogen systems (14), which are also thought to play important roles in angiogenesis. Tryptase has multiple functions, similar to the situations with thrombin and plasmin, and because tryptase is the predominant protein product of mast cells, understanding these functions is critical to our knowledge of mast cells' roles in normal and pathological situations.

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