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DIFFERENTIAL SPECIFICITIES OF THROMBIN, PLASMIN AND TRYPSIN WITH REGARD TO SYNTHETIC AND NATURAL SUBSTRATES AND INHIBITORS

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SUMMARY

1. Thrombin (EC 3.4.4.13), plasmin (EC 3.4.4.14) and trypsin (EC 3.4.4.4) were examined with regard to their differential specificities toward arginyl and lysyl compounds.

2. A series of eight arginyl and lysyl peptides were synthesized and, together with an arginyl and lysyl ester, were compared as substrates and inhibitors of these enzymes. The compounds include N^{α}-tosyl-L-arginylglycine and its methyl ester, N^{α}-tosyl-L-arginylsarcosine and its methyl ester, the lysine analogues of these peptides, and the methyl esters of N^{α}-tosylarginine and N^{α}-tosyllysine.

3. Both carboxy- and amino-terminal analyses were performed on fibrin digested by plasmin and trypsin and the bonds broken compared.

4. The results of these studies were consistent with the notion that thrombin has a high degree of specificity for the arginyl side chain and plasmin a preference for lysyl residues. Trypsin did not display a significant preference, hydrolyzing arginyl and lysyl bonds equally well.

INTRODUCTION

The work of many laboratories has made it clear that trypsin (EC 3.4.4.4), thrombin (EC 3.4.4.13) and plasmin (EC 3.4.4.14) (as well as certain other proteases) have descended from a common ancestral type[1,2]. Of these three, thrombin has the narrowest natural specificity, attacking only four arginylglycine bonds when clotting fibrinogen*. On the other hand, trypsin hydrolyzes virtually all arginyl and lysyl

Abbreviations: TLG, N^{α}-tosyl-L-lysylglycine; TAG, N^{α}-tosyl-L-arginylglycine; TLS, N^{α} tosyl-L-lysylsarcosine; TAS, N^{α}-tosyl-L-arginylsarcosine; TLGME, TAGME, TLSME and TASME the methyl esters of TLG, TAG, TLS and TAS, respectively; TAME, N^{α}-tosyl-L-arginine methyl ester; TLME, N^{α}-tosyl-L-lysine methyl ester; TPCK, tosyl-L-phenylalanine chloromethylketone; TATG, thioacetylthioglycolic acid.

* Thrombin has other biological activities besides the clotting of fibrinogen, such as the activation of Factor XIII[3], but the exact nature of the linkages cleaved in these ancillary situations is still unknown.

bonds when digesting proteins. Between these two extremes, plasmin hydrolyzes significantly fewer bonds during the prolonged digestion of fibrin than does trypsin under comparable circumstances[4].

We now report a systematic difference in the inhibition of thrombin, plasmin and trypsin by a series of arginyl and lysyl derivatives, as well as in the hydrolytic specificities of these enzymes towards an arginyl and lysyl ester. We have also found the same preferential specificity to extend to the digestion of fibrin by plasmin as opposed to trypsin.

The compounds synthesized and compared in the present study include the dipeptides N^{α}-tosyl-L-lysylglycine (TLG), N^{α}-tosyl-L-arginylglycine (TAG), N^{α}-tosyl-

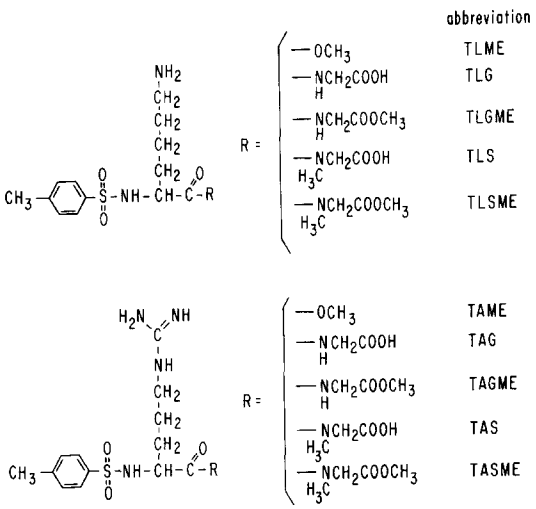


Fig. 1. Structures and abbreviations for compounds used in this study.

L-lysylsarcosine (TLS), N^{α}-tosyl-L-arginylsarcosine (TAS), and the methyl esters of these peptides (TLGME, TAGME, TLSME and TASME). In addition, N^{α}-tosyl-L-arginine methyl ester (TAME) and N^{α}-tosyl-L-lysine methyl ester (TLME), first studied by SHERRY *et al.*[5,6], were reexamined (Fig. 1).

We have found that bovine thrombin is inhibited to a much greater degree by arginyl (compared with lysyl) derivatives when catalyzing the conversion of fibrinogen to fibrin. In contrast, the fibrinolytic action of plasmin is inhibited strongly by lysyl (compared with arginyl) esters and peptide esters. Trypsin is inhibited by lysyl peptides somewhat more strongly than arginyl peptides, but equally well by the arginyl and lysyl esters examined. The arginylsarcosine peptides, which were not hydrolyzed by any of the enzymes, may be potentially useful as *in vivo* anticoagulants and/or regulators.

The preferences of thrombin for the arginine side chain and plasmin for that of lysine, (in contrast to the ability of trypsin to attack both almost equally rapidly), are also reflected in the esterolytic activities of the enzymes and, in the case of plasmin, its specificity towards fibrin. At the time a fibrin gel is rendered completely fluid (lysis time) by plasmin, a maximum of 8–9 and perhaps as few as 4, lysyl bonds per

mole of fibrin* (mol. wt. 340 000) have been broken. No significant cleavage of arginyl bonds has occurred at this time. Trypsin produces the same liquefying effect after cleaving approximately 20 equivalents per mole of fibrin—6 arginyl and 12–14 lysyl bonds.

MATERIALS AND METHODS

Nitro-L-arginine and dicyclohexyl carbodiimide were purchased from Aldrich Chemical Company. *p*-Toluene sulfonyl chloride was a product of Eastman Organic Chemicals. Sarcosine was obtained from Mann Chemical Company, glycine and N^{α}-tosyl-L-arginine methyl ester from Calbiochem, N^{ε}-benzyloxycarbonyllysine from Pierce Chemical Company, and N^{α}-tosyl-L-lysine methyl ester from Cyclo Chemical Company. All other chemicals were of reagent grade.

Parke–Davis bovine thrombin was purified according to the method of BAUGHMAN AND WAUGH[7] through the DEAE-Cellex P step and had an activity of approximately 1700 NIH units/mg protein.

Plasminogen was prepared by affinity chromatography following the procedure of DEUTSCH AND MERTZ[8], and stored as a lyophilized powder. When activated with 500 streptokinase units/CTA plasmin unit, it had a specific caseinolytic activity of 5 CTA units per absorbance unit using the assay of JOHNSON *et al.*[9]. Human Standard CTA Plasmin in 50% glycerol with an activity of 10 CTA units/ml was supplied by the American National Red Cross and was used in the esterolytic studies.

Bovine fibrinogen (Fraction I of bovine plasma obtained from the Armour Pharmaceutical Co.), was purified by the $(NH_4)_2SO_4$ fractionation procedure of LAKI[10]; it always contained more than 95% clottable protein.

Tosyl-L-phenylalanine chloromethylketone (TPCK)-treated trypsin (214 units/mg) was purchased from Worthington Biochemicals, as were carboxypeptidase B (187 units/mg), and soy bean trypsin inhibitor (5 times crystallized).

Synthesis of arginyl and lysyl peptides

N^{α}-Tosylnitro-L-arginine, N^{α}-tosyl-N^{ε}-benzyloxycarbonyl-L-lysine, glycine and sarcosine benzyl ester benzene sulfonates were prepared according to methods described in GREENSTEIN AND WINITZ[11].

The lysyl and arginyl peptides were prepared as follows: to a vigorously stirring solution of methylene chloride and 1 equiv of either glycyl or sarcosyl benzyl ester benzene sulfonate, was added 1.5 equiv of 5% $NaHCO_3$. The solution was stirred at room temperature for 0.5 h, the methylene chloride phase separated, dried with Na_2SO_4, and filtered.

* Fibrinogen molecules (mol. wt. 340 000) are dimeric, consisting of two half-molecules of 170 000 mol. wt. each. Each half-molecule is made up of three non-identical polypeptide chains designated α, β and γ. Before removal of the fibrinopeptides by thrombin, the amino terminals of bovine fibrinogen are glutamic acid, pyrrolidone carboxylic acid, and tyrosine. After removal of the fibrinopeptides (A and B from the α and β chains, respectively), glycine becomes the new amino terminal of both the α and β chains, while tyrosine remains the amino terminal of the γ chain. The term "fibrin monomer" has traditionally referred to the entire fibrinogen molecule stripped of its fibrinopeptides, but before polymerization (or after depolymerization by fibrin-dispersing solvents such as concentrated urea solutions). In this paper all of our analyses are based on the molecular weight of the parent molecule and are expressed as moles per 340 000 mol. wt.

The above solution was added to 1 equiv of either N^{α}-tosylnitro-L-arginine or N^{α}-tosyl-N^{ε}-benzyloxycarbonyl-L-lysine in tetrahydrofuran, and a 10% excess of dicyclohexyl carbodiimide was added slowly with stirring. The reaction mixture was stirred at room temperature for 4 h, or in the case of the sarcosyl derivatives, overnight. A few drops of acetic acid were added to stop the reaction and the solution filtered to remove the dicyclohexyl urea.

The methylene chloride solution was washed with water, 5% $NaHCO_3$, 2% HCl, and water, and dried. Yields ranged from 70 to 90%; intermediate products were not crystallized.

The intermediates were hydrogenated with 10% Pd–charcoal in acetic acid–water (90:10, v/v) for 4 h, or, in the case of the arginine peptides, until H_2 was no longer taken up. The solution was filtered and dried down under vacuum. Tosylarginylsarcosine acetate was converted to its hydrochloride salt to permit crystallization. Peptide yields were between 70 and 80%; after repeated crystallization all showed single spots on silica gel thin-layer chromatography (chloroform–methanol–acetic acid (75:20:5, v/v/v)).

N^{α}-Tosyl-L-lysylglycine·H_2O. Crystallized from water, m.p. 237–238° (lit.[7] 242–244°). (Found: C, 48.6; H, 6.7; N, 11.2; S, 8.5%. $C_{15}H_{23}O_5N_3S_1 \cdot H_2O$ requires C, 48.0; H, 6.7; N, 11.2; S, 8.5%.)

N^{α}-Tosyl-L-lysylsarcosine·H_2O. Crystallized from water–acetone, m.p. 169–170°. (Found: C, 49.3; H, 6.9; N, 10.8; S, 8.1%. $C_{16}H_{25}O_5N_3S \cdot H_2O$ requires C, 49.3; H, 6.9; N, 10.7; S, 8.2%.)

N^{α}-Tosyl-L-arginylglycine. Crystallized from methanol, decomp. 245–255°. (Found: C, 46.7; H, 5.9; N, 18.1; S, 8.3%. $C_{15}H_{23}O_5N_5S$ requires C, 46.7; H, 5.9; N, 18.1; S, 8.3%.)

N^{α}-Tosyl-L-arginylsarcosine·HCl, H_2O. Crystallized from methanol–ether, m.p. 142–143°. (Found: C, 42.1; H, 6.1; N, 15.3; S, 7.0; Cl, 8.0%. $C_{16}H_{25}O_5N_5S \cdot HCl$, H_2O requires C, 42.3; H, 6.1; N, 15.4; S, 7.0; Cl, 7.9%.)

The methyl esters were formed by reacting the peptides with 2 equiv of thionyl chloride in methanol, letting the solution stand for 1 h at 40° and 20 h at room temperature, and then evaporating the solution to dryness. The esters were crystallized several times from ether–ethanol or methanol solutions. TLSME did not crystallize, but could be precipitated with ethyl acetate as a hydroscopic amorphous powder. All showed major spots on thin-layer chromatography corresponding to the esters and very faint ones from the free acid. All the sarcosine peptides developed a characteristic pink color on silica gel thin-layer sheets when exposed to ultraviolet light.

TAGME·HCl	m.p. 148–149°
TASME·HCl	m.p. 204–205°
TLGME·HCl	m.p. 143–144°
TLSME·HCl	unable to crystallize

Enzymatic hydrolysis of esters

The hydrolysis of the ester bond in N^{α}-tosylarginine and N^{α}-tosyllysine methyl esters (TAME and TLME) by plasmin, trypsin and thrombin was followed by titration on a Sargent pH-stat. In a typical run, 4.0 ml of an appropriate amount of ester dissolved in 0.1 M KCl were titrated with 5 mM NaOH at 30°; the solutions were

maintained at pH 8.0 for thrombin and trypsin and at pH 7.5 for plasmin. The trypsin experiments were performed by adding 0.025 ml of a 0.05 mg/ml trypsin solution to the preparation. Plasmin determinations were made by adding 0.25 CTA unit of Human Standard Plasmin for each analysis. In the case of thrombin we arbitrarily set our unit as the amount of enzyme which hydrolyzed 0.1 μmole TAME per min, starting with an initial concentration of $9.1 \cdot 10^{-3}$ M TAME. For each TAME hydrolysis we used 1.45 of these units; for the TLME determinations we used 7.25 units each time.

Enzymatic hydrolysis of synthetic peptides

Paper electrophoresis was used to separate hydrolysis products produced by the hydrolytic action of thrombin, plasmin and trypsin on the various synthetic peptides. This procedure was used primarily as an initial qualitative screening of the peptide digests to determine which peptides were indeed susceptible to enzymatic hydrolysis. The concentrations of enzymes used were as follows: 10 CTA units of plasmin per ml 0.005 M phosphate buffer, pH 7.4; 1 mg/ml trypsin in 0.2 M Tris–HCl, pH 7.3; and approximately 1000 NIH units of Parke–Davis thrombin in 0.2 M Tris–HCl, pH 7.3. Peptide digestion was carried out by mixing 0.01 ml of an enzyme solution with 0.1 ml of 0.01 M peptide solution. Aliquots (0.01 ml) were withdrawn at 1-h intervals over a period of 4 h and applied directly to electrophoresis paper. High-voltage electrophoresis was performed at pH 2 (formic acid-acetic acid–water (2:8:90, v/v/v)) on Whatman No. 1 paper for 45 min at 2 kV; the papers were stained with 0.25% ninhydrin in acetone. After this qualitative screening, certain of those peptides found to be hydrolyzed were studied using the quantitative ninhydrin method of HIRS *et al.*[13]; the same concentrations of enzyme and peptides were used in both the qualitative and quantitative experiments.

Clotting and lysis inhibition studies

Thrombin inhibition was determined by the abilities of the various compounds to prolong the transformation of fibrinogen into fibrin. Bovine fibrinogen solutions (0.27% in 0.06 M NaCl plus 0.05 M phosphate buffer, pH 7.3) were pipetted out in 0.3 ml lots, 0.1 ml of inhibitor solution of appropriate concentration added, and finally 0.1 ml of a thrombin solution (approx. 0.3 NIH unit) blown in. Clotting times were taken as the time when a gel remained rigid when its tube was tilted 90°.

The effectiveness of these compounds as inhibitors of plasmin and trypsin was determined by observing the delay in the lysis of fibrin gels. A 0.2-ml aliquot of a 0.18% bovine fibrinogen solution (0.073 M NaCl *plus* 0.015 M phosphate buffer, pH 7.4) was mixed with 0.1 ml of inhibitor solution. Purified thrombin (approx. 20 NIH units) in 0.1 ml of the same buffer was added simultaneously with either 0.1 ml of a 0.05 mg/ml trypsin solution or 0.1 ml of a 0.5 CTA unit/ml streptokinase-activated plasmin solution. Lysis time was taken as the complete disappearance of the gel.

Carboxy-terminal determinations

Carboxy-terminal lysine and arginine residues exposed during fibrin liquefaction by plasmin or trypsin were determined by a method employing carboxypeptidase B. Samples (0.5 ml) containing 5–6 mg fibrin were digested with plasmin or trypsin,

proteolytic activity being stopped at appropriate intervals by the addition of 0.1 ml soy bean trypsin inhibitor solution (1.5 mg/ml 0.1 M NH_4HCO_3). Subsequently 6 units of carboxypeptidase B in 0.005 ml 0.1 M NH_4HCO_3 were added and the mixtures incubated at 37° for 60 min, after which absolute ethanol was added to a final concentration (v/v) of 80%. The samples were chilled on ice to aid the precipitation of protein and then centrifuged; the precipitates were washed once with 80% ethanol and the supernatant fluids combined and dried. The residues were dissolved in pH 2.2 buffer and analyzed on the short column of a modified (6.6 mm cuvette) Spinco Model 120B amino acid analyzer. Free arginine and lysine resulting from the action of plasmin or trypsin alone (as opposed to the carboxypeptidase B) were determined in the same way except for the omission of the carboxypeptidase.

Amino-terminal determinations

Amino-terminal analyses were performed using thioacetylthioglycolic acid (TATG) as an activating agent for the removal of amino-terminal residues[16]. The advantage of this method lies in the fact that most of the naturally occurring amino acids can be regenerated from the thiazolinones which are released during the process and measured directly on an amino acid analyzer. Threonine, serine and tryptophan are troublesome exceptions, however, and glutamine and asparagine are converted into glutamic acid and aspartic acid during the hydrolytic step.

Most determinations were made on 10–12 mg of fibrinogen or fibrin. Typically, 1.0 ml of a fibrinogen solution or fibrin lysate was mixed with 1.0 ml of pyridine and 20 mg TATG added. The pH was adjusted to 9.0–9.5 with triethylamine and the mixture set at 40° for 60 min. Preparations were then washed with benzene 3 times and acetone 3 times. After aspiration of the final traces of acetone, about 1 ml of water was added and the protein residue freeze-dried overnight. Cleavage was achieved with 0.3 ml of trifluoroacetic acid at 40° for 20 min. The resulting thiazolinones were extracted with ether (three extractions of about 2 ml each), mixed with 0.1 ml of 6 M HCl, and the ether evaporated with a stream of nitrogen. The residues were hydrolyzed by the addition of 0.2 ml more 6 M HCl, sealing the tubes and heating at 126° for 4 h. Samples were dried *in vacuo* and then analyzed on a Spinco Model 117 Automatic Analyzer employing a single column procedure. A slight modification of this method was used on occasion in order to insure that the amino-terminal residues of small peptides, including the fibrinopeptides released by thrombin and even any free lysine released by plasmin, were not lost during the washing procedures. In the alternate method all acetone washes were omitted; instead, the final acid hydrolysates were extracted with ether to remove yellow side products.

The efficiency of the TATG method on native bovine fibrinogen averaged about 60% of theoretical in terms of the actual moles of amino acids recovered. Because the amounts of amino-terminal tyrosine did not vary during the course of fibrin digestion, all amino acid values were normalized to a theoretical 2.0 moles of tyrosine per 340 000 mol. wt.

RESULTS

Enzymatic hydrolysis experiments with arginyl and lysyl esters and peptides

The hydrolysis rates of the methyl esters, TAME and TLME, for thrombin,

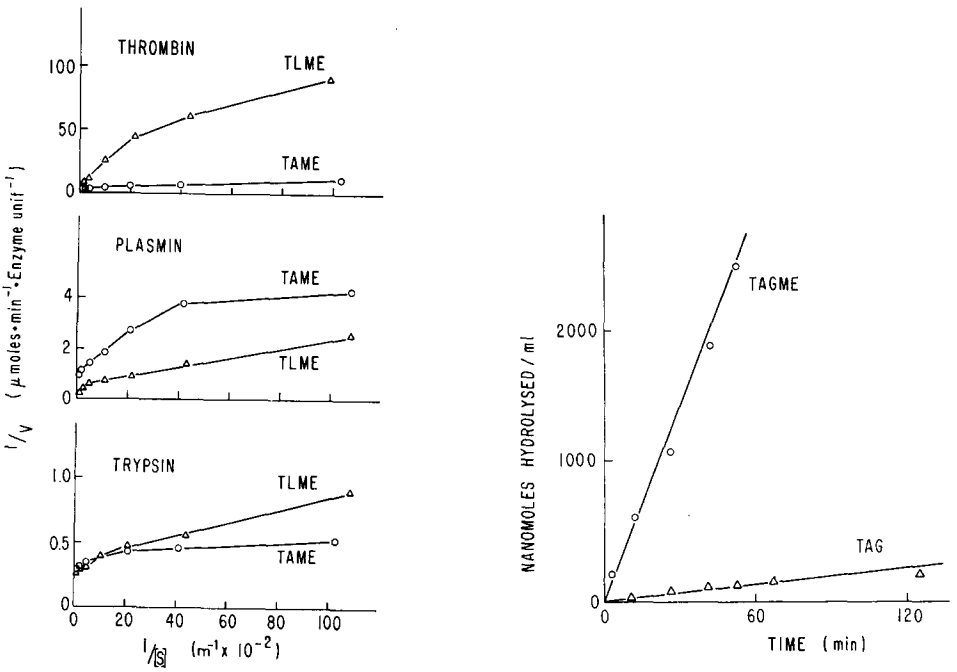


Fig. 2. Hydrolysis of TAME and TLME by thrombin, plasmin and trypsin. Rates were determined at pH 8.0 for thrombin and trypsin, and pH 7.5 for plasmin. All measurements made in 0.1 M KCl at 30°. All points are the average of two or more observations.

Fig. 3. Hydrolysis of TAGME and TAG by trypsin. Peptide concentration, $9.1 \cdot 10^{-3}$ M; trypsin, 0.09 mg/ml; pH 7.3; temp., 27°. The reaction progress was followed by quantitative ninhydrin determinations.

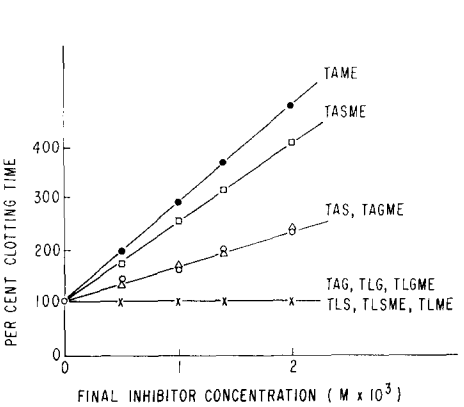
plasmin and trypsin are depicted using Lineweaver–Burk plots in Fig. 2. All three enzymes display the phenomenon of "substrate activation"[14,15] with both substrates, *i.e.* a faster rate of hydrolysis is observed at high substrate concentrations than one would expect from extrapolation of data obtained at lower concentrations. These results obviously complicate the determination of a realistic K_m or v_{max}. It can be seen, however, that thrombin hydrolyzes TAME much more rapidly than it does TLME over a broad concentration range. Plasmin, on the other hand, hydrolyzes TLME considerably faster than it does TAME in this same concentration range. Trypsin exhibits considerably less preference in hydrolyzing these two esters, attacking them at approximately equal rates at concentrations between $1 \cdot 10^{-2}$ and $5 \cdot 10^{-4}$ M (Fig. 2).

Under our experimental conditions, neither plasmin nor thrombin significantly hydrolyzed any of the synthetic dipeptides. In contrast, trypsin hydrolyzed both the arginyl and lysyl glycine peptides (TAG and TLG) and their methyl esters (TAGME and TLGME). Quantitative studies revealed that TAGME was hydrolyzed about 20 times faster than TAG at the concentrations employed (Fig. 3). As anticipated, not even trypsin hydrolyzed either of the sarcosine peptides (TAS and TLS) or their methyl esters (TASME and TLSME).

Inhibition of fibrin formation and fibrinolysis by synthetic arginyl and lysyl compounds

Fibrin gel formation, induced by the thrombin-catalyzed removal of fibrinopeptides, is strongly inhibited by arginyl (compared with lysyl) derivatives (Fig. 4). The lysyl compounds were uniformly ineffective in delaying clot formation. The N^{α}-tosylarginylsarcosine derivatives are particularly potent inhibitors, TASME being approximately 80% as effective as TAME in this regard. The arginyl free acid peptides, TAS and TAG, were significantly less effective than their methyl esters; in fact, TAG showed virtually no anticlotting activity.

The distinction between arginyl and lysyl compounds with regard to the inhibition of fibrin lysis is not as dramatic as that found for clot inhibition, but a definite pattern of preferential lysine specificity by plasmin is apparent (Fig. 5). Thus, N^{α}-tosyllysine methyl ester (TLME) is approximately twice as effective as its arginyl analogue (TAME) in prolonging the time it takes to render a fibrin gel completely



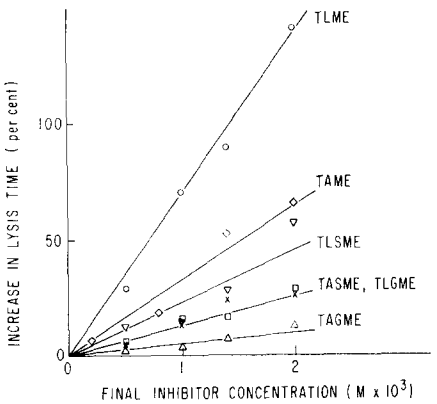


Fig. 4. Inhibition of thrombin-catalyzed fibrin gel formation. Each point is the mean of four determinations. With no inhibitor present, clotting occurred at 23 sec. TAG, TLG, TLGME, TLS, TLSME and TLME did not exhibit detectable inhibition.

Fig. 5. Inhibition of plasmin-catalyzed fibrin gel lysis. The peptides with free carboxyl groups had negligible inhibitory activity except for TAS, which was equivalent to TASME and TLGME. Each point is the mean of four determinations. With no inhibitor present, lysis occurs at 10.2 ± 0.6 min.

fluid. The various peptides did not inhibit lysis as well as either TLME or TAME, but the lysyl peptide esters (TLSME and TLGME) were better inhibitors than the corresponding arginyl compounds (TASME and TAGME) (Fig. 5).

The digestion of fibrin gels by trypsin was inhibited equally well by TAME and TLME (Fig. 6). All of the peptide derivatives were less effective than these simple esters, and no clear-cut preference for arginyl or lysyl derivatives was apparent (Fig. 6).

Carboxy-terminal amino acids exposed by trypsin and plasmin digestion of fibrin

Confirmation of the natural specificity of plasmin for lysyl bonds during fibrinolysis was obtained by studying the carboxy-terminal groups exposed during the lytic process. At the time a fibrin gel is just liquefied (lysis time), 8–9 moles of lysine and none of arginine have been exposed per mole of fibrin (mol. wt. 340 000) (Fig. 7).

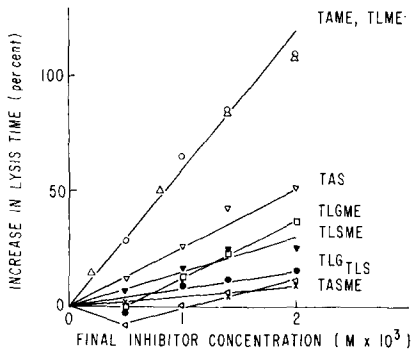
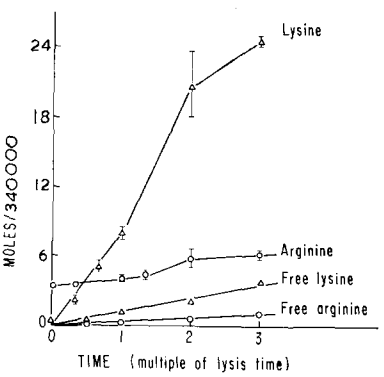


Fig. 6. Inhibition of trypsin-catalyzed fibrin gel lysis. TAG and TAGME produced no delay in lysis. With no inhibitor present, lysis occurs at 9.5 ± 0.5 min. Each point is the mean of four determinations.

In contrast, trypsin was able to produce the same liquefying effect only after splitting 12–14 lysyl bonds and 6 arginyl bonds (Fig. 8).

The preference of plasmin for lysyl bonds, compared with arginyl bonds, continues well after the moment of liquefaction. Hence, at 3 times the lysis time only 2 new equivalents of arginine have been rendered accessible to the carboxypeptidase B by plasmin digestion, compared with more than 20 equivalents of lysine. At this same point (3 × lysis), trypsin has exposed approximately 12 arginines and 30 lysines per mole of fibrin (Fig. 8).



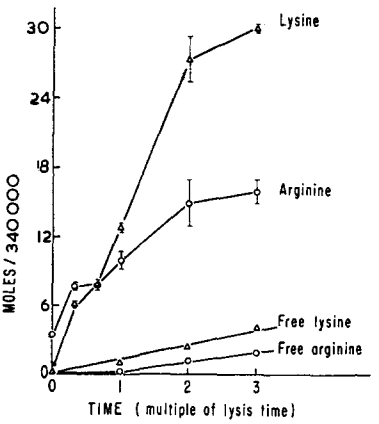


Fig. 7. Carboxy-terminal amino acids exposed by the digestion of bovine fibrin with plasmin. For the curves labeled Arginine and Lysine, amino acid analyses were performed on the supernatant of fibrin samples digested first with plasmin, then with carboxypeptidase B. The amount of free arginine and lysine released by plasmin were found by eliminating the carboxypeptidase B step.

Fig. 8. Carboxy-terminal amino acids exposed by the digestion of bovine fibrin with trypsin. The same experimental conditions were employed as those used with plasmin (Fig. 7).

TABLE I

NEW AMINO-TERMINAL RESIDUES EXPOSED BY PLASMIN DURING LIQUEFACTION OF FIBRIN

Bovine fibrinogen has 2 moles each of glutamic acid, pyrrolidone carboxylic acid and tyrosine at its amino terminals, for every 340 000 mol. wt. Pyrrolidone carboxylic acid, having no α-amino group, is not detected by these procedures. During the transformation of fibrinogen into fibrin, thrombin releases two pairs of peptides (fibrinopeptides) from the glutamic acid and pyrrolidone carboxylic acid chains, respectively, producing four new glycine amino terminals. The actual yields of the TATG method averaged about 60% of theoretical for the tyrosine and glutamic acid in fibrinogen. Because no new tyrosine endgroups were exposed during the clotting or fibrinolysis, it was found convenient to normalize all data to a theoretical 2.0 moles tyrosine per 340 000 mol. wt. Zero-time values were determined by adding pyridine to the fibrinogen solution before the addition of thrombin and plasmin; lysis time measurements were established by adding pyridine to lysing clot preparations at the moment they became completely fluid. The zero-time results are the average of three determinations; the lysis time results are the average of two determinations.

Amino acid	*Residues per 340 000 mol. wt.*		
	Zero time	*Lysis time*	*Δ*
Aspartic acid	0.4	1.0	0.6
Glutamic acid	2.1	2.4	0.3
Glycine	0.4	(4.9)*	0.5
Alanine	–	2.1	2.1
Methionine	–	1.8	1.8
Tyrosine	2.0	2.0	–
Lysine	–	1.4	1.4
Total residues	4.9	(15.6)*	6.7

* Includes 4 moles of glycine exposed by thrombin during clotting phase.

Amino-terminal amino acids of fibrin exposed by plasmin at lysis time

In order to determine if the lysine residues exposed by plasmin during the liquefaction process were originally bonded to a variety or just a few kinds of amino acids, we also examined the new amino-terminals produced during the lytic phenomenon. For every mole of fibrin (mol. wt. 340 000) approximately 2 moles each of alanine and methionine were revealed by lysis time (Table I). In addition, lesser amounts of aspartic acid, glutamic acid, glycine and lysine were also unmasked during the lysis process. The amount of lysine found (approx. 1.4 moles) was about equivalent to the free lysine found upon direct analysis of plasmin-digested fibrin (Fig. 7).

It should be noted that the sum of the amino-terminal endgroups exposed during lysis (about 6–7 per 340 000) is significantly less than the number of carboxy-terminal end groups exposed (about 8–9 per 340 000). There are at least two possible explanations for these results. If the bonds broken by plasmin are actually of the sequence ...Lys–Lys–amino acid..., then cleavage of the Lys–amino acid bond would make 2 moles of lysine available to carboxypeptidase B for those bonds. This explanation would also be consistent with the finding of fractional amounts of free lysine in the lysis preparations. Alternatively, some of the new amino terminals exposed may involve those amino acids which give poor yields with the TATG method (*viz.* threonine, serine or tryptophan).

DISCUSSION

The data reported in this study support general notions that thrombin has a

natural preferential specificity for the arginyl side chain, plasmin for the lysyl group, and trypsin an approximately equal affinity for both. In the past, these suppositions have been based on several lines of evidence. In all vertebrate species examined to date, for example, only certain arginylglycine bonds are broken by thrombin when it catalyzes the release of fibrinopeptides from fibrinogen[17]. Similarly, thrombin was found to cleave only arginyl bonds in the few cases where it has been used as a general protease[18,19]. The specificity of plasmin for the lysyl side chain has been inferred from the widely observed inhibitory effect of ε-aminocaproic acid, a lysine analogue, on fibrinolysis[20]. Furthermore, amino acid sequence determinations performed on plasmin-produced fragments of sulfitolyzed fibrinogen, have yielded oligopeptides containing internal arginines, but, for the most part, having lysine at their carboxy-terminal ends[21]. The ability of trypsin to hydrolyze peptide and ester bonds adjacent to both arginyl and lysyl side chains has been known for many years[22]. Our studies on the rates of hydrolysis by these enzymes and the differential inhibitory effects of the various synthetic compounds on these proteases, combined with the analysis of newly exposed carboxy-terminal amino acids after digestion of fibrin with plasmin and trypsin, extend and fully support these general observations.

Enzymatic hydrolysis of arginyl and lysyl esters

The kinetics of ester hydrolysis we observed (Fig. 2) are similar, in many respects, to those reported by SHERRY *et al.*[5,6] for the hydrolysis of N^{α}-tosylarginine methyl ester (TAME) and N^{α}-tosyllysine methyl ester (TLME) by thrombin, plasmin and trypsin. The values of maximum hydrolysis rate (v_{max}) reported in these earlier works indicated that human plasmin hydrolyzes lysyl esters more rapidly than arginyl esters, while trypsin cleaves both at approximately equal rates[6]. Thrombin, on the other hand, was found to have a v_{max} for TLME more than twice that for TAME, and a Michaelis constant (K_m) for TLME approximately 5 times greater than for TAME[5]. Our results clearly show, however, that TLME is a much poorer substrate for thrombin than TAME over a wide concentration range.

This apparent difference in results may be explained by the non-linear relationship between the reciprocals of velocity and substrate concentration, the so-called "substrate activation" effect, which is evident for both esters in the case of all three enzymes. Substrate activation, a rate of hydrolysis at high substrate concentrations greater than expected from extrapolation of rates at low concentrations, has been observed to occur with trypsin[14,15] and chymotrypsin[23], among other enzymes. It has been suggested by CURRAGH AND ELMORE[24] that thrombin also displays this effect.

The values of v_{max} and K_m derived by SHERRY *et al.*[5,6], were obtained from hydrolysis rates measured at relatively high ester concentrations (0.05 to 0.005 M). At lower concentration ranges ($2 \cdot 10^{-3}$ to $0.5 \cdot 10^{-3}$ M), thrombin has a K_m for TAME approximately 20 times smaller than for TLME, plasmin a K_m for TLME two times smaller than for TAME, and trypsin a K_m of nearly equal value for both esters. It should be noted that the concentrations used in our subsequent inhibition studies fall into the latter range.

Enzymatic hydrolysis of arginyl and lysyl peptides

The synthetic peptides were hydrolyzed by trypsin at rates in accord with those obtained by other investigators in analogous studies[25,26]. Trypsin, for example,

hydrolyzes N^{α}-tosyl-L-arginylglycine methyl ester (TAGME) 20 times faster than the free acid peptide TAG (Fig. 3). This observation is not unexpected since digestion by chymotrypsin is known to be inhibited by a free carboxyl group adjacent to the hydrolyzable bond of a substrate[25]. The rate of hydrolysis of TAGME by trypsin is 800 times slower than that of the ester TAME under the same conditions. This observation is in agreement with the finding that trypsin cleaves ester bonds much more rapidly than analogous amides[26].

Under the conditions employed in the present study, neither thrombin nor plasmin hydrolyzed any of the synthetic peptides. The finding that thrombin does not catalyze the hydrolysis of TAG and TAGME is in accord with previously published reports[27,28]. In a recent study by ANDREATTA *et al.*[29], however, synthetic oligopeptides with amino acid sequences similar to those found around the arginylglycine bond of bovine fibrinopeptide A have been reported to be cleaved after prolonged digestion with thrombin.

Inhibition of proteolytic activity by arginyl and lysyl derivatives

The differential inhibition of thrombin, plasmin and trypsin by the various peptides and esters followed patterns, for the most part, predictable from the natural proteolytic specificity of the enzymes. Thus, the thrombin-catalyzed conversion of fibrinogen to fibrin was inhibited only by the arginyl compounds and more strongly by those having blocked amino and carboxy groups (Fig. 4). Similarly, plasmin was found to be inhibited to a greater degree by the lysyl peptides and lysyl ester than by the corresponding arginyl analogues (Fig. 5). The lysis of fibrin gels by trypsin (Fig. 6) is inhibited equally well by the arginyl and lysyl esters (TAME and TLME), but the lysyl and arginyl peptide inhibition pattern was not easily interpreted. In this regard, TAS was more effective than TLS, but TLSME was better than TASME.

It has previously been reported[27] that N^{α}-tosyl-L-arginylglycine (TAG) is a good inhibitor of fibrin gel formation, but our experiments do not confirm this observation. On the other hand, the ability of the arginyl ester, TAME, to inhibit clot formation agrees well with similar observations by SHERRY *et al.*[5].

N^{α}-Tosyl-L-arginylsarcosine methyl ester (TASME) proved to be the best thrombin inhibitor of the peptides examined in this study. Sarcosine, which is structurally analogous to glycine, has previously been shown to form peptide bonds which are resistant to hydrolysis by carboxypeptidase[30]. The stability of TASME to attack by the three enzymes tested, combined with the high affinity displayed by thrombin, suggests that TASME may be a useful *in vivo* thrombin inhibitor and/or regulator. A full description of the effects of these lysyl and arginyl compounds on the overall process of blood coagulation will appear elsewhere[31].

Nature of the bonds broken by plasmin during fibrinolysis

For a more quantitative appraisal of the preferential specificities of plasmin and trypsin for lysyl and/or arginyl side chains, the appearance of these amino acids at newly exposed carboxy-terminal positions was followed during the course of fibrinolysis. Trypsin was found to hydrolyze both arginyl and lysyl bonds, 6 arginyl and 12–14 lysyl equivalents per mole of fibrin (mol. wt. 340 000) having been exposed by the time of lysis (Fig. 8). These values do not include the four arginyl residues of the two pairs of fibrinopeptides released by thrombin. It should be pointed out that the

number of arginine residues in fibrin is only about three-fourths that of lysine[33,34].

The preferential specificity of plasmin for lysyl residues during fibrinolysis is striking, only lysyl bonds having been broken by the time of lysis (Fig. 7). The data indicate that a maximum of 8–9 lysine bonds per mole of fibrin (mol. wt. 340 000) are all that have to be broken to liquefy a fibrin gel.

In fact, our quantitative amino-terminal analyses, which are in good qualitative agreement with those of previous investigators[35,36], suggest that the number of key bonds split during fibrinolysis may be as few as 4 per mole of fibrin (mol. wt. 340 000). This minimum number is based on the supposition that the fractional amounts of aspartic acid, glutamic acid, glycine and lysine found are the result of less specific cleavage than is involved in the rupture of the two pairs of lysylalanine and lysylmethionine bonds. It would be expected that these latter bonds are symmetrically located in the dimeric halves of each parent molecule, presenting an interesting analogy to the disposition of the two pairs of fibrinopeptides in fibrinogen.

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