Mechanism of Action of Thrombin on Fibrinogen, I. 
Synthesis of Fibrinogen-like Peptides, and Their 
Proteolysis by Thrombin and Trypsin

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ABSTRACT In a study of the action of thrombin on fibrinogen and a comparison of this enzyme with trypsin, several fibrinogen-like oligopeptides were synthesized. The hydrolysis of the arginyl-glycine bond in these peptides, by both of these enzymes, is examined and compared.

Bovine fibrinogen has a molecular weight of 340,000 (1), and consist of three chains $\alpha(A)$, $\beta(B)$, and $\gamma$ (2–6) in the dimeric form $[\alpha(A)\beta(B)\gamma]_2$. In the blood clotting process, thrombin (EC 3.4.4.13) transforms fibrinogen into fibrin monomer (1), which subsequently forms the fibrin clot. When thrombin acts on fibrinogen, the N-terminal portions of the $\alpha(A)$ and $\beta(B)$ chains, called fibrinopeptide A and B, respectively, are split off (7–10). The release of fibrinopeptide A from fibrinogen seems to be of greater importance for fibrin formation than that of fibrinopeptide B. For example, fibrinopeptide A is released faster than the B-peptide from bovine fibrinogen (9); in addition, the rate of release of the A-peptide parallels the rate of formation of fibrin, whereas the rate of release of fibrinopeptide B becomes maximal when the formation of fibrin is complete (11). Furthermore, the enzyme reptilase, which hydrolyzes only the $\alpha(A)$ chain, nevertheless causes the clotting of fibrinogen (12).

The action of thrombin on fibrinogen is particularly remarkable because of its high specificity. Only four peptide bonds in fibrinogen are hydrolyzed by thrombin, i.e., the arginyl-glycine bonds linking the fibrinopeptides to the rest of the molecule (7–10). However, thrombin does not cleave the Arg-Gly bond in the isolated B-chain of insulin or in intact insulin itself (13), nor the Arg-Ala bond in glucagon (14). The two Arg-Gly bonds in hen egg white lysozyme have also been reported to be unaffected by thrombin (15).

The specificity of thrombin cannot be attributed to the three-dimensional native structure of fibrinogen, since thrombin can also release fibrinopeptides A and B from the separated $\alpha(A)$ and $\beta(B)$ chains (16). Nor can the specificity be ascribed to the limited sequence of amino acids around the active site, since the same sequence occurs in several enzymes (thrombin, trypsin, chymotrypsin, and elastase) of different specificity (17, 18). Thrombin resembles trypsin (EC 3.4.4.4) in its action at arginyl bonds but, whereas both enzymes will cleave the same Arg-Gly bonds in fibrinogen, trypsin will split all the 376 peptide bonds in fibrinogen that follow basic groups (arginine and lysine), although not at the same rate (19, 20); on the other hand, the action of thrombin seems confined essentially to specific Arg-Gly bonds of fibrinogen. Also, both thrombin and trypsin will cleave esters such as tosyl-arginine methyl ester (TAME) (21, 22). The primary purpose of this series of papers is, thus, to understand the mechanism of action of thrombin on fibrinogen and, at the same time, to account for the differences in specificity between thrombin and trypsin.

It has been suggested that the reason for the narrow specificity of thrombin may lie in the primary sequence of the fibrinopeptides (23), especially fibrinopeptide A. This suggestion has arisen mainly from the fact that fibrinopeptide A has changed very little during mammalian evolution (24), as well as from the fact that the fibrinopeptides and some synthetic peptide analogs of fibrinopeptide A act as inhibitors of thrombin (9, 25, 26).

Rather than limit ourselves only to the fibrinopeptides, we have considered the possibility that the sequence of amino acids on both sides of the Arg-Gly bond may be responsible for the limited specificity of thrombin. This reasoning follows from the work of Berger et al. (27–29) on the specificity of action of papain and elastase. Further, for the reasons stated above, we have confined our initial work to the $\alpha(A)$ chain. Therefore, we have synthesized a number of small peptides containing the Arg-Gly bond of the $\alpha(A)$ chain of bovine fibrinogen, and have compared the action of thrombin and trypsin on them.

The amino acid sequence of bovine fibrinopeptide A is reported (30) to be:

H-Glu-Asp-Gly-Ser-Asp-Pro-Pro-Ser-Gly-Asp-Phe-Leu-Thr-
Glu-Gly-Gly-Val-Arg-OH

In addition, the initial portion of the N-terminal sequence of the $\alpha$ chain of bovine fibrin monomer is thought (31) to be: H-Gly-Pro-Ala-OH. We have, therefore, synthesized oligopeptides with alanine amide as the C-terminal group, starting with the tripeptide H-Gly-Pro-Ala-NH₂ and continuing through the octapeptide H-Gly-Gly-Gly-Val-Gly-Pro-Ala-NH₂. We used the purified peptides as substrates for purified thrombin and trypsin, respectively, to examine the
variability among these peptides and enzymes with respect to
the hydrolysis of the Arg-Gly bond, in which H-Gly-Pro-Ala-
NH₂ is released as one of the products in all cases.

MATERIALS AND METHODS
Preparative aspects
The solid-phase technique (32-34), essentially that of Mar-
shall and Merrifield (34), was used for the synthesis of the fol-
lowing peptide amides related to the a(A) chain of bovine
fibrinogen:

H-Gly-Pro-Ala-NH₂ (I)
H-Arg-Gly-Pro-Ala-NH₂ (II)
H-NO₂Arg-Gly-Pro-Ala-NH₂ (III)
H-Val-Arg-Gly-Pro-Ala-NH₂ (IV)
H-Gly-Val-Arg-Gly-Pro-Ala-NH₂ (V)
H-Gly-Gly-Val-Arg-Gly-Pro-Ala-NH₂ (VI)
H-Gly-Gly-Gly-Val-Arg-Gly-Pro-Ala-NH₂ (VII)

Boc-alanine was esterified to chloromethyl-copoly(styrene-
2% divinylbenzene), giving Boc-alanyl resin. The Boc-amino
acids were then added in cycles. The cycle for each amino acid
consisted of removal of the Boc group by 1 N HCl in acetic
acid, neutralization of the resulting hydrochloride with tri-
ethylamine in CHCl₃, followed by coupling of the next Boc-
amino acid to the free base, with dicyclohexykarbodiimide as
the condensing agent. An aqueous solution of the triethyl-
ammonium chloride, collected after each neutralization step,
was titrated for chloride ion by the Volhard method. The re-
sults of these titrations gave a measure of the extent of the
reaction in each cycle.

The stepwise synthesis was first carried through two cycles,
with Boc-proline and Boc-glycine, giving a tripeptide resin
compound. Part of this tripeptide resin was withdrawn for
later liberation of the tripeptide H-Gly-Pro-Ala-NH₂. The
remainder was carried through one cycle at a time (with with-
drawals after each cycle) up to the octapeptide resin.

The protected peptide chains were cleaved from the resin
by ammonolysis of the benzyl ester linkage through which the
peptides are attached to the polymer support (35); this gave
the protected oligopeptides NO₂Arg-Gly-Pro-Ala-NH₂ etc.
The N-terminal amino group was free in all intermediates
withdrawn. The remaining protecting group, the nitro group
of arginine, was removed by catalytic hydrogenation over a
freshly prepared palladium catalyst. The course of hydrogena-
tion was monitored by thin-layer chromatography.

The crude preparations (I-VII) all contained one major
component and 1-2 minor contaminants, which were then
removed by column chromatography on carboxymethyl-cellu-
lose with an ammonium acetate-pH gradient. The fractions
common to the major peak were pooled and lyophilized.

Homogeneity of the purified products was determined by
chromatography on thin layers and paper in several systems.
Acid hydrolysis showed the expected amino acid composition,
as determined with a Technicon amino acid Autoanalyzer.
Digestion (36) with aminopeptidase M, followed by analysis in
the same manner as for the acid hydrolysatate, gave the con-
stituent amino acids in the expected amounts and ratios
together with the dipeptide H-Gly-Pro-OH, which was found
to be totally resistant towards this enzyme under the condi-
tions used. Complete liberation of the C-terminal alanine, as
well as one mole of ammonia from the C-terminal amide, may
have arisen because of the possible presence of a carboxypepti-
dase as well as other contaminants in the commercial amin-
peptidase M preparation.

Purification of thrombin and trypsin
Bovine prothrombin from Sigma Chemical Co. (Lot 75B-8200)
was partially purified by isoelectric precipitation, as described
by Ingwall and Scheraga (37). The precipitates that showed
the prothrombin band in polyacrylamide gel electrophoresis
were pooled and activated to thrombin in 25% citrate. Sam-
ple were taken every few hours and tested for thrombin
activity by clotting tests until no increase in clotting activity
was observed. The resulting thrombin solutions were sub-
dsequently purified on BioRex 70 (38) and DEAE-Sephadex
columns. The purified enzyme preparations traveled homo-
geously in polyacrylamide gel electrophoresis. Esterase
activity towards TAME was determined by the method of
Ehrenpreis and Scheraga (39).

Bovine trypsin (Worthington, lyophilized) was purified by
precipitation of the denatured fraction with 1 M NaCl, ac-
cording to the procedure of Yon (40). Its activity towards
TAME was also measured.

Details of the synthesis of the oligopeptides and the purifi-
cation of these materials and also the enzymes will be pre-
ented elsewhere.

Qualitative hydrolysis experiments
Small amounts of the peptides (I-VII) were dissolved in 1.5
ml of 0.05 M Tris buffer, pH 8.6, to give approximately 0.01
M peptide solutions. The solutions were divided into three
0.5-ml portions. One of the portions was left undisturbed as a
control. To the second portion, 0.5 ml of a thrombin solution
(10 TAME units/ml) was added, and to the third portion
trypsin (13 TAME units/ml) was added in five aliquots of 0.1
ml over a period of 24 hr. All the samples were left at room
temperature; a drop of toluene was added to each sample.
Samples were applied to thin-layer chromatography plates
after 2 hr, 18 hr, and 30 hr. The plates were developed in two
different solvent systems, and sprayed with ninhydrin and
with Sakaguchi reagents, respectively, to locate the peptides
and to determine those that contained arginine. The control
was run to determine the RT values of the starting material.
In all cases, one of the hydrolysis products would be the same,
namely H-Gly-Pro-Ala-NH₂. Since this peptide was run as a
control, the appearance of a spot for this product could be
identified, and was an indication that hydrolysis had oc-
curred.

RESULTS AND DISCUSSION
The action of thrombin and trypsin on peptides I-VII are
summarized in Table 1, in which a plus sign indicates that the
tripeptide H-Gly-Pro-Ala-NH₂ was detected after the stated
hydrolysis time, and a minus sign indicates no detectable
tripeptide. From this table, we see that, after a sufficient
amount of time, thrombin will cleave the Arg-Gly bond in all
of the peptides, even those in which the guanidino group is
protected. On the other hand, trypsin hydrolyzes only those
peptides in which Arg or NO₂Arg is not the N-terminal amino
acid. A comparison of the action of thrombin and trypsin on
peptides IV-VII will be deferred to a later paper in which
quantitative kinetic data will be discussed; for the present, the
positive action of trypsin on peptides IV–VII and its lack of action on peptides II and III is in accord with expected behavior (41), in which trypsin acts only as an endopeptidase, splitting "internal peptide bonds," in substrates where the positive charge of the basic residue is not masked.

On the other hand, it appears, first of all, that thrombin can act as both an exo- and endopeptidase, since peptides II and III, in addition to IV–VII, were hydrolyzed by thrombin. This exopeptidase action of thrombin cannot be due to contamination by an aminopeptidase, since the hydrolysis products for peptides IV–VII showed only two new ninhydrin-positive spots.

A second surprising result is that peptide III, containing NO₂Arg instead of Arg, is also a substrate for thrombin; it appears that the positive charge on Arg is not required for thrombin action on this peptide. Since the hydrolysis products gave a negative Sakaguchi test for arginine (see Table 2), the hydrolysis of peptide III is probably not due to the loss of the nitro protecting group; also the hydrolysis products gave positive ninhydrin tests at Rf values for nitroarginine and the tripeptide I.

It is of interest to note the difference in reaction rates between the hydrolysis of these peptides and that of the ester TAMe. A TAMe unit is defined as the amount of thrombin necessary to hydrolyze 0.1 μmol in a 0.01 M TAMe solution (pH 8) in 1 min at 25°C (39). This means that TAMe at the same concentration as the peptides with the same amount of thrombin added, would have been completely hydrolyzed in less than 2 hr. However, our results indicate that, after 2 hr, no significant amount of hydrolysis had occurred. This difference in rates is not altogether surprising, since trypsin has been found to hydrolyze esters approximately 60 times as fast as similar amides (21). Our results are also consistent with other data reported in the literature. Tosylarginine glycine has been reported to produce a marked inhibition if present in clotting mixtures, but no free tosylarginine or glycine was detectable after several hours of incubation (42). Recently Blombäck et al (26) found that the hexapeptide H-Phe-Val-Arg-Gly-Pro-Arg-OCH₃ showed a much lower inhibition than H-Phe-Val-Arg-OCH₃. It seems, then, that arginyln esters are better substrates as well as inhibitors than similar peptides containing the Arg-Gly bond.

Another surprising result is the hydrolytic behavior of peptide IV toward thrombin. In the ninhydrin test, both thrombin and trypsin lead to H-Gly-Pro-Ala-NH₂ as one of the products; however, the second products give different Rf values in both systems, although both are ninhydrin-positive. In the Sakaguchi test, only the trypsin hydrolysate gives a second arginine-positive spot, with the same Rf as the second product in the ninhydrin test. Further work on the identification of these products is now in progress.

While it is premature to assign a mechanism for the different actions of thrombin and trypsin on these peptides, we note the presence of the Gly-Gly-Gly sequence, which persists in the fibrinopeptides of most mammals (15). At the present time, we speculate that these three glycines permit the entry of the α(A) chain into the (near) active-site cleft of thrombin, which does not allow the binding of chains with bulkier amino acids; presumably, trypsin has a wider cleft, and can accommodate peptide chains with amino acids larger than glycine. If the fibrinogen chain enters the thrombin site in the form of, say, a hairpin (accommodated only because of the Gly-Gly-Gly sequence), then shorter chains (like II–IV) can also serve as substrates; this would be compatible with the exopeptidase action of thrombin on II and III. These speculations are being tested by substitution of other amino acids for glycine in peptides V–VII and in related peptides.

**CONCLUSION**

The general scheme of the synthesis of fibrinogen-like peptides by the solid-phase method as well as their proteolysis by thrombin and trypsin have been described. We have found that thrombin, unlike trypsin, can also act as an exopeptidase. Further work is in progress to study the kinetics of hydrolysis of these peptides, as well as analogs of these peptides, with thrombin and trypsin, in order to be able to characterize the binding behavior of these peptides to thrombin and trypsin, and to understand some of the differences in the mode of action of these two enzymes.

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**TABLE 2. Presence* of arginine in incubation mixtures† of oligopeptides with thrombin and trypsin after 30 hr**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Blank (no enzyme added)</th>
<th>Action with thrombin</th>
<th>Action with trypsin</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
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<tr>
<td>III</td>
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<tr>
<td>IV</td>
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<td>+</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VII</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* By Sakaguchi test on thin-layer chromatography.
† Tests were performed on the second new ninhydrin-positive spot, the first new spot being the tripeptide I.