# CHEMICAL CHARACTERIZATION OF FRAGMENTS PRODUCED IN THE CONVERSION OF BOVINE PROTHROMBIN TO THROMBIN BY ACTIVATED FACTOR X

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## 1. Introduction

Bovine prothrombin of 72 000 daltons is activated to the clotting enzyme, thrombin, by Factor Xa in the presence of Factor V, phospholipids and calcium ion, yielding other fragments [1–3]. The thrombin molecule of 37 000 daltons is released from the C-terminal region of the precursor protein [4]. Previously, we found that a slow activation of prothrombin with Factor Xa in the absence of Factor V and phospholipids yields two large fragments of 18 700 and 57 000 daltons, in which the former is derived from the N-terminal region of the parent molecule and the latter is an intermediate which results in the formation of thrombin [5]. This paper describes further studies on the isolation and characterization of fragments produced when prothrombin is converted to α-thrombin by Factor Xa.

## 2. Materials and methods

Bovine prothrombin purified from citrated fresh plasma [5] was used. The specific activity of the preparation measured by the method of Magnusson [6] was 1200 NIH units per mg protein. The purified material had N-terminal alanine as determined by the PITC [7] and cyanate methods [8]. Purified bovine Factor X1 [9] was a generous gift from Dr. K. Fujikawa, Dept. of Biochemistry, University of Washington, Seattle. The material was activated with the venom of *Vipera russelli* under the conditions described previously [5]. Sephadex G-150 was a product of Pharmacia, Uppsala, Sweden. Disc polyacrylamide gel electrophoresis in the presence or absence of SDS was made essentially by the methods of Weber and Osborn [10] and Davis [11]. The gels were stained with Coomassie brilliant blue R-250. Amino acid analysis was performed by the method of Spackman et al. [12] with an amino acid analyzer, Model JLC-5AH from Japan Electron Optics Lab., Ltd. Hexose and hexosamine were determined, respectively, by the methods of Dubois [13] and Gardell [14]. For determination of sialic acid, the periodate–thiobarbituric acid method of Warren [15] was used. N-terminal analysis was performed by the cyanate [8], DNP [16] and PITC methods [9], and the PTH-derivatives were estimated quantitatively from their ultraviolet absorption at 269 nm and identified by thin layer chromatography [16]. Molecular weights were estimated from electrophoretic mobilities on 10% SDS-gels, referring to calibration curves prepared with bovine serum albumin, ovalbumin, α-chymotrypsinogen A, myoglobin, cytochrome *c* and α-bungarotoxin.

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*Abbreviations:* SDS, Sodium dodecyl sulfate; PITC, Phenyl-*iso*thiocyanate; PTH, Phenylthiohydantoin; NIH, National Institute of Health; DNP, 2,4-dinitrofluorobenzene.

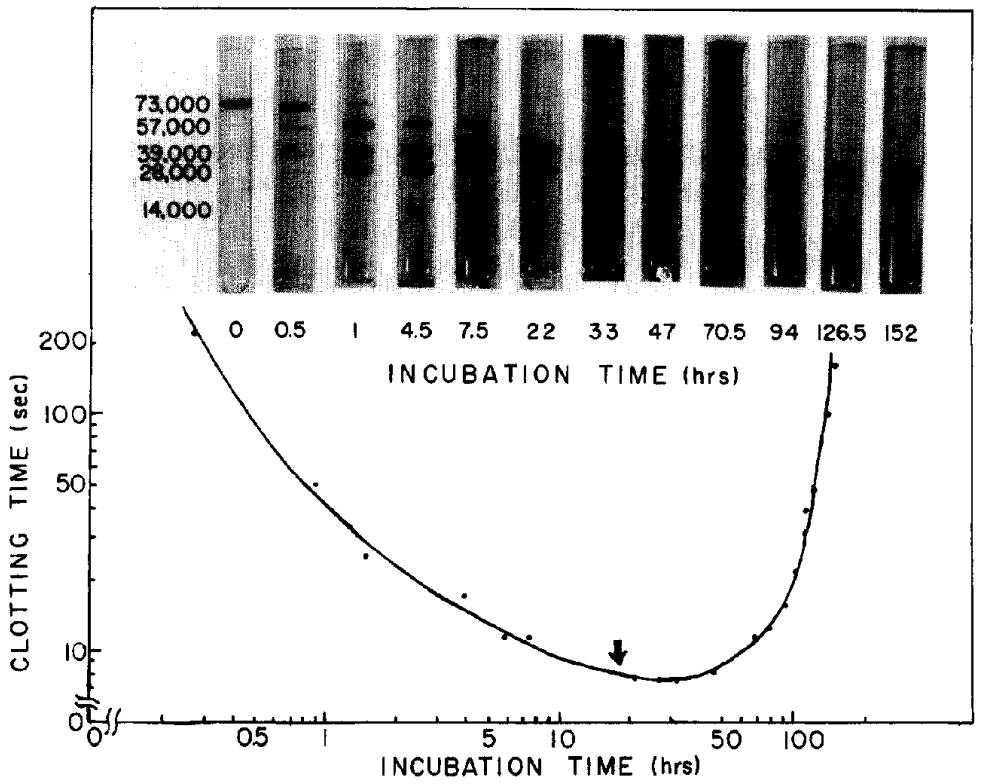


Fig. 1. Time course of formation of thrombin from prothrombin with Factor Xa and SDS-gel electrophoresis of the reaction products at various stages. The conditions used were described in the text. Thrombin activity was measured at 37°C, using 0.3% bovine fibrinogen (fraction I-4) [18] and the time for clot formation was recorded with a Fibrometer®, Baltimore, Biochemical Lab., Maryland. Electrophoresis was made in 8% SDS-gel for 6 hr using a current of 7 mA per tube.

## 3. Results

### 3.1. *Isolation of fragments produced during activation of prothrombin by Factor Xa*

Prothrombin (0.6 mg) dissolved in 0.15 M NaCl was incubated at pH 7.2 at 37°C with Factor Xa (5 μg) in a total volume of 0.35 ml. Aliquots of the mixture were taken at intervals. Half of each was used to estimate formation of thrombin and the other half was subjected to SDS-gel electrophoresis after dialysis and lyophilization [5].

The results are shown in fig. 1. In parallel with the rapid formation of thrombin, prothrombin was fragmented into four fragments of 57 000, 39 000, 28 000 and 14 000 daltons and then into three fragments. The maximum formation of thrombin under the conditions used was achieved after incubation for 30 hr. Based on these results, large scale fragmentation of thrombin with Factor Xa was performed and the resulting products were separated by chromatography on a Sephadex G-150 column. The results are shown in fig. 2. As revealed by their electrophoretic patterns on SDS-gels, the fragment of 39 000 daltons was eluted in the first peak, that of 27 000 daltons in the second peak and that of 14 000 daltons in the last peak. The fractions indicated by solid bars were combined and lyophilized. The yields of these fragments were 69.7 mg (first peak), 51.4 mg (second peak) and 31 mg (last peak).

### 3.2. *N-Terminal residues and amino acid and carbohydrate compositions of each fragment*

#### 3.2.1. Fragment of 39 000 daltons (± 2000)

This fragment gave a single band on SDS-gel electrophoresis, but the SDS-gel pattern after reduction

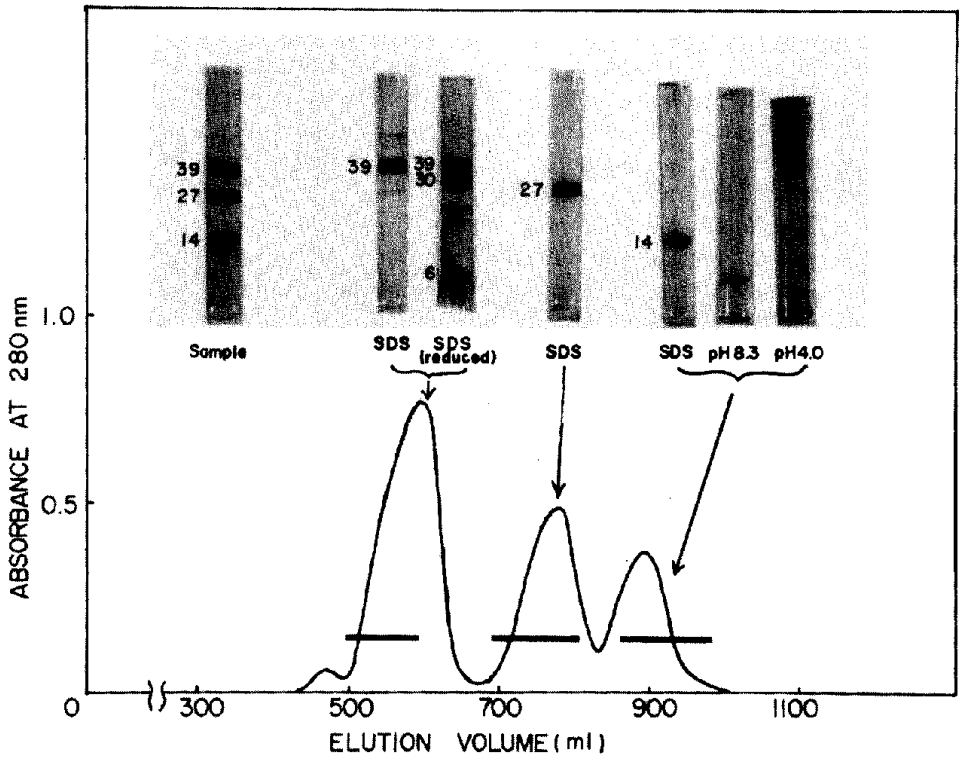


Fig. 2. Separation of reaction products of bovine prothrombin with Factor Xa on a Sephadex G-150 column. Purified prothrombin (200 mg) dissolved in 100 ml of 0.15 M NaCl was incubated at 37°C with 33.4 ml of solution containing 940 μg of Factor X1, 16.7 μg of *Vipera russelli* venom, 0.05 mmoles of $CaCl_2$ and 5 mmoles of Tris–HCl buffer, pH 7.2. After incubation for 18 hr, the reaction was terminated by acetic acid to a final concentration of 30% and the mixture was lyophilized. The freeze-dried material was dissolved in 20 ml of 30% acetic acid and applied to a column (3.8 × 134 cm) of Sephadex G-150, equilibrated with 30% acetic acid. Elution was performed with the same solution at a flow rate of 53 ml/hr. The fractions indicated by the solid bars were pooled. SDS-gel electrophoresis of samples after and before reduction was done as described for fig. 1. Disc-gels at pH 8.3 and 4.0 were subjected to electrophoresis under the conditions described by Davis [11]. Migration was from top (cathode) to bottom (anode).

of the sample showed the presence of three major fragments, which were estimated to have molecular weights of 39 000, 30 000 and 6 000 (fig. 2). Among them, the fragment of 39 000 daltons, which was the same as that of the unreduced sample, appeared to be α-thrombin precursor (intermediate 2 in fig. 3) consisting of a single polypeptide chain, and the other two seemed to correspond to the A-chain of 58 000 daltons and B-chain of 31 200 daltons of the α-thrombin molecule [19]. The amino acid composition of the unreduced sample was indistinguishable from that of α-thrombin (table 1). Moreover, N-terminal analysis of the unreduced sample revealed the presence of threonine and isoleucine, which must be derived, respectively, from the N-terminal ends of the A- and B-chains of α-thrombin.

3.2.2. Fragment of 27 000 daltons (± 2000)

This fragment gave a single band on electrophoresis on SDS-gel and disc-gel at pH 8.3. The N-terminal residue was alanine and further analysis revealed the sequence Ala–Asn–Lys–. Moreover, the amino acid and carbohydrate compositions (table 1) were identical to those previously found in the N-terminal fragment of prothrombin [5].

3.2.3. Fragment of 14 000 daltons (± 2000)

This fragment gave a single band on disc-gel

electrophoresis at pH 8.3, and on electrophoresis on SDS-gel (fig. 2). However, the electrophoretic pattern on disc-gel at pH 4.0 indicated the existence of three components with slightly different mobilities. These components were partially separated by an isoelectric focusing method but on examination their amino acid compositions were found to be the same. Thus, the difference in their electrophoretic mobilities may be due to microheterogeneity in their carbohydrate moieties. The fragment contained N-terminal serine, as identified by the DNP method, while stepwise Edman degradation revealed the sequence Ser–Gly–Gly. Its amino acid and carbohydrate compositions were found to be quite different from those of the two fragments described above (table 1).

Table 1 also shows the amino acid and carbohydrate compositions of bovine prothrombin and α-thrombin obtained by two other groups. The data indicate that the sum of the total amino acid residues of the three fragments is in good agreement with those of the parent molecule analyzed by the three groups.

## 4. Discussion

The results presented here indicate that three major fragments are produced when prothrombin is converted to α-thrombin by Factor Xa. One of these fragments, which has a mol. wt of 39 000, must be α-thrombin, as judged from its N-terminal residues and amino acid composition. On reduction with β-mercaptoethanol, this fragment yields two major fragments of 6000 and 30 000 daltons, which seem to be derived from the α-thrombin molecule. The fragment contains an additional component, consisting of a single polypeptide chain of the same size as that of α-thrombin. This component (intermediate 2 in fig. 3) could be a precursor molecule of α-thrombin, as its S-carboxymethyl derivative has threonine as the single N-terminal (unpublished data).

The second fragment of 27 000 daltons must be derived from the N-terminal region of prothrombin, because the first three amino acids in its N-terminal sequence and its amino acid and carbohydrate compositions are identical with those of the N-terminal fragment of prothrombin characterized previously [5].

The third fragment of 14 000 daltons with N-terminal serine seems to be derived from the N-terminal portion of the intermediate of 57 000 daltons (intermediate 1 in fig. 3) reported previously [5]. This was concluded because production of the fragment was accompanied by disappearance of the intermediate (fig. 1) and because the N-terminal sequence of Ser–Gly–Gly– of the intermediate was identical to that of the intermediate 1 (unpublished data). The fragment had a relatively high carbohydrate content, suggesting the presence of one oligosaccharide chain per mole of fragment.

From these results, it is concluded that these three major fragments are probably located in the parent molecule as shown in fig. 3. Thus, there should be at least three peptide-bond cleavages associated with the activation of prothrombin yielding α-thrombin, an N-terminal fragment and an inner fragment. The results also indicate that there can be no disulfide bridges linking these three fragments, and that of the four oligosaccharide chains in the prothrombin molecule

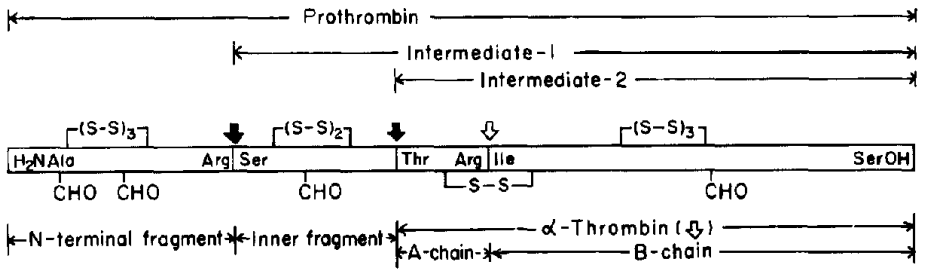


Fig. 3. Gross structural model of bovine prothrombin. The solid lines and CHO represent the fragments produced by Factor Xa and a carbohydrate side chain. The N-terminal and inner fragments and α-thrombin correspond to the fragments of 39 000, 27 000 and 14 000 daltons, respectively.

Table 1
Amino acid and carbohydrate compositions of bovine prothrombin and its fragments.

| Amino acids and carbohydrates | Prothrombin | | Present authors[a] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mann et al. [19] | Cox and Hanahan [21] | Pro-thrombin | Sum of three fragments | N-terminal fragment | Inner fragment | Intermedi-ate 2 + α-thrombin | α-Thrombin Mann et al. [19] |
| | Residues per mole | | | | | | | |
| Aspartic acid | 59 | 55 | 57 | 59 | 11 | 16 | 32 | 30 |
| Threonine | 27 | 26 | 26 | 26 | 7 | 4 | 15 | 15 |
| Serine | 32 | 32 | 32 | 32 | 8 | 8 | 16 | 14 |
| Glutamic acid | 71 | 67 | 67 | 67 | 17 | 13 | 37 | 41 |
| Proline | 34 | 32 | 35 | 33 | 8 | 8 | 17 | 19 |
| Glycine | 46 | 44 | 47 | 47 | 9 | 10 | 28 | 27 |
| Alanine | 33 | 31 | 33 | 33 | 8 | 9 | 16 | 15 |
| 1/2Cystine | 17 | 17 | 18 | 18 | 6 | 4 | 8 | 6 |
| Valine | 35 | 32 | 33 | 35 | 7 | 5 | 23 | 21 |
| Methionine | 6 | 5 | 6 | 7 | 1 | trace | 6 | 4 |
| Isoleucine | 18 | 18 | 18 | 20 | 3 | 1 | 16 | 14 |
| Leucine | 46 | 42 | 45 | 46 | 8 | 9 | 29 | 28 |
| Tyrosine | 19 | 17 | 18 | 17 | 3 | 3 | 11 | 11 |
| Phenylalanine | 19 | 18 | 20 | 20 | 3 | 3 | 14 | 16 |
| Histidine | 9 | 8 | 9 | 10 | 2 | 1 | 7 | 8 |
| Lysine | 29 | 29 | 32 | 30 | 4 | 3 | 23 | 25 |
| Arginine | 42 | 43 | 45 | 40 | 12 | 7 | 21 | 20 |
| Tryptophan | 11 | 11 | (11) | (11) | 3 | | (8) | 8 |
| Total | 553 | 527 | 552 | 551 | 120 | 104 | 327 | 322 |
| Hexoses | 4.5%[b] | | 3.7% | | 11.94% | 10.48% | | 1.56%[d] |
| Hexosamine | 5.5%[b] | | – | | 6.84% | 4.89% | | 1.7%[d] |
| Sialic acid | 4.7%[b] | | – | | 4.45% | 1.15% | | 1.8%[d] |
| Mol. wt estmated by SDS gels | 75 000 | | 73 000 | – | 27 000 | 14 000 | 39 000 | 40 000 |
| Mol. wt calculated from chemcal analyses[c] | 72 000 | | 72 400 | 71 700 | 18 600 | 13 300 | 39 800 | 39 000 |

[a] The amino acid compositions of the fragments estimated by us were calculated from extrapolated or average values estimated on samples of 24, 48 and 78 hr hydrolyzates, except for that of prothrombin which was calculated from the values analyzed on a sample of the 24 hr hydrolyzate.

[b] Taken from the data of Nelsestuen and Suttie [20].

[c] The molecular weights based on chemical analyses were obtained from the sum of the total amino acid and carbohydrate residues.

[d] Taken from the data of Magnusson [1].

[20], two must be located in the N-terminal fragment and the other two in the inner fragment and α-thrombin, respectively. Moreover, based on these and previous results [5], the processes of prothrombin activation with Factor Xa in the absence of Factor V and phospholipids may be deduced to be as follows:
Step 1. Prothrombin → Intermediate 1 + N-Terminal fragment;
Step 2. Intermediate 1 → Intermediate 2 + Inner fragment;
Step 3. Intermediate 2 → α-Thombin.

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