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Release of Human and Horse Fibrinopeptides

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N-Terminal amino acids are determined with Edman's phenylisothiocyanate method during conversion of human and horse fibrinogen to fibrin with homologous thrombin.

The results indicate that, in both species, peptide A is released at a high initial rate, and that this release initiates clotting, whereas peptide B is released at a slow initial rate. The results also show that the yield of peptide B from horse fibrinogen is low.

In all mammalian fibrinogens hitherto studied, the molecule is built up of three different peptide chains, denoted as A, B and C.[1,2] During the action of the proteolytic enzyme thrombin on fibrinogen, the fibrinopeptides A and B are released from the A- and B-chains of the fibrinogen molecule. The proteolytically changed fibrinogen subsequently polymerizes to fibrin. The third chain, C, is not attacked by thrombin.[3–7]

In fibrinogens, the A- and B-chains end with a different amino acid in different species. The human A-chains end with alanine [8,9] and the B-chains with pyroglutamic acid.[10] The A-chains of horse fibrinogen have threonine in the N-terminal position, whereas no reacting N-terminal amino acid is present in the B-chains.[9] All mammals studied have tyrosine as N-terminal amino acid in the C-chains. Fibrins of all mammals have glycine at the end of the A- and B-chains, and tyrosine at the C-chains.[9]

During the action of bovine thrombin on bovine fibrinogen, the fibrinopeptides A and B are released at different initial rates. Peptide A is split off far more rapidly than peptide B. Furthermore, the release of peptide A parallels the formation of fibrin.[11,12] This also seems to apply to the action of bovine and rabbit thrombin on rabbit fibrinogen,[13] as well as of bovine and human thrombin on human fibrinogen.[14]

Studies on the fibrinopeptides have revealed that the amino acid sequence in peptide A near the point of thrombin cleavage is similar in many mammalian species. Moreover, no apparent species specificity of several mammalian

Abbreviations:
NIH: National Institutes of Health.
PTC: Phenylthiocarbamyl, PTH: Phenylthiohydantoin, Tris: Tris(hydroxymethyl)aminomethane.

thrombins is recognized with regard to the thrombin-fibrinogen reaction.[15] If peptide A contains specific structures for the rapid reaction between thrombin and fibrinogen, one would expect this peptide to be released at the highest rate during fibrinogen-fibrin conversion in all mammalian species.

In the present work, the release of peptides A and B from human and horse fibrinogen with homologous thrombin was followed. The release of the peptides was evaluated from determinations of N-terminal amino acids with Edman's phenylisothiocyanate method. In both species, fibrinopeptide A was released at a much higher initial rate than fibrinopeptide B, and the release of fibrinopeptide A seemed to initiate clotting. In horse fibrinogen, peptide B was released to a very small extent, if at all.

MATERIAL AND METHODS

Human fibrinogen was prepared from pooled plasma, as described by Blombäck and Blombäck.[16] Fraction I−2, with a coagulability of 90 %, was used.

Horse fibrinogen, from one animal, was also prepared as described by Blombäck and Blombäck.[16] The coagulability of fraction I−4 was only 68 % and the fibrinogen was, therefore, further purified by precipitation with sodium chloride.[17] One volume of saturated sodium chloride solution was slowly added to one volume of fraction I−4 in 0.3 M sodium chloride, protein concentration 5 mg/ml, at 0°C. The mixture was centrifuged and the sediment discarded. To the supernatant was added once more the same volume of saturated sodium chloride at 0°C, and the mixture was allowed to stand overnight at 0°, after which it was centrifuged. This sediment was also discarded, and the supernatant was kept at room temperature overnight. During this time, a third precipitate appeared, which was collected by centrifugation and used for the experiments. It was dissolved in 0.3 M ammonium acetate and dialyzed against this solution. After dialysis, the coagulability of the protein was 99 %.

Human thrombin was prepared as described earlier.[15] The specific activity was 46 NIH units/mg.

Horse thrombin. Horse prothrombin was prepared as previously described.[15] Activation of prothrombin to thrombin was made in 25 % trisodium citrate according to Seegers *et al.*[18] Further purification of the thrombin was the same as for human thrombin. The specific activity was 43 NIH units/mg.

N-Terminal amino acid analysis. The method of Edman [19] was used. For application of the method to fibrinogen, the techniques described by Blombäck and Yamashina [9] were followed. The N-terminal amino acids were identified by paper chromatography according to Edman and Sjöquist [20] and Sjöquist.[21,22] After coupling with phenylisothiocyanate, the protein was washed three times with benzene. The protein was then precipitated in 80 % acetone and washed three times with aqueous acetone. This procedure removed the PTC derivatives of amino acids and peptides. The cyclization time was 60 min.

In the experiment with human fibrinogen, PTH-tyrosine was determined from chromatograms developed with solvent III, PTH-glycine with solvent II and solvent III, PTH-alanine with solvent II, and PTH-aspartic acid with solvent III. Two – four chromatograms were run, and the figures for yield are mean values. The figures were corrected on the basis of the yield of free amino acids added to the fibrinogen (see below).

In the experiment with horse fibrinogen, PTH-tyrosine, PTH-glycine and PTH-aspartic acid were determined from chromatograms developed with solvent F, and PTH-glycine, PTH-dehydrothreonine and PTH-leucine with solvent G.[9] The figures are mean values of 2−4 determinations and are corrected for yield.

Yield of free amino acids. Determinations of yield of different amino acids were made according to Blombäck and Yamashina.[9] To 75 mg of human fibrinogen in tris-HCl buffer, pH 8.5, ionic strength 0.15, protein concentration 1.7 mg/ml, were added one μmole each of alanine, glycine, threonine, leucine, aspartic acid, and tyrosine. The same fibrinogen solution without addition of amino acids was used as blank. N-Terminal

Table 1. Yield of different amino acids added to fibrinogen.

Amino acid	Yield %	Mean %
Alanine	73, 72, 76	74
Glycine	86, 70, 75, 80, 79	79
Threonine as PTH-dehydrothreonine	61, 54, 64	60
Leucine	74, 71	73
Aspartic acid	55, 44	49
Tyrosine (60′ cyclization)	77	
» (30′ »)	79	82
» (15′ »)	81	(extrapolated)
» (extrapolated to 0′ time cyclization)	82	

analyses were performed as described above, except that precipitation and washings with acetone were omitted and replaced by five washings with benzene. This procedure had to be adopted as the PTC derivatives of free amino acids are soluble in aqueous acetone. The cyclization time of alanine, glycine, threonine, leucine, and aspartic acid was 60 min. For tyrosine it was 15, 30, and 60 min. The PTH derivatives of tyrosine and glycine were determined from chromatograms developed in solvent F, and the PTH derivatives of alanine, glycine, dehydrothreonine, leucine, and aspartic acid in chromatograms developed in solvent G. At least two determinations were made with each two chromatograms.

The yields of the added free amino acids are listed in the table. It was stated earlier that free tyrosine, but not protein-bound tyrosine, is destroyed during cyclization.[9] The figures for yield of tyrosine were, therefore, extrapolated to zero-time cyclization. For the other amino acids, the mean values were calculated. The yield of protein-bound amino acid is assumed to be the same as that of free amino acid.

EXPERIMENTS AND RESULTS

Human thrombin-fibrinogen reaction. Fibrinogen, sodium chloride solution and tris-imidazole-HCl buffer were placed in each of six beakers, so that the final volume was 45 ml, fibrinogen concentration 1.7 mg/ml, tris and imidazole concentration each 0.02 M, ionic strength 0.2, and pH 8.8—8.9. 1.0 ml of a solution of human thrombin in distilled water was then added. The thrombin concentration in the reaction mixture was 0.02 NIH units/ml. Only distilled water was added to the zero-time sample. After 6, 20, 40, 60 min and 8 h, 46 ml of pyridine and 2.0 ml of phenylisothiocyanate were added, which interrupted the thrombin action. If a clot had formed, it dissolved when pyridine was added.

The N-terminal amino acid pattern during conversion of human fibrinogen to fibrin is shown in Fig. 1. As expected, the amount of N-terminal tyrosine did not change during incubation with thrombin. The mean value for tyrosine

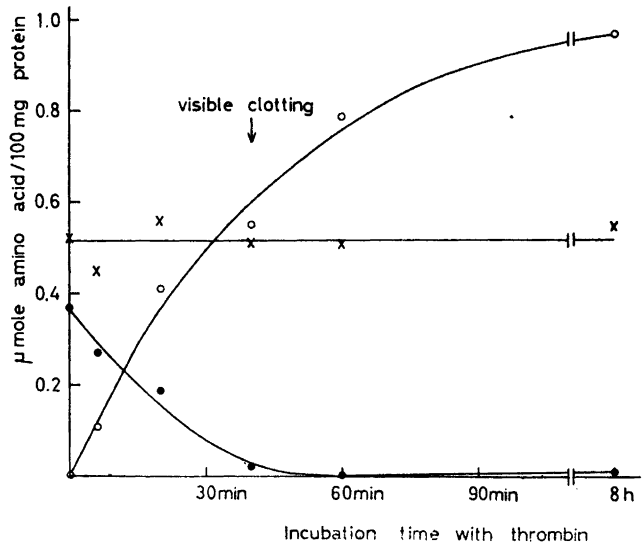

Fig. 1. N-Terminal amino acids during conversion of human fibrinogen to fibrin with homologous thrombin. Clotting is observed after 40 min of incubation. × Tyrosine, ○ glycine, ● alanine.

yield was 0.52 mole/10^5 g protein. Assuming a molecular weight for human fibrinogen of 340 000 [23] and a purity of 90 % of the used protein, this value corresponds to 2.0 tyrosyl residues per molecule of fibrinogen.

The N-terminal alanine disappeared during incubation with thrombin, indicating a release of fibrinopeptide A. Since the PTC derivative of peptide A is soluble in acetone, it was removed by precipitating the PTC protein with acetone. The amount of alanine in the zero-time sample was 0.37 mole/10^5 g protein, which corresponds to 1.4 alanyl residues per molecule of fibrinogen.

The N-terminal amino acid of the B-chains is pyroglutamic acid which does not react with phenylisothiocyanate. The release of peptide B cannot, therefore, be followed directly.

Shortly after the addition of thrombin, glycine appeared as N-terminal amino acid in the reaction mixture. The yield of glycine after 8 h incubation was 0.97 mole/10^5 g protein, corresponding to 3.6 glycyl residues per molecule of fibrinogen, as compared to the expected 4.

Fig. 2 shows the increase in glycine and the decrease in alanine. In the beginning of incubation, the curves ran close together. It is evident that the amount of N-terminal glycine continued to increase after disappearance of the N-terminal alanine. It can, therefore, be concluded that peptide A is released at a higher initial rate than peptide B. It is to be noted that clotting occurred in the system after 40 min incubation when only a small amount of peptide B had been released.

In addition to the amino acids mentioned above, traces of N-terminal aspartic acid were found in a yield of 0.1 mole/10^5 g protein, which corresponds

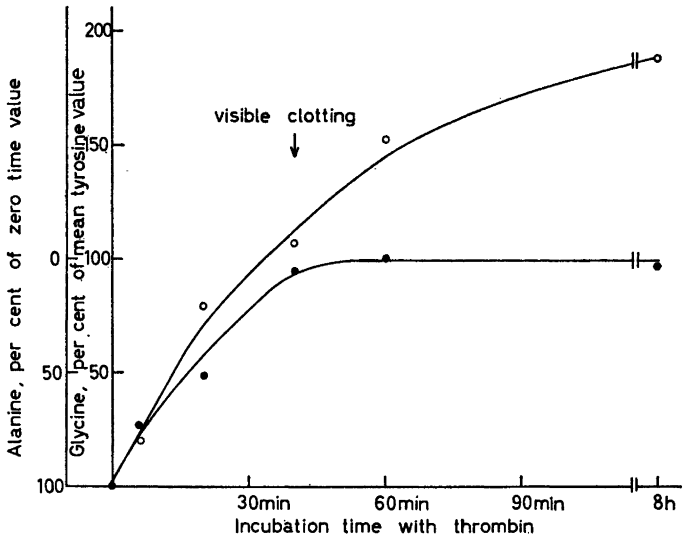

Fig. 2. Disappearance of N-terminal alanine and appearance of N-terminal glycine during conversion of human fibrinogen to fibrin with homologous thrombin. Alanine is expressed in per cent of the zero time value, which is 100 %. Glycine is expressed in per cent of the average N-terminal tyrosine value. Clotting is observed after 40 min of incubation. ○ Glycine, ● alanine.

to 0.3 residues of aspartic acid per molecule of fibrinogen. The aspartic acid disappeared during incubation with thrombin.

Horse thrombin-fibrinogen reaction. Horse fibrinogen was dialyzed against tris-HCl buffer, pH 8.5, ionic strength 0.15. The fibrinogen solution was diluted with buffer and divided into six samples, so that the final volume of each sample was 45 ml, and the fibrinogen concentration 1.7 mg/ml. To five samples was added 1.0 ml of a solution of horse thrombin in tris buffer to a final thrombin concentration of 0.02 NIH units/ml. Only buffer was added to the zero-time sample. After 7, 15, 30, 90 min and 9 h, 46 ml of pyridine and 2 ml of phenylisothiocyanate were added.

The N-terminal amino acids during fibrin formation are illustrated in Fig. 3. The amount of tyrosine did not change during the experiment. The average tyrosine yield was 0.49 mole/10^5 g protein, *i.e.*, about the same as found for human fibrinogen. This value is higher than previously reported.[11]

The N-terminal threonine of the A-chains was isolated and determined as PTH-dehydrothreonine. The zero-time value for threonine was 0.34 mole/10^5 g protein. During thrombin action, the amount of N-terminal threonine diminished to zero.

A spot on the chromatogram, corresponding to PTH-leucine and/or PTH-isoleucine, was recognized. Since leucine has been found in the N-terminal position of the isolated peptide B from horse fibrinogen,[24] this spot was probably due to N-terminal leucine of the corresponding B-chains. The zero-time value

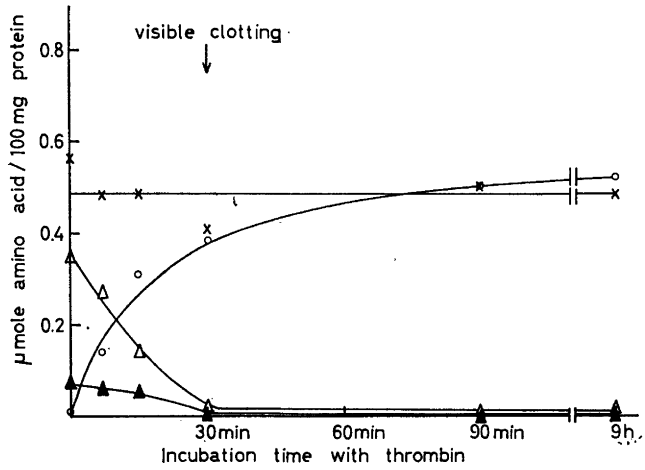

Fig. 3. N-Terminal amino acids during conversion of horse fibrinogen to fibrin with homologous thrombin. Clotting is observed after 30 min of incubation. × Tyrosine, ○ glycine, △ threonine, ▲ leucine.

for leucine was, however, only 0.08 mole/10^5 g protein. The small amount of leucine obtained in the first four samples was not present in the last two samples, indicating a release of peptide B.

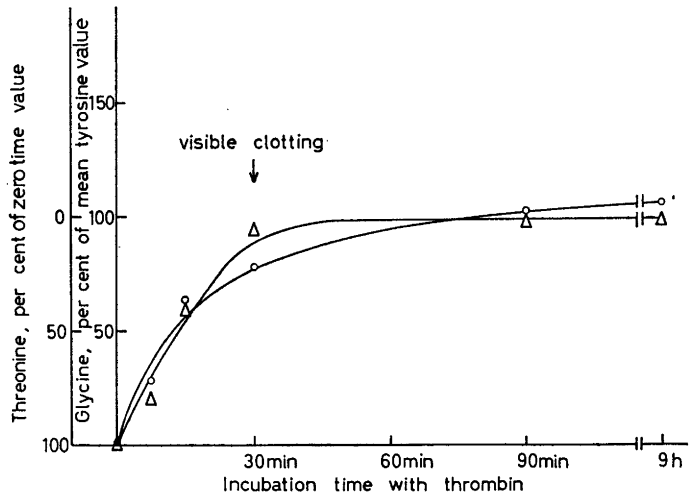

Fig. 4. Disappearance of N-terminal threonine and appearance of N-terminal glycine during conversion of horse fibrinogen to fibrin with homologous thrombin. Threonine is expressed in per cent of the zero time value, which is 100 %. Glycine is expressed in per cent of the average N-terminal tyrosine value. Clotting is observed after 30 min of incubation. ○ Glycine, △ threonine.

During the conversion, glycine appeared in the reaction mixture as N-terminal amino acid. The final amount of glycine was 0.52 mole/10^5 g protein, which is 106 % of the tyrosine value. Fig. 4 shows the increase in glycine and the decrease in threonine. The curves run close together. Clotting was observed after 30 min incubation.

The N-terminal pattern of the horse fibrinogen-fibrin mixture is interpreted as follows. Fibrinopeptide A is released early during incubation. Peptide B is released only to a small extent, if at all. Clotting occurs irrespective of whether any measurable amount of fibrinopeptide B have been released.

In addition to the amino acids mentioned above, traces of N-terminal aspartic acid, not exceeding 0.08 mole/10^5 g protein, were detected. There was no measurable difference in the amount of N-terminal aspartic acid during incubation with thrombin.

DISCUSSION

The results of other investigations,[9,11,13,14] as well as the present one, indicate that the rapid release of peptide A from fibrinogen and the correlation between visible clotting and splitting of the A-chains are valid for different species, also with homologous thrombin. Under the conditions used here, visible clotting occurs before any significant amount of peptide B is split off. The clotting time in a thrombin-fibrinogen system might therefore conveniently be used as a measure of the rate of release of fibrinopeptide A. The findings also support the view that the structures in the peptide A area are more favourable for the action of thrombin than those in the peptide B area.

The amount of N-terminal tyrosine, alanine, and glycine in human fibrinogen and fibrin is in accordance with the figures found by others,[9,14,25] and the results are in agreement with the general view that human fibrinogen consists of three pairs of peptide chains. As in some other investigations,[14,25] small amounts of N-terminal aspartic acid were detected. The aspartic acid was present to an amount of 19 % of the value for tyrosine, and diminished to zero during conversion to fibrin. Consequently, the aspartic acid cannot easily be ascribed to protein impurities, but seems rather to be derived from chains corresponding to peptide Y. This peptide is similar to peptide A, except that it has lost its N-terminal alanine, and is thus one amino acid shorter and ends in aspartic acid. Peptide Y occurs to an amount of about 10 % in human fibrinogen.[26] The low yield of alanine, 1.4 residues/molecule of fibrinogen, as compared to tyrosine, 2.0 residues/molecule of fibrinogen, can partly be accounted for by the occurrence of such Y-chains.

The molecular weight of horse fibrinogen has been determined with different methods with somewhat divergent results. The value 490 000 was obtained with a light-scattering technique,[11] while 340 000 was found by means of ultracentrifugal analyses.[27] It is, therefore, at present somewhat difficult to interpret the yields of the different amino acids in terms of amino acid residue per molecule of fibrinogen. It is nevertheless probable that horse fibrinogen, like other mammalian fibrinogens, consists of three different peptide chains, as Henschen showed that horse fibrinogen and fibrin, after sulphitolysis and electrophoresis, can be separated into three different fractions.[28] Consequently,

one would expect the amount of N-terminal amino acids from the different chains, *e.g.* threonine from the A-chains, leucine from the B-chains and tyrosine from the C-chains, to be equimolar. This was not, however, the case. The amount of leucine was much too low to fit this model. The exceptionally low yield of N-terminal leucine indicates that the N-terminal amino acid of the B-chains is only partly accessible to the phenylisothiocyanate reagent, or that part of the molecule is devoid of B-chains.

N-Terminal aspartic acid was also found in small amounts in horse fibrinogen. This residue might have derived from impurities, or it might represent an N-terminal amino acid in fibrinogen. The isolated peptide B has aspartic acid next to leucine in the N-terminal position.[24] Perhaps different B-chains are present in horse fibrinogen, analogous with the finding of different A-chains in human fibrinogen.

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