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Short Communications

Action of Thrombin on Plasmin Digested Fibrinogen

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During the last years convincing evidence has been presented indicating that the effect of thrombin on fibrinogen is a proteolytic reaction [1]. At this proteolysis glycine appears as a new N-terminal residue. The reports concerning the amount of glycine residues, released from one mole fibrinogen, have varied. Recently published investigations [2] carried out by means of Edman's phenyl*iso*thiocyanate method have indicated that 4 moles of glycine appear at the proteolysis of one mole of fibrinogen with thrombin. From the supernatant after clotting Bettelheim and Bailey isolated two peptides, A and B, by electrophoresis [3]. The A peptide contained glutamic acid in N-terminal position and its isoelectric point was lower than that of the B peptide in which no N-terminal amino acid could be demonstrated. Recently Blombäck and Vestermark [4] have been able to isolate the A and B peptides by means of chromatography on 2 % crosslinked Dowex-50.

The purpose of this work was to study the effect of thrombin on the split products from the digestion of fibrinogen by plasmin. One way of doing this is to determine if addition of thrombin to a sample of digested fibrinogen causes any difference in the N-terminal amino-acid composition as compared with a similar sample to which no thrombin had been added. In an earlier work [5] it was shown that Edman's phenyl-*iso*thiocyanate method is very well suited for the study of the fibrinogenolysis.

The fibrinogen used was bovine fraction I-4 of Blombäck and Blombäck [6]. This highly purified fibrinogen contains traces of plasminogen which on activation gives rise to enough plasmin to make the fibrinogen unclottable within a relatively short time. As activator urokinase was used. It was prepared from human urine according to a method described by us earlier [5]. The thrombin used was citrate-activated prothrombin, prepared according to the method of Seegers [7,8]. It contained about 800 NIH units per mg.

The digestion mixture consisted of 1 g of bovine fibrinogen and 15 mg urokinase dissolved in 100 ml 0.02 M phosphate buffer, pH 7.2, containing NaCl to a concentration of 0.15 M. This mixture was incubated at 37°C. Clots obtained by adding 10 NIH units of thrombin to one ml of the reaction mixture dissolved in about 15 min at 37°C. At different times two 10 ml portions were removed from the digestion mixture. To one of them was added 1 ml of a thrombin solution containing 200 NIH units. Both aliquots were incubated for 15 min at 37°C. The enzyme activity was interrupted by adding 10 ml of pyridine containing 0.75 ml phenyl*iso*thiocyanate. The coupling with phenyl*iso*thiocyanate and the conversion of the PTC-amino acids to the corresponding PTH derivates were performed as described by Blombäck and Yamashina [2] and by us in an earlier communication [5]. The PTH-amino acids were identified and quantitatively estimated by the method of Edman and Sjöqvist [9]. The solvents used for the chromatography were those of Edman and Sjöqvist [9] and Sjöqvist [10].

During the fibrinogenolysis quantitative estimations of the following N-terminal amino acids were made: aspartic acid, glutamic acid, glycine, alanine, valine, threonine, tyrosine and lysine. Probably there are some additional N-terminal amino acids, which have not been studied. At least 150 bonds seem to be split by plasmin at maximal digestion.

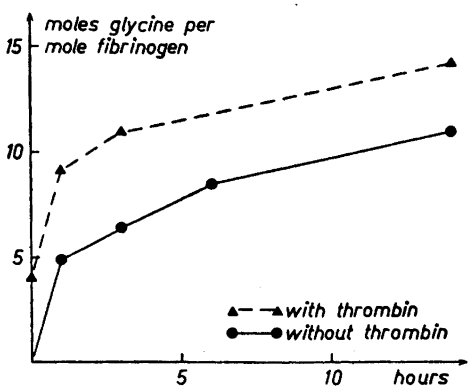

Fig. 1. Increase of N-terminal glycine in a fibrinogenolytic mixture at the addition of thrombin. (The values are corrected for a yield of 80 %.)

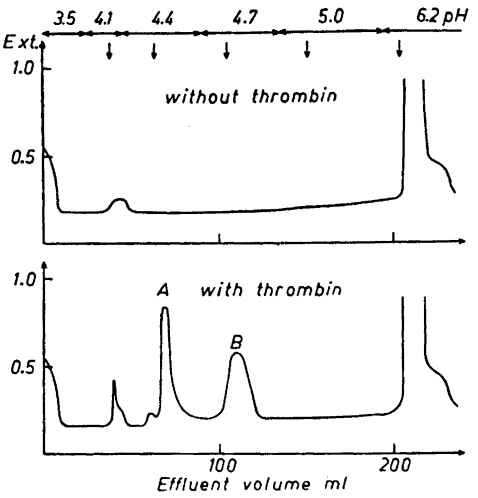

Fig. 2. Release of fibrinopeptides at the addition of thrombin to a fibrinogenolytic mixture. The extinction was read at 700 mμ. The following buffers were used: Na-formate at pH 3.5, Na-acetate at pH 4.1 to 5.0 and Na-phosphate at pH 6.2. The ionic strength of all buffers was 0.1. The vertical arrows indicate the points at which the same pH, as that of the eluting buffer, was obtained.

In the tests to which thrombin was added to the digestion mixture there was an immediate increase in N-terminally bound glycine as compared to the samples taken at the same time but to which no thrombin had been added. This increase in glycine corresponded to nearly 4 moles per mole fibrinogen in the original mixture. This was true even after a digestion time of 14 h when nearly 50 peptide bonds were split (Fig. 1). After still longer digestion times, however, the increase of glycine after the addition of thrombin gradually decreased but seemed to reach the level of about 2 additional moles of glycine at maximal digestion.

In order to determine whether the bonds split by thrombin are the same in the digested as in the intact fibrinogen, an attempt was made to isolate the peptides liberated by the action of thrombin on the digestion mixture described above. The method used was that described by Blombäck and Vestermark [4]. After a digestion time of 5 h two 20 ml samples were removed from the digestion mixture. To one of them 400 NIH units thrombin dissolved in 2 ml were added. Both samples were incubated for 15 min at 37°C. The enzyme action was interrupted by adjusting the pH of the samples to 2 with formic acid. After being kept over night at —20°C they were thawed and the pH adjusted to 4.5. A heavy precipitate formed and was removed by centrifugation. After adjusting the pH of the supernatants to 3.1 they were put on Dowex-50 × 2 columns, 0.9 × 40 cm, previously equilibrated with sodium formate buffer, pH 3.1, of ionic strength 0.1. The columns were eluted with sodium buffers of ionic strength 0.1 with varying pH as seen from Fig. 2. The flow rate of the columns was about 1.5 ml/h. Hourly fractions were collected in a fraction collector. The peptide contents in the fractions were estimated with the colour reaction of Lowry *et al.*[11]

In the chromatograms of peptides from plasmin-digested fibrinogen eluted at pH 4.4 to pH 5.0, *i. e.* in the region where the fibrinopeptides A and B should appear [4], no peptides were found. However, when thrombin was added to the digestion mixture there appeared one peptide at pH 4.4 (A) and another peptide at pH 4.7 (B). With these exceptions the two chromatograms showed an identical pattern. Further it was shown that the first of these peptides (A) contained glutamic acid in N-terminal position, whereas no N-terminal residue could be detected in the second one (B). It is most probable that the peptides A and B, which are released through the action of throm-

bin on the fibrinogenolytic mixture, are identical to the peptides in the supernatant after clotting.

Our results indicate that through the action of thrombin the same peptide bonds are split in fibrinogen irrespective of whether its molecule is intact or has been digested with plasmin. Recently Niewarowski and Kowalski [12] have reported that at the digestion of pure fibrin and fibrinogen by plasmin there appears antithrombin activity in the digestion mixture. It is possible that the protein fragments which are formed during fibrinogenolysis and which are substrates for thrombin can act as competitive inhibitors in the reaction between thrombin and fibrinogen and thus contribute to the antithrombin activity. Work is in progress to study the conditions at maximal plasmin digestion and to isolate the protein fragments, that can be proteolysed by thrombin. A more detailed paper on this subject is to be published in *Arkiv för Kemi*.

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Chromic Acid Esters
A Correction

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In a recent paper evidence for the existence of 1:1 complexes of alcohols or acetaldehyde with acid chromate ion was presented. The equilibrium was written $X + HCrO_4^- = X \cdot HCrO_4^-$, where X stands for methanol, ethanol, propanol, 2-propanol, 2-butanol or acetaldehyde. But the equilibrium constants became incorrect as no allowance was made for the change of the total number of moles per liter with the alcohol or acetaldehyde concentration.

According to Westheimer *et al.*[2,3] the complexes formed in the alcoholic solutions are esters.

$$ROH + HCrO_4^- = ROCrO_3^- + H_2O$$

In solutions containing acetaldehyde the complex formed may be pictured as chromic acid ester of ethylideneglycol.

The equilibrium constant is

$$K_e = \frac{c_e \cdot \gamma_e \cdot c_w \cdot \gamma_w}{c_l \cdot \gamma_l \cdot c_a \cdot \gamma_a} \qquad (1)$$

c_e, c_w, c_l and c_a being molar concentrations of the ester, water, acid chromate and the alcohol, respectively. γ_e, γ_w, γ_l and γ_a are acticity coefficients. For some of the above-mentioned alcohols γ_w and γ_a are known [4,5], but since we do not know γ_e and γ_l we assume that $\gamma_l = \gamma_a \cdot \gamma_l$ and neglect variations in γ_w. This enables us to calculate values of K_e from the reported measurements of $\Delta\varepsilon$[1]. $\Delta\varepsilon$ is the difference between the extinctions at 375 mμ of two solutions both containing 10^{-4} M acid chromate and 10^{-3} M perchloric acid, and one of them also an alcohol or acetaldehyde in varying concentration. Assuming that the extinction coefficients are independent of alcohol or acetaldehyde concentration, we have $\Delta\varepsilon = \Delta\varepsilon_{KI} \cdot l \cdot c_e$, where l is the length of the optical path in cm and $\Delta\varepsilon_{KI}$ the difference between the extinction coefficient of the ester and that of the acid chromate ion at 375 mμ. On introduction of $\Delta\varepsilon$ and the total chromate concentra-