DETERMINATION OF *C*-TERMINAL AMINO ACID RESIDUES OF BOVINE FIBRINOGEN BY HYDRAZINOLYSIS

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1. Introduction

Fibrinogen has a molecular weight of 340,000 and is composed of three pairs of polypeptide chains, designated α, β and γ [1, 2]. The chemical structure of *C*-terminal regions had not been studied so extensively as that of the *N*-terminal regions. Recently Gerbeck et al. briefly reported that they found proline and valine from whole bovine fibrinogen and only valine from its γ-chain by hydrazinolysis [3]. However they did not present any quantitative data. Mills and Liener [4], and Chen and Doolittle [5], using carboxypeptidase A, also reported valine as *C*-terminal amino acid of the γ-chain of human and bovine fibrinogen. *C*-terminal amino acids of α- and β-chains have remained undetermined. The present paper describes a detailed study of the hydrazinolysis of bovine fibrinogen and suggests that, of α- and β-chains, one has *C*-terminal valine and the other *C*-terminal proline.

2. Materials and methods

2.1. *Purification of bovine fibrinogen*

Commercial fibrinogen (Cohn fraction I from bovine plasma, Armour Pharmaceutical Co.) was fractionated with ammonium sulfate according to Laki [6] and further purified by precipitation in the presence of L-lysine according to Mosesson [7]. Clottability of this preparation was 97%. The amount of protein was determined by measuring the absorbance at 280 nm of a weighed amount of protein dissolved in 8 M urea using $A^{0.1\%}_{1\,cm} = 1.51$ [8].

2.2. *Preparation of S-carboxymethylated fibrinogen*

Fibrinogen was reduced with dithiothreitol in the presence of 5 M guanidine hydrochloride, and was alkylated with monoiodoacetic acid. Amino acid analysis suggested that almost all cystine residues had been converted to *S*-carboxymethylcysteine residues. Details will be published elsewhere. Performic acid oxidation was carried out according to Hirs [9].

2.3. *Hydrazinolysis*

In the absence of catalyst. About 10 mg (30 nmoles) of fibrinogen and 50 nmoles of norleucine, which was added as an internal standard, were placed in a test tube (1 × 12 cm) and dried overnight *in vacuo* over P_2O_5. The content of the tube was dissolved in 0.5 ml of anhydrous hydrazine which had been prepared by distillation of an azeotropic mixture with toluene over calcium oxide [10]. After the sample had been frozen in a freezing mixture, the tube was vacuum-sealed and placed in an oven at 100° for several hours. After removal of hydrazine in vacuo over concentrated H_2SO_4, free amino acids were separated from the bulk of hydrazides with an Amberlite CG-50 (H^+), type II, column as described by Fraenkel-Conrat and Tsung [11]. The water eluate which contained acidic and neutral amino acids was directly analyzed on a Hitachi KLA-3B amino acid analyzer equipped with a scale-expanded recorder (0.1 A full scale). The ammonium acetate eluate (0.1 M, pH 7.0) which contained basic amino acids was analyzed after benzaldehyde treatment in order to eliminate ninhydrin positive material. Amounts of amino acids were corrected by the overall recovery of norleucine, which was 50–70%.

In the presence of catalyst [12]. The procedure

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was essentially the same as described above except that the reaction mixture contained about 50 mg of dry Amberlite CG-50 (H^+), type II, and was heated at 80° for longer periods (24–72 hr). After removal of hydrazine, the content of the tube, including the resin, was suspended in a small quantity of water and transferred onto the Amberlite CG-50 column and treated as described above.

3. Results and discussion

Fig. 1 shows the result of hydrazinolysis of native fibrinogen at 100° in the absence of catalyst. Amino acids found in a significant amount were aspartic acid, serine, proline, glycine and valine. An unknown ninhydrin positive peak (tentatively designated X), the amount of which was comparable to that of other amino acids, was observed between valine and methionine, slightly overlapping with valine, under the standard elution system [13] of the amino acid analyzer. However, the amount of neither amino acid exceeds one mole per 340,000 *g* of fibrinogen even after hydrazinolysis for 5 hr. Serine and glycine might be due to by-products because they had often been found in the hydrazinolysate of proteins having no *C*-terminal serine or glycine [12, 14]. Amino acids found in a negligible amount (less than 0.2 moles per mole of fibrinogen) were threonine, alanine and phenylalanine. No other amino acid was detected.

Since no definite conclusion was reached from the

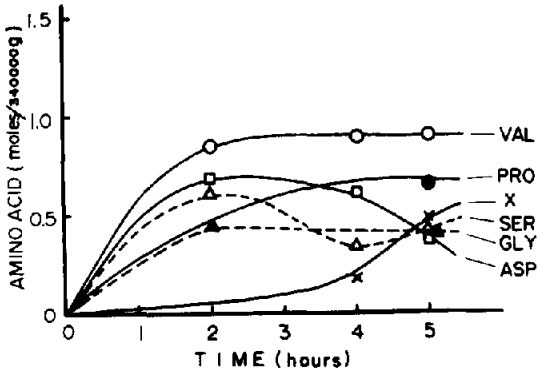

Fig. 1. Free amino acids released during hydrazinolysis of bovine fibrinogen at 100° in the absence of catalyst.

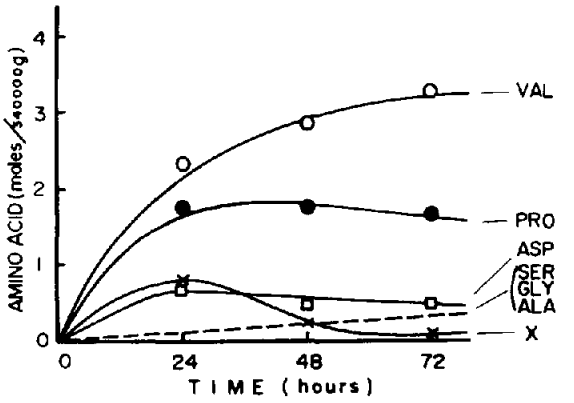

Fig. 2. Free amino acids released during hydrazinolysis of bovine fibrinogen at 80° in the presence of Amberlite CG-50 (H^+).

the experiments described above, hydrazinolysis in the presence of Amberlite CG-50 was attempted. The elution system of the amino acid analyzer was also slightly modified so as to elute valine in the first buffer (pH 3.25). This allowed complete separation of valine from X.

As shown in fig. 2, a remarkable improvement was achieved. Proline and valine became clearly distinguishable from other amino acids. Even after 24 hr, 1.8 moles of proline per one mole of fibrinogen was liberated and this value did not change until 72 hr. The amount of valine was about 2.3 moles after 24 hr, but increased to 3.3 moles after 72 hr. On the other

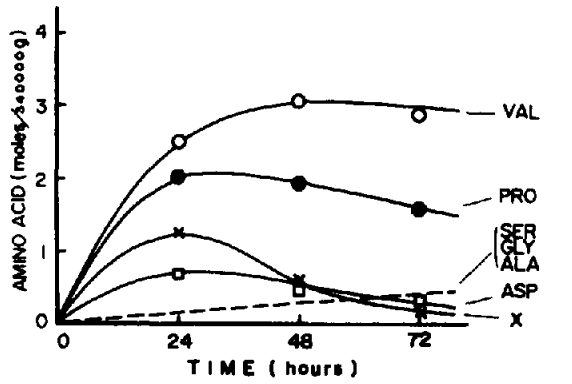

Fig. 3. Free amino acids released during hydrazinolysis of S-carboxymethylated bovine fibrinogen at 80° in the presence of Amberlite CG-50 (H^+).

hand, the amount of X became maximum after 24 hr and then decreased, suggesting that it may be a peptide including *C*-terminal valine (the molecular extinction coefficient of X was tentatively assumed to be the same as that of valine). Aspartic acid, the amount of which reached maximum at 24 hr and decreased gradually, is noticeable among other amino acids. It can not be attributed to a by-product. Braun et al. reported that free aspartic acid disappears slowly during hydrazinolysis [12]. Other amino acids may be regarded as by-products. The possibility of *C*-terminal phenylalanine, which once had been suggested [15], was completely excluded. Its amount was less than 0.2 mole per mole even after 72 hr.

Since cysteine and cystine are destroyed during hydrazinolysis, experiments on two derivatives of fibrinogen containing modified cysteine residues were performed. The result on *S*-carboxymethylated fibrinogen, shown in fig. 3, was essentially the same as that on native fibrinogen. The absence of free *S*-carboxymethylcysteine excluded the possibility of *C*-terminal cysteine or cystine. On hydrazinolysis of performic acid-oxidized fibrinogen also no cysteic acid was detected.

Possibilities of *C*-terminal asparagine and glutamine were examined by paper chromatography of a dinitrophenylated hydrazinolysate according to Kawanishi et al. [16], but no clear result was obtained.

These results strongly suggest the presence of four moles of *C*-terminal valine and two moles of *C*-terminal proline in one molecule of bovine fibrinogen. Apparently one of the two types of chains, α and β, has proline and the other had valine as *C*-terminal, since the γ-chain is known to have valine [3–5]. The fact that the amount of valine was somewhat lower than 4 moles and aspartic acid was always found in a significant amount might suggest the presence of micro-heterogeneities in fibrinogen as reported recently [3, 17]. Mills et al. and Chen et al. deduced the *C*-terminal sequence of γ-chain to be –Asp–Val–OH from experiments using carboxypeptidase A. However these experiments cannot exclude the possible existence of molecules which have *C*-terminal aspartic acid. To reach a definite conclusion, further investigations on the isolated chains would be required.

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