CARBOXYL-TERMINAL RESIDUES OF FIBRINOGEN AND FIBRIN

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SUMMARY: The carboxyl-terminal residues of bovine fibrinogen, fibrin and the chain peptides, α(A), β(B) and γ, isolated from it were determined by hydrazinolysis, digestion with carboxypeptidase and selective tritium labelling. The C-terminal ends of both fibrinogen and fibrin were identified as proline and valine, in the ratio of approximately 1:2. Proline was identified as the C-terminus of the α(A)-chain isolated, and C-terminal valine was found on both the β(B)- and γ-chain. On hydrazinolysis after selective tritium labelling of fibrinogen radioactive C-terminal valine was obtained.

Fibrinogen and fibrin from mammalian species are known to contain six, or rather three pairs of, N-terminal amino acids per molecule, assuming the molecular weight to be 340,000 (1-3). In these molecules one pair of N-terminal residues is tyrosine. The remaining two pairs differ in different mammalian fibrinogens, but are always glycine in fibrins (1).

There have been many studies on the N-terminal residues of fibrinogen and fibrin, but very few on the C-terminal residues. Bovine fibrin and fibrinogen have been suggested to have C-terminal phenylalanine (4,5). However, there is little information available on the C-terminal residues of individual chain peptides in the fibrinogen molecule. This paper reports the identification and quantitative estimation of the C-terminal ends of bovine fibrinogen and its derivatives.

MATERIALS AND METHODS

Bovine fibrinogen, which is about 98% coagulable, was prepared from pooled plasma by the glycine method (6), and fibrin was obtained by clotting the fibrinogen with human thrombin (7). DFP-treated carboxypeptidase A[EC 3.4.2.1] (68 units per mg protein) was purchased from Worthington Biochemical Corp., New Jersey. Bovine pancreatic ribonuclease A(lot, R70B-059) was a product of Sigma Chemical Co. Water containing 1.0 Ci per vial of tritium was obtained from New England Nuclear Corp., Boston. After sulfitolysis (3) of fibrinogen the α(A)-, β(B)- and γ-chain peptides were separated by column chromatography on CM-cellulose (8). The chain peptides isolated each gave essentially a single protein band on disc electrophoresis on polyacrylamide gel (9,10). Each chain peptide was also identified by the Edman method (1), and the well known N-terminal amino acids of the α(A)- and γ-chains were confirmed. The amino acid released by hydrazinolysis or digestion with carboxypeptidase was determined with an amino acid analyzer, model JLC-5AH, Japan Electron Optics Lab. Ltd., by the method of Spackman *et al.* (11).

RESULTS

Hydrazinolysis. Table I shows the results of hydrazinolysis (12) of fibrinogen and its derivatives. After hydrazinolysis of fibrinogen, proline and valine predominanted in the medium, and their quantities remained almost constant during hydrazinolysis. Other amino acids, including aspartic acid, glycine and serine were also found in the medium. However, their concentrations varied and for instance no aspartic acid was detected in the medium after 23 hours hydrazinolysis. Glycine and serine seemed to be

TABLE I

Hydrazinolysis of Bovine Fibrinogen and Its Derivatives[a)]

Material	Time (hours)	Val	Pro	Gly	Ser	Asp
		(mole per mole protein)				
Fibrinogen	3	1.62	0.84	0.52	-	-
	10	0.82	0.54	0.66	0.29	0.18
	23	1.02	0.53	0.36	0.17	-
Fibrin	23	0.94	0.37	0.46	0.22	0.33
α(A)-chain	12	0.13	0.41	0.20	0.14	0.12
	23	0.13	0.28	0.07	0.10	-
β(B)-chain	12	0.17	-	0.17	0.09	0.06
	23	0.19	-	-	-	-
γ-chain	3	0.43	-	-	0.15	0.17
	23	0.43	-	-	0.08	-
Ribonuclease[b)]	5	0.62	-	0.08	0.08	0.12

Material (0.1 μmole) was dissolved in 0.5 ml of distilled anhydrous hydrazine and heated in an evacuated, sealed tube at 100°C for the indicated times. Excess hydrazine was removed and the medium was passed through a column (1 x 3 cm) of Amberlite IRC-50 (H^+ form). The eluate with water (15 ml) was collected and freeze-dried. The residue was analyzed using an amino acid analyzer. a) Values are uncorrected and are calculated assuming the following molecular weights (8); fibrinogen (340,000), α(A)-chain (63,500), β(B)-chain (56,000) and γ-chain (47,000). b) Pancreatic ribonuclease with C-terminal valine was used as a control.

by-products formed during hydrazinolysis, because they were often found in the medium on hydrazinolysis of proteins which had no C-terminal serine or glycine. Thus, the presence of these amino acids was disregarded.

The quantities of valine and proline were maximal after hydrazinolysis for 3 hours (Table I). The uncorrected values for the yields of both amino acids were poor, but the molar ratio of valine to proline was always approximately 2 to 1. This suggests that two chain peptides in

the fibrinogen molecule probably have C-terminal valine while one has proline. The C-terminal amino acids of fibrin were identical to those of the parent fibrinogen molecule.

The S-sulfo-derivatives of the α(A)-, β(B)- and γ-chains isolated were subjected to hydrazinolysis (Table I). Proline was released predominantly from the α(A)-chain and valine from the β(B)- and γ-chains. Thus, all the C-terminal amino acids found in whole fibrinogen were found as main C-terminal components of the individual chain peptides.

Digestion with Carboxypeptidase A. Undenatured fibrinogen was digested with carboxypeptidase A (13). As shown in Table II, the predominant amino acids released in the initial stage of digestion were valine, aspartic acid and

TABLE II

Amino Acids Released from Fibrinogen by Treatment with Carboxypeptidase A[a)]

Time (hours)	Val (mole per mole protein)	Asp	Ala
0	trace	-	-
0.5	0.23	0.07	-
1	0.24	0.11	0.10
3	0.25	0.13	-

Material (1 μmole) was dissolved in 0.2 M N-ethylmorphorine acetate, pH 8.5, at a concentration of 5 mg protein per ml. Carboxypeptidase A(230 units) was added and the mixture was incubated at 37°C. At the indicated times volumes of the mixture containing 0.1 μmole of material were removed. The reaction was terminated by adding 9 ml of cold ethanol. After centrifugation the supernatant was evaporated to dryness and amino acids in the residue were analyzed in an amino acid analyzer.

a) Values were calculated taking the molecular weight of fibrinogen as 340,000.

alanine, although their recoveries were poor. Valine was released fastest. This amino acid must be liberated from the C-terminal end of the β(B)- or γ-chain peptide. As expected, digestion of the S-sulfo-γ-chain with carboxypeptidase A under the conditions described in Table II gave most rapid release of valine, and this was identified by two dimensional thin layer chromatography. No significant

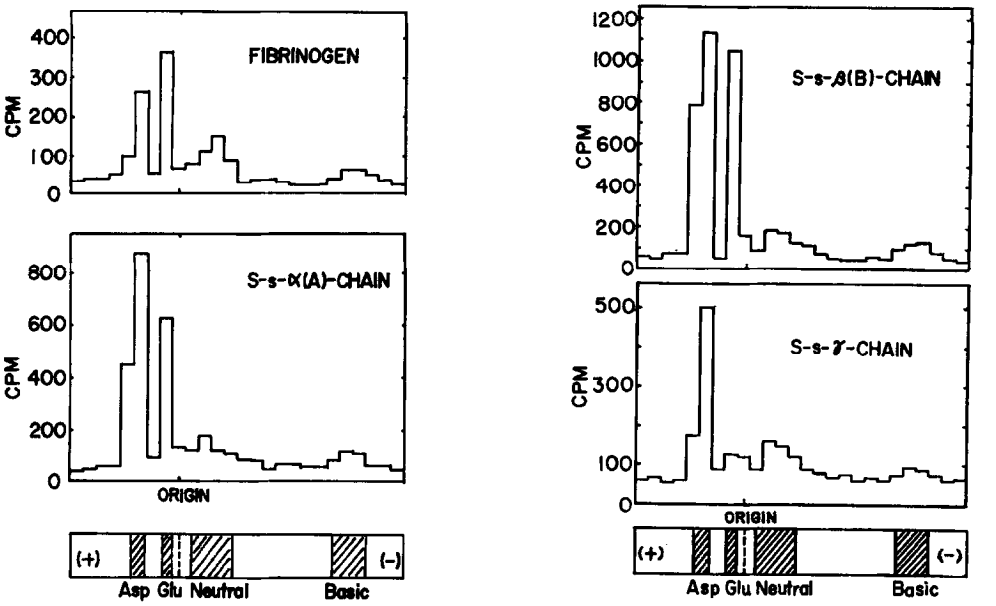

Fig. 1. Radioactive amino acids in hydrolyzates of fibrinogen and its derivatives after the tritium labelling procedure. Material (0.03-0.1 μmoles) was dissolved in 0.3 ml of tritium water (200 mCi/ml) containing 5M guanidine. Then 0.2 ml each of pyridine and acetic anhydride were added and the mixture was incubated for 2 hours at 25°C. The gel which formed was crushed with a glass rod and the same volumes of pyridine and acetic anhydride as before were again added. The gel suspension was then stood for 12 hours. Then, it was dialyzed extensively against distilled water and freeze-dried. The residue was hydrolyzed in 1 ml of constant boiling HCl at 110°C for 24 hours. The hydrolyzate was subjected to high voltage electrophoresis (50 volts per cm, pH 3.5) to identify the radioactive amino acid. For measurement of radioactivity, the chromatogram was cut into small pieces and each piece was counted directly in 10 ml of counting solution (5 g of PPO and 0.1 g of dimethyl-POPOP per liter of toluene) with a Beckman Liquid Scintillation Spectrometer, model LS250.

amount of any amino acid was released from fibrinogen or the α(A)-, β(B)- and γ-chain peptides by carboxypeptidase B.

Selective Tritium Labelling of C-terminal Amino Acid. The base catalyzed method of tritium labelling (14-16) was used to analyse the C-terminal amino acids, and results are shown in Fig. 1. After acid hydrolysis of the labelled proteins significant amounts of radioactivity were found in aspartic and glutamic acid. A small amount of radioactivity was also found in the region of the neutral amino acids on paper electropherograms. To identify labelled neutral amino acid, ^{3}H-fibrinogen (0.1 μmole) was subjected to hydrazinolysis, and then passed through a short column of Amberlite IRC-50 (H^{+} form). The fraction containing neutral and acidic amino acids was subjected to thin layer chromatography. As shown in Fig. 2, valine was identified as the radioactive compound. Proline, identified as the C-terminal residue of the α(A)-chain, was not labelled and this is reasonable, since C-terminal proline does not appear to form an oxazolone by reaction with acetic anhydride under the conditions used.

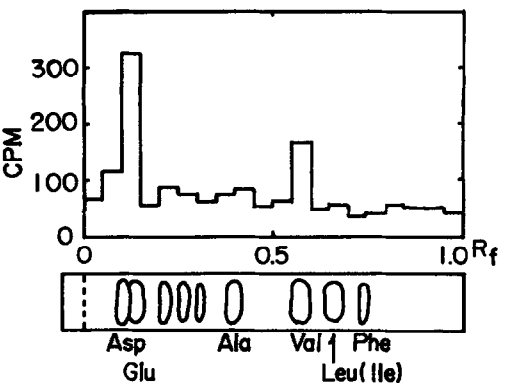

Fig. 2. Radioactive amino acids found in the medium after subjecting fibrinogen to the tritium labelling procedure and hydrazinolysis. The solvent for thin layer chromatography was phenol-water (75:25, w/w).

DISCUSSION

In 1955, Bailey and Bettelheim (4) reported that the amino acids liberated from bovine fibrinogen and fibrin by carboxypeptidase are similar, phenylalanine being the main component. This was later confirmed by Khodorava et al. (5) by hydrazinolysis. However, the present data do not support the above results, and no C-terminal phenylalanine was detectable in fibrinogen, fibrin or three chain peptides isolated from fibrinogen. We found C-terminal proline in the α(A)-chain and C-terminal valine in the β(B)- and γ-chains isolated. These findings are supported by the result that the ratio of C-terminal proline to valine released from whole fibrinogen by hydrazinolysis was calculated as approximately 1:2. Recently, C-terminal valine has been suggested to be present in the γ-chain peptide in bovine and human fibrinogens by Mills and Liener (17) and Gerbeck et al. (18). Moreover, Chen and Doolittle (19) studied the amino acid sequence of the C-terminal segment of bovine γ-chain, and identified valine as the C-terminus of the γ-chain. These findings are in good agreement with our own.

The finding of strongly labelled aspartic and glutamic acid after base-catalyzed selective tritium labelling was unexpected, since a number of proteins have previously been submitted to this method and only their C-terminal residues have been selectively labelled (cf. 14,15,18,19). Moreover, on hydrazinolysis after tritium labelling of fibrinogen labelled C-terminal valine was obtained. The radioactivity in the aspartyl and glutamyl residues of fibrinogen and its derivatives suggest that a peptide bond linked with the β- and γ-carboxyl groups of aspartyl and

glutamyl residues may be involved in the molecule. Theoretically, such isoaspartyl and isoglutamyl bonds could be labelled by the selective tritiation method in addition to the C-terminal end of protein (21). Further experiments are required to test this possibility.

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