ACTA CHEMICA SCANDINAVICA 20 (1966) 716–721

On the Nature of the Bovine E-Fibrinopeptides

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In addition to the main fibrinopeptides A and B a small fraction denoted "peptide E" is present in the clot-supernatant from bovine fibrinogen. This fraction has in the present paper been shown to consist of a mixture of fibrinopeptide A with blocked α-amino group and fibrinopeptide B lacking the C-terminal arginine.

In 1952 Lorand[1] and Bettelheim and Bailey[2] reported the isolation of a peptide fraction from the clot supernatant following the action of thrombin on bovine fibrinogen. Bettelheim and Bailey found this fraction to consist of two electrophoretically separable peptides which they called A and B. Using column chromatography on Dowex 50X2 Blombäck and Vestermark[3] isolated the A and B-fibrinopeptides and obtained also a more acidic fraction, comprising 10—15 % of the total, which they designated peptide E. The amino acid sequences of the A and B-peptides have been determined.[4,5] The present communication offers evidence that the E-peptide fraction is composed of two peptides which are variants of the A and B-fibrinopeptides.

Lyophilized E-peptide fractions, isolated earlier by Dowex 50X2 chromatography[3] and stored for 7 to 8 years, were pooled, dissolved in 0.1 M pyridine, desalted on Sephadex G-25 columns, lyophilized, dissolved in 0.1 M ammonium formate, pH 3.0 and rechromatographed as before[3] except that the pH was lower at the beginning and the stepwise increases were more gradual. From pH 3.0 through pH 4.0 (effluent volume 600 ml) three fractions were obtained (Fig. 1); further increases in pH to 4.8 through a total effluent volume of 1750 ml did not elute additional ninhydrin-reacting material.

The chromatographic fractions were subjected to horizontal paper electrophoresis for 4 h at room temperature in 0.1 M pyridine acetate, pH 4.1, at a potential gradient of 8.5 V cm^{-1}. The paper strips were stained using ninhydrin-[7] and Sakaguchi-[8] reagents. Fraction I (a brown, hygroscopic material) did not give spots with either reagent. Fraction II migrated 6 mm toward

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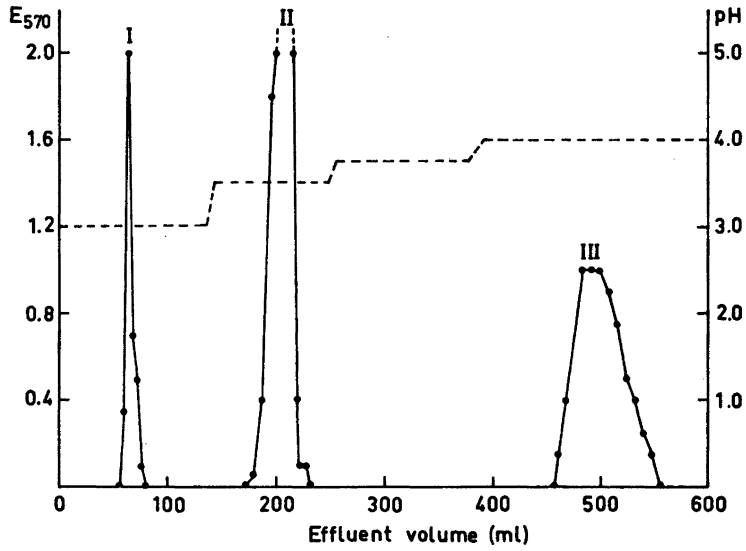

Fig. 1. Elution diagram of bovine E-fibrinopeptide fraction. Column: 0.9 × 90 cm. Dowex 50 × 2, 200–400 mesh, equilibrated with 0.1 M ammonium formate buffer, pH 3.0. Stepwise elution with 0.1 M ammonium formate, pH 3.0 to 4.0. Flow rate: 16 ml/h. Ninhydrin analysis after alkaline hydrolysis, *cf.* Refs. 4 and 6. Ninhydrin color ——, pH - - - -.

the cathode to give a broad, ninhydrin-positive band which was Sakaguchi negative. Fraction III moved 9 to 13 mm toward the anode in a narrow band which was blue with the ninhydrin reagent and was Sakaguchi positive. A reference sample of bovine A-fibrinopeptide migrated 4 mm toward the anode. Fractions I and II were not studied further.

Amino acid analysis of chromatographic fraction III (Table 1, column a) showed that it contained all of the amino acids of both A and B-fibrinopeptides.[9] It was assumed that the fraction was a mixture of the two peptides.

Since one group of amino acids (alanine, tyrosine, and lysine) occurs only in the B-peptide and another group (threonine, valine, leucine, and phenylalanine) occurs in unimolar amount in both A and B-peptides, it was possible to calculate amino acid compositions for hypothetical A and B-peptide components of fraction III which could then be compared with the values found for the whole fraction. Details of the calculation are given in the footnotes to Table 1. The degree of correspondence between values found and calculated is given in column e of Table 1, where a/d = 1 indicates perfect agreement. If normal structures are assumed for A and B-peptide components of the fraction, agreement is satisfactory for all residues except arginine, where the usual total of three residues gives a calculated value that is too high, so that a/d = 0.63. However, good agreement (a/d = 1.03) can be obtained by the assumption that the A and B components each contain one arginine residue. Thus, the data are consistent with a mixture of normal A-fibrinopeptide and a

Table 1. Amino acid composition and calculated peptide composition of bovine E-fibrinopeptide (fraction III).

Column	a	b		c		d	e
Peptide	Fraction III [a]	A component		B component		A + B component	a/d
Amino acid	μmole/mg found	R	μmole/mg calculated	R	μmole/mg calculated	μmole/mg calculated	
Aspartic acid	1.53	3	0.45	4	1.04	1.49	1.03
Threonine	0.39 [c]	1	0.15	1	0.26	0.41	0.95
Serine	0.31	2	0.30	0	0	0.30	1.03
Glutamic acid	1.11	2	0.30	3	0.78	1.08	1.03
Proline	0.66	2	0.30	2	0.52	0.82	0.81
Glycine	1.60	5	0.75	3	0.78	1.53	1.05
Alanine	0.26 [b]	0	0	1	0.26	0.26	1.00
Valine	0.41 [c]	1	0.15	1	0.26	0.41	1.00
Leucine	0.42 [c]	1	0.15	1	0.26	0.41	1.03
Tyrosine	0.24 [b]	0	0	1	0.26	0.26	0.92
Phenylalanine	0.42 [c]	1	0.15	1	0.26	0.41	1.03
Lysine	0.27 [b]	0	0	1	0.26	0.26	1.04
Arginine	0.42	1	0.15	2	0.52	0.67	0.63

[a] Hydrolyzed in 5.7 N hydrochloric acid for 22 h at 110°. Analysis based on the method of Moore and Stein, using a Technicon automatic system.

[b] Assumed to come, mole for mole, from B peptide only; mean (Ala, Tyr, Lys) = 0.26 μm 'e B peptide component/mg fraction III.

[c] Assumed to come, mole for mole of each peptide, from A + B peptides; mean (Thr, Val, Leu, Phe) = 0.41 μmole A + B peptide components/mg fraction III.

Thus, 0.41 μmole A + B peptide components −0.26 μmole B peptide component = 0.15 μmole A peptide component/mg fraction III.

R = residues of amino acids assumed for each peptide component of fraction III; *cf.* Ref. 4.

R × μmole component peptide = μmole component amino acid (columns b and c).

B-fibrinopeptide possessing one arginine instead of the usual two (see below). E-peptide fractions prepared from two other pools and analyzed in identical fashion gave the same results, except for variation in the proportions of the two components (B-peptide component: 46 %, 60 %, and 63 % on a molar basis).

Fig. 2. Amino acid sequence of bovine B-fibrinopeptide.[10] [GLU = pyroglutamic acid residue. TYS = tyrosine-O-sulfate residue. Arrow indicates tryptic cleavage site.

Evidence indicating which of the two expected arginine residues (Fig. 2) was missing from the B-peptide component was provided by analysis of fragments produced by tryptic digestion of fraction III. Such a treatment would

split the Lys-Val bond of the bovine B-fibrinopeptide,[4] but leave the A-peptide intact. A 100 μl digestion mixture containing 1 mg of peptide, 0.02 mg of trypsin,[11] 0.1 M triethylamine acetate buffer, pH 8.1, 0.05 M calcium chloride, and a drop of toluene was incubated for 17 h at 38°C and then subjected to paper electrophoresis as above. Three fractions with the characteristics given in Fig. 3 were obtained. Strips bearing these fractions were cut from the electrophoresis paper after drying at 60°C for 25 min and were eluted with water. The eluates were lyophilized, hydrolyzed (5.7 N hydrochloric acid, 110°, 22 h) and analyzed in a Technicon automatic amino acid analyzer. The compositions of the three fractions, are given in Table 2 along with previously published [4] normal compositions for A-peptide and for tryptic fragments of B-peptide. Fraction 2 has an amino acid composition characteristic of peptide A. The other two fractions are apparently fragments of a B-peptide lacking one arginine residue in the C-terminal end (Fig. 2). Fraction 1 has the amino acid composition of an N-terminal tryptic fragment of a B-peptide.

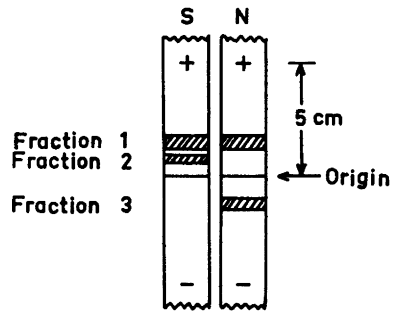

Fig. 3. Paper electrophoresis of tryptic digest of E-fibrinopeptide fraction. Conditions given in text. S = Sakaguchi. N = ninhydrin.

Fig. 3 records the unexpected finding that the A-peptide component (Fraction 2) was ninhydrin negative, suggesting that its terminal amino group was blocked in some way. This was substantiated by the fact that the E-peptide fraction did not contain an N-terminal amino acid accessible to degradation by the Edman phenylisothiocyanate method.[12]

That the association of the A and B-peptide components did not involve strong bonding was demonstrated by prolonged paper electrophoresis (15 h), using the conditions described above. The B-peptide component moved 24 mm toward the anode while the A-peptide component moved 19 mm. It was then possible, by amino acid analysis of eluates from electrophoretic strips, to verify directly that the intact B-peptide component lacked one arginine residue.

Thus, the bovine E-fibrinopeptide fraction is apparently a mixture of A-peptide with a blocked N-terminal glutamic acid residue and B-peptide lacking its usual C-terminal arginine. The relatively acidic chromatographic and electrophoretic behavior of the fraction is consistent with these alterations.

The chemical basis for blockage of the N-terminal amino acid of the A-peptide component is not known. One possibility is the formation of a pyrrolidone ring such as has been shown to be normally present at the amino terminus of the human B-fibrinopeptide [13] and most likely also in the bovine fibrino-

Table 2. Amino acid composition of tryptic fragments of fraction III.

Fraction	1 [a]		Try 2 B-1 [b]	3 [a]		Try 2 B-2 [b]	2 [a]		Peptide A [b]
Amino acid	μmole/mg	R	R	μmole/mg	R	R	μmole/mg	R	R
Aspartic acid	0.36	4	4	—	0	0	0.19	3	3
Threonine	0.09	1	1	—	0	0	0.06	1	1
Serine	—	0	0	—	0	0	0.10	2	2
Glutamic acid	0.27	3	3	—	0	0	0.13	2	2
Proline	0.19	2	2	—	0	0	0.12	2	2
Glycine	0.11	1	1	0.12	2	2	0.29	5	5
Alanine	—	0	0	0.05	1	1	—	0	0
Valine	—	0	0	0.05	1	1	0.06	1	1
Leucine	—	0	0	0.05	1	1	0.06	1	1
Tyrosine	0.08	1	1	—	0	0	—	0	0
Phenylalanine	0.09	1	1	—	0	0	0.06	1	1
Lysine	0.09	1	1	—	0	0	—	0	0
Arginine	0.09	1	1	—	0	1	0.06	1	1

[a] Refers to number assigned in Fig. 3. [b] From Ref. 4 included for comparison. R = residues.

peptide B.[5] Bettelheim [14] reported the formation of a similar peptide when a solution containing peptides A and B was taken to dryness in the presence of salt. This peptide was ninhydrin negative, unreactive with fluorodinitrobenzene, relatively acidic on electrophoresis and had the predominant amino acid pattern of the A-peptide, although B-peptide amino acids were present in lesser amount. Bettelheim considered pyrrolidone ring formation less likely than condensation of α-amino groups of A-peptide with free carboxyl groups of the A- or B-peptide. The last possibility can be excluded in the case of the E-peptide fraction, since, as noted above, its A and B components can be separated completely by electrophoresis. In any case, Bettelheim's observations suggest that the A-peptide component of the E-peptide fraction was probably derived from normal A-fibrinopeptide by modification during isolation.

The origin of the B-peptide component of the E-peptide fraction is also obscure. If it were derived from a normal B-peptide, enzymatic (*e.g.*, carboxypeptidase) cleavage of the Ala-Arg linkage (Fig. 2) might be suspected. Thrombin is unlikely as the responsible enzyme because of the narrow specificity of its proteolytic action. For the same reason, it is improbable that thrombin would have split an Ala-Gly bond between the parent fibrinogen molecule and a B-peptide biosynthetically lacking a C-terminal arginine. It is of interest that B-peptides lacking C-terminal arginine have now been found in small amount also in the cases of reindeer, pig, and bison.[15]

Acknowledgements. We are indebted to Mrs. Birgit Hessel for skillful assistance. This investigation was supported, in part, by Grant No. HE 7379—03 from *National Institutes of Health*, U.S.A.

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Received November 22, 1965.