The Sequence of Amino Acids at the N-terminal End of Bovine Fibrinopeptide B

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Earlier reports on the sequence of amino acids in bovine fibrinopeptide B have not been in agreement. Sjöquist *et al.* [1] established the sequence of amino acids in the peptide with the exception of the N-terminal tetrapeptide. The N-terminal amino acid of the B-peptide was not accessible to degradation by the Edman method [2], and the suggestion was made that the terminal amino group was either covered by some accessory group or that a ring structure existed. However, repeated N-terminal analyses on the same peptide sample revealed small amounts of glutamic acid. On subsequent degradations, trace amounts of different amino acids in the following order appeared: phenylalanine, proline, threonine. In each step accumulation of the preceding amino acids occurred. This finding suggested that a glutamic acid derivative was N-terminal in the tetrapeptide and that the sequence of the amino acid residues was: Glu-Phe-Pro-Thr. In a later preliminary report Folk and Gladner [3] reported that the N-terminal sequence of the bovine fibrinopeptide B was N-acetyl-Thr-Glu-Phe-Pro-. Those authors have since referred to the preliminary note as the final word in the structural determination of the peptide [4].

Recently evidence for the occurrence of an N-terminal pyroglutamyl residue in human fibrinopeptide B has been put forward [5]. As regards the bovine B-peptide, the results by Sjöquist *et al.* suggest that such a residue might also be N-terminal in this peptide. The correct sequence of the bovine fibrinopeptide B took on added importance during our study of the sequence of fibrinopeptides from related species in an effort to chart the nature of amino acid substitutions during evolution [6,15].

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Repeated attempts by us to reproduce the experiments of Folk and Gladner [3], utilizing pepsin digests of desulphated bovine fibrinopeptide B, have failed. Under no conditions have we been able to isolate the critical fragments which those authors reported. For these reasons, a variety of other methods were employed in an effort to establish the nature of the terminal tetrapeptide.

Bovine fibrinopeptide B was isolated after chromatography on Dowex 50 × 2 columns as described previously [7]. The terminal tetrapeptide, which is ninhydrin negative, was readily obtained by two independent methods. In the first these, it was produced by hydrolysis [8] of the fibrinopeptide in 1 % acetic acid (pH = 2.8) in a sealed tube at 100°C for 16 h. Only aspartic acid links were appreciably split under these conditions. Paper electrophoresis of the hydrolysate in 0.1 M pyridine-acetate buffer, pH 4.1, revealed several ninhydrin positive components. On chlorination [9] an additional band was disclosed. The ninhydrin-negative fragment was isolated in good yield after column chromatography on a 0.9 × 70 cm Dowex 50 × 2 column (Fig. 1). All other portions of the degraded fibrinopeptide in the hydrolysate could be accounted for after two-dimensional paper electrophoresis-paper chromatography (0.1 M pyridine-acetate, pH 4.1, butanol-acetic acid-water, 4:1:5). Alternatively, the tetrapeptide was isolated after a 24 h incubation with subtilisin as described in the original report by Sjöquist *et al.*[1] The fragments obtained with subtilisin could also be isolated in good yield by column chromatography.

After acid hydrolysis the tetrapeptide yielded approximately equimolar amounts of glutamic acid, phenylalanine, proline and threonine. The peptide was resistant to enzymatic degradation by chymotrypsin, carboxypeptidase A (Sigma), pronase, and pepsin (Worthington). The resistance to chymotrypsin and carboxypeptidase (*cf.* Ref. [10],) is consistent with both of the previously proposed structures [1,3]. In the case of pepsin, however, the results are in direct contrast to the splitting claimed by Folk and Gladner [3], who cited splits on both sides of the phenylalanine residue.

Partial acid hydrolysis of 1 mg tetrapeptide in 100 μl 5.7 N HCl for 20 min in a boiling water-bath yielded each of the constituent amino acids together with a variety of ninhydrin positive peptides which were isolated after two-dimensional paper electrophoresis-paper chromatography, eluted with water, hydrolyzed in 5.7 N HCl and analyzed for

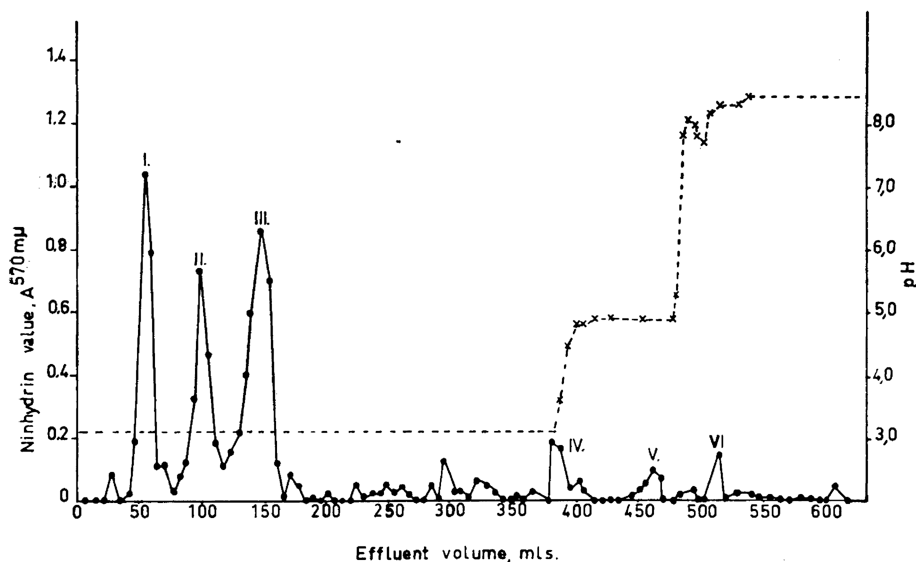

Fig. 1. Chromatogram of an acetic acid hydrolysate of bovine fibrinopeptide B. Columns: Dowex 50 × 2, 200−400 mesh, 0.9 × 70 cm. The column was equilibrated with 0.1 M ammonium formate buffer, pH 3.0 Elution was performed stepwise with the following buffers: 1) 0.1 M ammonium formate, pH 3.0. 2) 0.1 M ammonium formate, pH 5.0 and 3) 0.1 M ammonium carbonate, pH 8.0. The ninhydrin values are obtained after alkaline hydrolysis of an aliquot of the effluent fraction (see Ref.[1]). Peak II contains the N-terminal tetrapeptide. Ninhydrin; ●—●, pH; × — — — ×.

amino-acids in the two-dimensional system described. Of the different fragments isolated, the tripeptide composed of phenylalanine, proline and threonine was of particular interest because it was irreconcilable with the structure of Folk and Gladner but in conformity with the structure proposed by Sjöquist *et al.* All other fragments identified were also consistent with the structure proposed by the latter authors.

The terminal tetrapeptide (0.52 mg) was subjected to hydrazinolysis [11] with anhydrous hydrazine (100 μl) in a sealed tube at 100° for 10 h. After removing excess hydrazine in vacuo over H_2SO_4, the residue was dissolved in water and subjected to two-dimensional paper electrophoresis-paper chromatography. The only free amino acid obtained was threonine. The implication was that threonine was C-terminal. The separation of hydrazides in the hydrazinolysate by paper chromatography in lutidine-water (5:3) [12] or paper electrophoresis at pH 4.1 with subsequent development with ammoniacal $AgNO_3$ was less clear, however, but no trace of acetyl hydrazide could be observed. Furthermore, no evidence for the presence of an acetyl group could be obtained after methanolysis [13] of 2.2 μ-moles of fibrinopeptide B (Table 1). Before methanolysis the fibrinopeptide had been freed from trace contaminations of acetate by passage over a Sephadex G-25 column (2 cm^2 × 40 cm; flow-rate: 0.5 ml/cm^2/min) equilibrated with 0.05 M pyridine.

The final elucidation of the amino acid sequence of the peptide was attained utilizing experimental conditions described by Dekker *et al.* [14] in their examination of the tripeptide fastigiatin, which has pyroglutamic acid as N-terminal residue. These authors were able to open the terminal pyrrolidone ring by mild alkaline hydrolysis without breaking the rest of the peptide chain. Accordingly, 8.3 mg of bovine fibrinopeptide B were dissolved in 0.5 ml 1 N NaOH and left at room temperature in a stoppered tube. After 24 h, 0.9 ml of 0.5 N acetic acid were added to partially neutralize the mixture. The preparation was desalted by passage through Sephadex G-25 equilibrated with 0.05 M pyridine. After freeze-

Table 1.

Sample	Added µmole	Methyl acetate recovered	
		µmole	Mole per mole sample
B-peptide	2.23*	0.15	0.07
N-acetyl phenylalanine	2.05	2.10	1.03
» »	4.25	4.29	1.01
N-acetyl glutamic acid	1.96	1.04	0.53
» » »	4.29	4.25	0.99

* Mol. wt. 2400.

drying, two major components could be identified on paper electrophoresis in 0.1 M pyridine-acetate buffer, pH 4.1; one with an anodic mobility greater than that of the untreated fibrinopeptide and one slower moving anodic component. Qualitative amino acid analysis of both bands after elution and acid hydrolysis revealed the presence of all the constituent amino acids of the B-peptide. The mobilities of the two bands coincided with expectations of (1) a bovine fibrinopeptide B with its amide nitrogen removed and (2) a deamidinated fibrinopeptide which had a N-terminal pyrrolidone ring opened. N-acetylglutamic acid and pyroglutamic acid were also exposed to the same treatment with 1 N NaOH. The N-acetyl-glutamic acid remained ninhydrin negative but could be detected by chlorination. It moved with the same mobility as its control. The alkali treated pyroglutamic acid exhibited two components, one of which was ninhydrin positive and corresponded to glutamic acid, and a second ninhydrin-negative component that moved with the same mobility as the control pyroglutamic acid.

The desalted alkali treated peptide mixture (3.1 mg) was subjected to N-terminal analysis by the Edman stepwise degradation procedure [2]. The first step yielded glutamic acid in 30 % yield based on the total amount of fibrinopeptides present (1.3 µ-mole). Two subsequent residues were obtained in 100 and 42 % yield respectively of the preceding steps. The residues obtained were phenylalanine and proline, respectively. The experimental data presented above suggest that the amino acid sequence of the N-terminal tetrapeptide of bovine fibrinopeptide B is: Pyr-Phe-Pro-Thr-, where Pyr represents a pyroglutamyl residue, probably formed by the internal condensation of a glutamine residue, either during fibrinogen biosynthesis or during isolation. Fig. 2 shows the suggested complete structure of bovine fibrinopeptide B as compiled from the data of the present authors and those of Sjöquist *et al.*

It is possible, of course, that intraspecific variations exist among the fibrinopeptides and that the fibrinopeptide B isolated by Folk and Gladner from cows in the United States has a different amino acid sequence than that obtained from cows in Sweden. Current studies [15] in our laboratory on the sequence of fibrinopeptides isolated from four other genera of the order Artiodactyla, however, indicate that the particular change in question would be unlikely.

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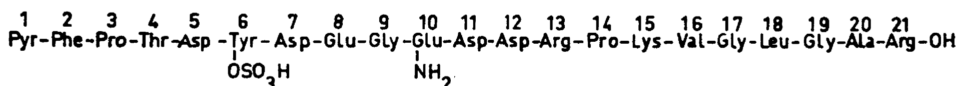

Fig. 2. Deduced amino acid sequence of bovine fibrinopeptide B.

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Amino Acid Sequence Studies on Fibrinopeptides from Several Species

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N-Terminal amino acid analyses of fibrinogen from several mammalian species indicate that the fibrinogen molecule is built up of several peptide chains [1-5]. A common feature in mammalian fibrinogens so far investigated is the occurrence of the same number of N-terminal tyrosyl residues per unit weight of protein. These tyrosyl residues seem to have replaced N-terminal seryl residues in certain lower vertebrates.[6] As regards the other N-terminal chains demonstrated in different fibrinogens, an extreme qualitative variability has been noted among the N-terminal amino acids from different species.[4-6] During the transformation in different species of fibrinogen to fibrin, produced by thrombin, peptides (fibrinopeptides) are split off from the N-terminal parts of the molecule.[7-14] As a result of this limited proteolysis, N-terminal glycine residues are exposed in the different fibrins.[1-6] The N-terminal tyrosyl chains are not involved in this proteolysis.

The amino acid sequences of the fibrinopeptides exhibit marked species specificity[11], although it has recently been shown[12,14] that the C-terminal regions of bovine and human fibrinopeptides show interesting similarities. Thus a C-terminal sequence of six amino acid residues is identical in human and bovine fibrinopeptide A. The present study was undertaken in order to determine the extent of structural differences among fibrinopeptides from a variety of mammals. Complete or partial amino acid sequences for fibrinopeptides from pig, sheep, goat, reindeer, and rabbit are contained in this report.

Fibrinogen was prepared [15] from pooled citrated plasma from a number of animals from each species. Clotting was effected in all cases with purified bovine thrombin (about 200 NIH units/mg), and the peptides released were isolated as described previously [10]. The amino acid compositions of the various peptides were mainly determined by column chromatography [16]. The amino acid composition of pig and rabbit fibrinopeptides have been reported earlier [11]. Amino acid sequences of whole peptides and peptide fragments were determined by the phenylisothiocyanate method of Edman [17] in its three-stage form [18], and the phenyl thiohydantions (PTH) identified by paper chromatography.[19,20]. In order to determine the complete amino acid sequence by the stepwise degradation method, 7–10 μ-moles of the peptides were used. When only small amounts of peptides were available the step-wise degradations were supplemented by enzymatic and chemical degradations followed by amino acid analyses of acid (HCl) hydro-

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