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A Phosphopeptide Isolated from Bovine α-Casein after Tryptic Hydrolysis

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Bovine α-casein has been subjected to a short-time tryptic hydrolysis. From the mixture of phosphopeptides in the hydrolysate an apparently homogeneous phosphopeptide has been isolated through zone electrophoresis. From the amino acid composition and phosphorus analyses the empirical formula is suggested to be: Asp_3, Glu_9, Gly_1, Ala_2, Val_2, $Ileu_3$, Met_2, Ser_5, Thr_1, Pro_1, Lys_{1-2}, $(PO_4)_6$. The N-terminal position is occupied by an aspartic acid residue and a lysine residue is probably in the C-terminal position.

Enzymic degradation of cow's casein with pepsin, followed by pancreatic enzymes, yields a mixture of phosphorus-containing peptides [1,2], which has been prepared and studied by several authors; the literature has been reviewed by Mellander [3] and Perlmann [4]. Attempts to purify such a mixture at this institute resulted in the isolation of a phosphopeptide consisting of eleven amino acids and three phosphate groups [5].

Peterson *et al.*[6] diminished the number of possible phosphopeptides resulting from hydrolysis of casein by considering the inhomogeneity of this protein and by using one specific enzyme for the degradation. These authors thus found that only one phosphopeptide could be recovered after treatment of β-casein with trypsin. On the other hand, they found that tryptic digestion of α-casein furnished at least three different phosphopeptides. Later, Pantlitschko and Gründig [7,8] reported the preparation of "phosphopeptones" from α- and β-casein.

As part of a study of the structure and metabolic significance [9,10] of phosphopeptides from bovine casein this paper describes the preparation and main composition of an apparently homogeneous phosphopeptide from α-casein. The isolation of this phosphopeptide was undertaken through zone electrophoresis of a crude phosphopeptide fraction, obtained from a short-time tryptic hydrolysate of the α-casein.

MATERIAL

α-Casein was prepared from bovine casein according to the method described by Hipp *et al.*[11] The product obtained was homogeneous in moving boundary electrophoresis (0.1 M veronal buffer, pH 8.7). The preparation showed an atomic ratio N/P of 32.9.

The trypsin was a commercial preparation (Armour, twice crystallized, containing 50 % $MgSO_4$).

METHODS

Tryptic hydrolysis and preparation of a crude phosphopeptide fraction. 30 g of α-casein were suspended in 1 litre of distilled water, and the pH adjusted to 8.0 by addition of N NaOH. The solution was incubated in a water bath at 25°. Two hundred mg of trypsin, dissolved in a small volume of distilled water, were added to the substrate. The hydrolysis was carried out at a constant pH recorded by a pH-meter, titrator TTT 1a (Radiometer, Copenhagen). The course of hydrolysis was followed by measuring the amount of 0.5 N NaOH necessary for maintaining the pH at 8.0 in the reaction mixture. The reaction was almost complete after 50 min, and interrupted by boiling the solution for 2 min. The solution was cooled and the pH rapidly adjusted to 4.6 by addition of glacial acetic acid. Small amounts of precipitate was filtered off through a fluted paper (Munktell 95). From the slightly opalescent filtrate, a crude fraction containing phosphopeptides was precipitated by adding saturated lead acetate [8]. The precipitate was suspended in distilled water and the pH adjusted to 7.5 with NaOH. The lead was removed by a stream of hydrogen sulphide, the lead sulphide centrifuged off, and the supernatant precipitated by addition of several volumes of absolute ethanol. After lyophilization, the yield of substance was 2.2 g. Analyses of nitrogen and phosphorus gave an atomic ratio N/P of 6.2. In order to find out whether any carbohydrate from α-casein [12] could be recovered in this fraction, analyses of hexose [13], hexosamine [14] and sialic acid [15] were carried out. Only small amounts (0.12 %) of hexose could be detected.

Electrophoresis. Moving boundary electrophoresis of the crude fraction was run in 0.1 M sodium acetate buffer at pH 4.6. Three peaks appeared. Paper electrophoresis (apparatus manufactured by LKB, Stockholm; Schleicher and Schüll paper 2043 B, 4 × 41 cm) in 0.2 M formic acid, adjusted to pH 2.8 with triethyl amine, revealed at least six ninhydrin-positive zones (0.3 % ninhydrin dissolved in ethanol:glacial acetic acid, 99:1, v/v) (Fig. 1A). At least four of these gave positive reaction with the phosphorus reagent described by Hanes and Isherwood [16] (Fig. 1B).

Isolation of a zone electrophoretic component. Small amounts (20—30 mg) of the fastest moving component was eluted from cut out strips of 40 electropherograms with distilled water, after localization of zones in ultraviolet light. Up to 3 mg of crude phosphopeptide could be applicated on every paper. Re-electrophoresis under the same conditions of the eluted fraction showed an essentially homogeneous component (Fig. 1C). Isolation of other components in pure form by this method was not very successful.

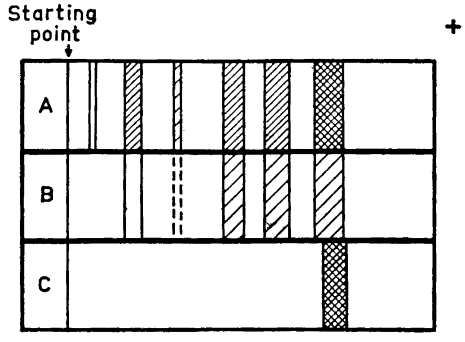

Fig. 1. Paper electrophoretic patterns. A. Crude phosphopeptide, ninhydrin color. B. Crude phosphopeptide treated with phosphorus reagent. C. Isolated component, ninhydrin color.

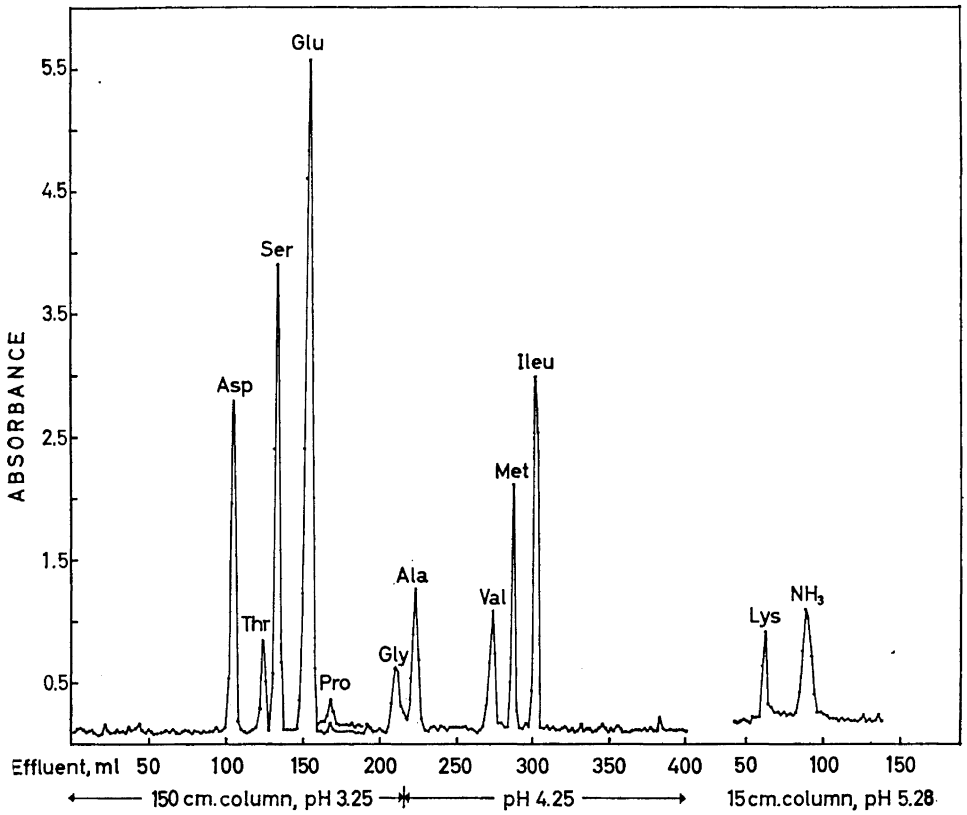

Fig. 2. Amino acid chromatogram according to Moore *et al.*[19] of the isolated component. The values of absorbance for lysine and ammonia should be multiplied with 2.6 to correspond to those of the other amino acids.

Analysis of the isolated component: N 8.56; P 3.20; S 1.1 % (dry weight, not corrected for ash content).* Atomic ratio N/P and N/S was calculated to 5.9 and 21.7, respectively.

N-Terminal amino acid residues were determined by Sanger's dinitrophenylation method[17]. The DNP-peptide was hydrolysed in 5.7 N HCl at 108° in sealed tubes for 10 and 16 h. Two-dimensional descending chromatography was carried out on Whatman No. 1 filter paper, using *tert*-amylalcohol: 0.1 M phthalate buffer, pH 6.0, in the first, and 1.5 M phosphate buffer, pH 6.0, in the second direction. This system was found to give satisfactory separation of DNP-amino acids. In the ether phase, DNP-aspartic acid only could be detected, whereas ε-DNP-lysine was present in the water phase.

Quantitative amino acid analysis. The amino acid composition of the isolated fraction was determined by the dinitrophenylation method as described by Koch and Weidel[18]. The chromatographic separation of the DNP-amino acids was, however, performed in the solvents mentioned above. Amino acid analysis was also performed by the chromatographic procedure on Amberlite IR-120, recently described by Moore *et al.*[19] A chrom-

* We are indebted to Analytiska Laboratoriet, Kemiska Institutionen, Lund, Sweden, for these analyses.

Table 1. Amino acid composition of the isolated phosphopeptide fraction expressed as molar ratios. The values are corrected for blank, and the molar ratios stated are based on the minimum glutamic acid content, taken as 9.00.

Method	Duration of hydrolysis, h	Molar ratios of amino acids											
		Asp	Glu	Gly	Ala	Val	Ileu	Met	Ser	Thr	Pro	Lys	NH_3
M and St *	22	3.40	9.00	1.07	1.86	1.57	2.91	1.56	4.70	0.93	1.12	2.45	7.73
K and W **	22	3.50	9.00	0.91	1.97	1.82	2.71	1.92	4.86	0.92	1.11	0.97	—
K and W	72	3.14	9.00	0.94	1.94	2.06	2.99	2.22	4.05	0.93	0.94	1.01	—
Probable minimum number of amino acid residues		3	9	1	2	2	3	2	5	1	1	1—2	—

* According to Moore and Stein [19].
** According to Koch and Weidel [18].

atographic run is shown in Fig. 2. In order to certify the identity of the three last peaks emerging from the 150 cm column, a known amount of leucine was added in one of the runs. This resulted in an extra peak, corresponding to the quantity of leucine added.

No tryptophan could be detected in the isolated fraction, using the method of Spies and Chambers [20].

RESULTS AND DISCUSSION

It is evident from the moving boundary electrophoresis and zone electrophoresis (Fig. 1) that a mixture of phosphopeptides is obtained by tryptic hydrolysis of α-casein. One of these zone electrophoretic components was isolated and its amino acid composition determined, the results of which are shown in Table 1. It is evident from this table that the same main amino acids, *i.e.* glutamic acid, serine, *iso*leucine, and valine are found in the present peptide, as earlier reported for phosphopeptides obtained from whole casein [5,10], as well as from α- and β-casein [6–8] after enzymic hydrolysis.

The results of paper electrophoresis, N-terminal amino acid analysis and quantitative amino acid analysis, indicate that the apparently homogeneous component may constitute a single peptide. If this is true, the minimum molecular composition of the phosphopeptide will be 30 amino acid residues and 6 phosphate groups, corresponding to a minimum molecular weight of about 4 000. The N-terminal position is occupied by aspartic acid and the C-terminal might be a lysine residue which would be in accordance with the specificity of trypsin.

The fact that the bovine α-casein fraction gives several phosphorus-containing peptides after tryptic hydrolysis has previously been pointed out by Peterson *et al.*[6], who found at least three peaks in boundary electrophoresis of the phosphopeptone fraction from a tryptic digest of α-casein. No data concerning these phosphopeptides were given by them. Boundary electrophoresis of our crude phosphopeptide preparation also revealed the existence of three or more components. Pantlitschko and Gründig [7] have recently reported the isolation of a "phosphopeptone" after tryptic digestion of α-casein.

It seems remarkable that no basic amino acids were found in their preparation and, furthermore, it contained a certain amount of acetylglucosamine. No certain criteria of homogeneity of the "phosphopeptone" are to be found in their communication. It seems also likely that α-casein, in accordance with our results, should furnish several phosphorus-containing peptides, as it is known that the α-casein fraction from cow's milk consists of at least two phosphoprotein components [21,22].

Further investigations concerning the separation of phosphopeptides from bovine α-casein by means of ion exchange chromatography and zone electrophoresis are in progress.

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