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Phosphopeptides from a Tryptic Hydrolysate of Human Casein

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Phosphopeptides have been isolated by anion exchange chromatography from a tryptic hydrolysate of human casein. The homogeneity of the phosphopeptide with the highest phosphorus content has been demonstrated and its amino acid composition and terminal amino acid residues determined. It contains 19 amino acid residues and 5 phosphate groups and appears to have the composition

$$Arg[Thr_2, Ser_5, Glu_5, Ileu_3, Leu, (PO_4)_5]Tyr, Lys$$

one of the glutamic acid residues possibly being in amide form.

Phosphopeptides from enzymatic hydrolysates of cow's casein have been isolated and characterized by several authors [1–8]. Phosphopeptides from human casein on the contrary have so far been investigated only by Mellander and collaborators [1–2], who studied the qualitative difference in the amino acid composition of phosphopeptone from cow, goat and human casein by a paper chromatographic method.

The aim of this investigation was to isolate phosphorylated peptides from a tryptic hydrolysate of human casein and to determine their amino acid composition.

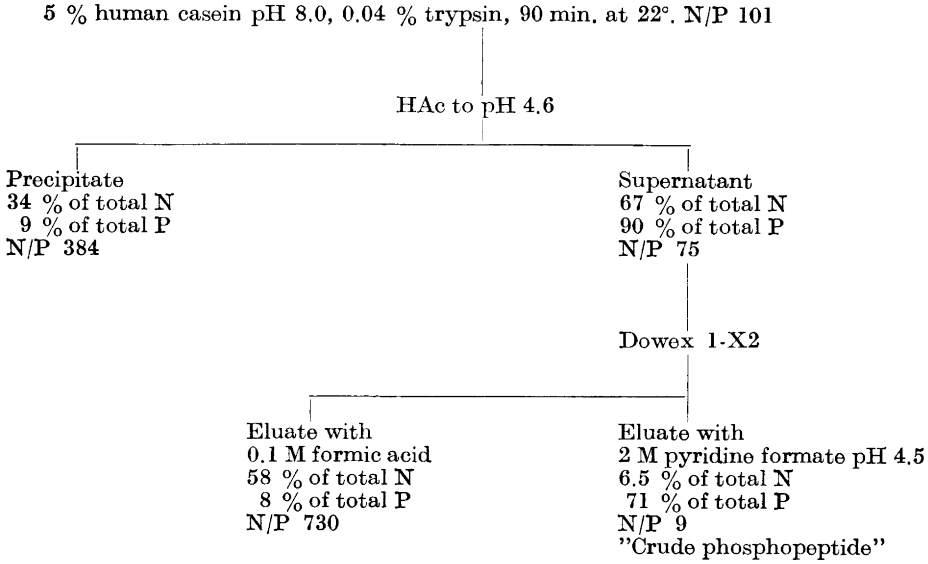
MATERIALS AND METHODS

Preparation of human casein. Human casein was prepared according to Johansson and Strid [9]. The technical procedure was as follows:

8 l pooled human milk was defatted by centrifugation and passed through a 3.5 × 30 cm column of Amberlite IRC 50 in Na^+-form equilibrated with 0.1 M NaAc–HAc buffer pH 5.8 in order to remove the main part of calcium. The flow rate was 30 ml per min. The casein was precipitated by acidification with glacial acetic acid to pH 4.6 and heating to 35° during 30 min. After centrifugation the casein was washed with 0.1 M NaAc–HAc buffer pH 4.6, followed by methanol:chloroform (1:1), acetone and ether. Finally the casein was filtered off and airdried.

Degradation of human casein by trypsin. 20 g human casein suspended in 400 ml water was dissolved by automatic addition of 0.1 M NaOH while the pH was kept constant at 8.0. A clear solution was obtained. Trypsin (50 % $MgSO_4$, 2 × cryst. Mann Lot No. B1401) was added to give a weight ratio enzyme/substrate of 1:130. The hydrolysis was performed at 22°. The pH was maintained at 8.0 by automatic addition of 0.1 M NaOH. After 90

PREPARATION SCHEME



min. (50 ml 0.1 M NaOH) the reaction was interrupted by addition of 1 M HAc to pH 4.6. The precipitate obtained was removed by centrifugation and the clear supernatant lyophilized.

Separation and isolation of phosphopeptides from the tryptic hydrolysate. Column I. 10 g lyophilized hydrolysate dissolved in 150 ml water was applied to a 2.8 × 7 cm column of Dowex 1-X2 in formate form. 0.1 M formic acid was added until the effluent gave a negative ninhydrin reaction. The column was then eluted with 2 M pyridine formate at pH 4.5, whereby a fraction containing 71 % of the casein phosphorus and 7 % of the nitrogen was obtained. This fraction was lyophilized.

Column II. To separate the phosphopeptides in the crude fraction, the method described earlier was used with small modifications [10]. 850 mg of the crude fraction was dissolved in 30 ml water and 1 N NaOH was added to pH 6. The solution was applied to a 1.6 × 40 cm column of Dowex 1-X2(200–400 mesh) in formate form. A gradient elution to 2 M pyridine formate pH 4.5 was immediately started. The mixing chamber (constant volume) was filled with 1 l 0.1 M formic acid. The flow rate was 1.5 ml per min. and fractions of 12 ml were collected. 0.5 ml of each fraction was analysed with ninhydrin according to Moore and Stein [11] in order to locate the peaks (see Fig. 1). The fractions of each peak were combined and lyophilized. To remove remaining pyridine the lyophilized material was dissolved in water and added to a 1 × 3 cm column of Dowex 50-X12 (H^+). The phosphopeptides were washed through with 0.1 M formic acid and then lyophilized.

Amino acid determination. Quantitative amino acid analysis was performed with column chromatography according to Moore *et al.*[12] Samples were hydrolysed for 22.5 and 90 h at 110° in 6 N HCl.

Two-dimensional paper chromatography in the solvent systems butanol:acetic acid:water (4:1:5) and phenol containing 0.1 % ammonia was used for qualitative amino acid determination.

Paper electrophoresis. The buffer used for paper electrophoresis was 0.1 M pyridine acetate pH 5.0 [13]. Satisfactory resolution of phosphopeptides was obtained after 3 h and 10 V/cm.

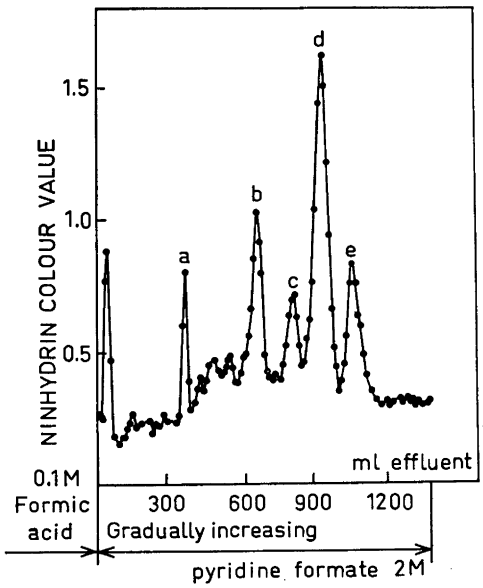


Fig. 1. Chromatogram obtained by the separation of the phosphopeptides from a tryptic hydrolysate of human casein on a 1.6 × 40 cm Dowex 1-X2 column. 0.5 ml of each fraction was used for the ninhydrin reaction [11].

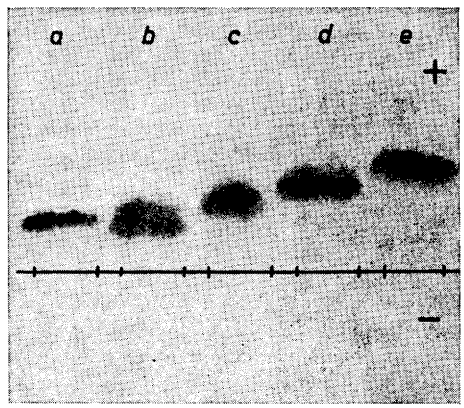


Fig. 2. Paper electrophoretic pattern of the isolated phosphopeptides, ninhydrin colour. Buffer: 0.1 M pyridine acetate pH 5.0 (10 V/cm, 3 h).

Determination of N-terminal amino acid residues. N-Terminal analysis was performed with the DNFB-method [14]. After hydrolysis liberated DNP-amino acids were identified by paper chromatography in the solvent system *tert.* amyl alcohol:biphthalate [15].

Determination of the atomic N/P ratio. Phosphorus was determined according to Dryer *et al.*[16] after combustion of the phosphopeptide with perchloric acid at 150°.

Nitrogen was determined with a colorimetric method similar to that described by Fels and Veatch [17]. 0.2 mg phosphopeptide was hydrolysed with 0.1 ml of the combustion-acid used in the Kjeldahl procedure. The hydrolysate was mixed with 3 ml 4 N sodium acetate pH 5.5 buffer [11] and finally diluted to 10 ml with water. 2 ml samples were taken for the ninhydrin reaction [11]. Ammonium sulphate was used as standard.

RESULTS AND DISCUSSION

From a 90 min. tryptic hydrolysate of human casein about 34 % of total N and 9 % of total P was precipitated by acidification to pH 4.6.

The material soluble at pH 4.6 was separated on column I in two fractions, one containing about 8 % of the total casein phosphorus and 58 % of the nitrogen and the other 71 % and 7 %, respectively. Separation of the later fraction on column II gave five well separated peaks (See Fig. 1). This showed that the previously described method for separation of small phosphopeptides involving an anion exchange resin eluted with pyridine formate [10] was applicable also to large phosphopeptides.

Table 1. Amino acid content, N/P atomic ratio and N-terminal amino acid residues of the isolated phosphopeptides.

	Phosphopeptide				
	a	b	c	d	e
Aspartic acid	Trace	Trace	Trace		
Threonine	Trace	+ +	+ +	+ +	+ +
Serine	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Glutamic acid	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Proline	+ +				
Alanine	Trace	Trace	+		
Valine + Methionine	Trace				
*Iso*leucine + leucine	+ + +	+ + + +	+ + + +	+ + + +	+ + + +
Tyrosine		+	+	+	+
Lysine		+	+	+	+
Arginine	+	+	+	+	+
Atomic ratio N/P	11	9.5	8.0	5.3	4.5
N-terminal amino acid residue	Ileu or Leu	Arg	Arg	Arg	Arg

The isolated materials from the peaks were tested by paper electrophoresis. From Fig. 2 it can be seen that the isolated compounds appeared to be electrophoretically homogeneous except peptide *b*, which gave a double-zone. The atomic ratio N/P was; fraction $a = 11$, $b = 9.5$, $c = 8.0$, $d = 5.3$ and $e = 4.5$.

N-terminal analysis showed that fraction *a* had leucine or *iso*leucine as the N-terminal amino acid residue, while the other four had arginine. The qualitative amino acid composition of the isolated compounds was determined by paper chromatography (Table 1).

The paper chromatographic amino acid analysis demonstrated that four of the five isolated phosphopeptides were very similar as regards their amino acid composition. These four were all found to have arginine as the N-terminal amino acid residue and seemed to differ only in their phosphorus content.

Phosphopeptide *e*, which had the highest phosphorus content, was selected for further investigations. Its quantitative amino acid composition was determined according to Moore *et al.*[12] after different times of hydrolysis. The results are given in Table 2. The peptide seems to contain 19 amino acid residues, 5 phosphate groups and possibly one amide group. Its minimum molecular weight is therefore 2 600.

The peptide contained one tyrosine residue and ought to be split by chymotrypsin in accordance with the specificity of this enzyme.

Chymotryptic hydrolysis of the phosphopeptide *e* (3 mg in 0.2 M THAM—HCl* buffer pH 8.7) gave two new components, which were isolated by paper elec-

* THAM = abbreviation for tris(hydroxymethyl)amino methan.

Table 2. Amino acid composition of phosphopeptide *e*.

	Duration of hydrolysis 6 N HCl, 110°C				Mean values		Molar ratio ***	Number of amino acid residues
	22.5 h		90 h					
	% amino acid	% N	% amino acid	% N	% amino acid	% N		
Threonine	7.20	0.846	5.66	0.666	7.71 *	0.906 *	1.92	2
Serine	14.65	1.952	9.16	1.222	16.48 *	2.195 *	4.65	5
Glutamic acid	25.50	2.430	24.05	2.287	24.77	2.358	5.00	5
*Iso*leucine	9.70	1.038	11.58	1.237	11.58 **	1.237 **	2.62	3
Leucine	5.10	0.543	4.87	0.520	4.99	0.531	1.11	1
Tyrosine	6.28	0.485	3.26	0.252	7.29 *	0.529 *	1.10	1
Lysine	4.96	0.953	5.19	0.995	5.07	0.974	1.03	1
Arginine	5.80	1.867	(5.01)	(1.610)	5.80	1.867	0.99	1
Ammonia	1.18	0.974	2.88	2.375	0.61	0.507	1.07	1
	80.37	11.088	71.66	11.164	84.30	11.104		

* Extrapolated to zero time.
** 90 h value.
*** Based on the glutamic acid content taken as 5.00.

trophoresis in a preparative scale. One of these moved very fast to the cathode. The other moved to the anode with somewhat higher mobility than the original phosphopeptide. Qualitative amino acid analysis showed that the compound moving to the cathode consisted of lysine only whereas the other component contained the same amino acids as the original phosphopeptide with the exception of lysine. This proved lysine to be the C-terminal amino acid residue in the phosphopeptide, which is in accordance with the specificity of trypsin and that the peptide probably contained only one lysine residue. This indicated that the molecular weight is 2 600 and not a multiple of it. The disappearing of phosphopeptide *e* in the electrophoretic pattern after chymotryptic hydrolysis appeared to indicate that this peptide fraction was homogeneous.

From a peptic hydrolysate of the phosphopeptide *e* a peptide containing only tyrosine and lysine was isolated. Thus, tyrosine appeared to be the residue next to lysine.

The results above indicated that the isolated phosphopeptide had the composition

$Arg[Thr_2, Ser_5, Glu_5, Ileu_3, Leu, (PO_4)_5]Tyr, Lys$

one of the glutamic acid residue possibly being in amide form.

A comparison between the phosphopeptide *e* and the phosphopeptides obtained in the same way from cow's casein [2-8] showed a resemblance in the high contents of glutamic acid, serine and *iso*leucine. It lacked aspartic acid, glycine

and valine, which usually are present in phosphopeptides isolated from cow's casein.

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