ISOLATION OF A PHOSPHOPEPTIDE AS MAGNESIUM COMPLEX FROM A TRYPSIN HYDROLYSATE OF α-CASEIN BY ANION EXCHANGE CHROMATOGRAPHY

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SUMMARY

A phosphopeptide has been isolated from a short-time hydrolysate of α-casein by anion exchange chromatography, using an eluent containing a complex-forming ion (Mg^{2+}). This peptide contains at least 16 % of α-casein phosphorus and is very similar to a peptide previously[1] isolated from this source by zone-electrophoresis. The empirical residue formula is suggested to be: HOOC–Lys_1, Asp_3, Thr_1, Ser_6, Glu_{10}, Pro_1, Gly_1, Ala_2, Val_2, Met_2, $Ileu_4$, Lys_1, Asp–NH_2, $(NH_3)_3$, $(PO_4H_3)_7$. The presence of two lysine residues is discussed in relation to trypsin resistant bonds involving a lysine carboxyl group.

INTRODUCTION

In a previous work[1] from this institute a strongly acidic phosphopeptide was isolated from a tryptic digest of α-casein by zone electrophoresis and its main composition determined*. Further studies of the structure and properties of this peptide require relatively large amount of material. Ion exchange chromatography seemed therefore in this case to imply a more convenient separation method.

Using conventional buffers, however, the separation of the most acidic phosphopeptides of a tryptic α-casein hydrolysate is not entirely satisfactory by this method, reasonably due to the great net negatively charge on these peptides. In preliminary experiments with Dowex 50-X2 and 0.01 *M* HCl as a buffer, these peptides were eluted as a mixture near to the front (*cf.* ref. 2, 3). On Dowex 1-X2 they were strongly bound and could not be recovered with 2 *M* sodium formate (*cf.* ref. 2).

A satisfactory separation was, however, obtained with anion exchange chromatography (Dowex 1-X2), when an eluent containing a complexing metal ion (Mg^{2+}) was used. Magnesium, which has an intermediate tendency to complex formation with O-phosphorylserine[4], was selected, since it seemed that it would provide sufficient blocking of charges, as well as allowing simple removal of bound metal ions. By chromatography of a trypsin digest of α-casein in this way a phosphopeptide was isolated, which contained at least 16 % of the α-casein phosphorus and is apparently the same peptide prepared previously[1]. Its composition has now been confirmed.

* Recent literature in this field was reviewed in ref. 1.

EXPERIMENTAL AND RESULTS

Trypsin digestion

α-casein (21.8 g, prepared according to HIPP *et al.*[5]; P: 0.99%; homogenous in moving boundary electrophoresis) was treated with Armour's twice recrystallized trypsin (containing 50% $MgSO_4$) for 65 min at 25° and pH 8.0. The procedure and apparatus employed were the same as described previously[1]. From the heat-inactivated digestion solution, a crude barium phosphopeptide fraction (6.1 g) (P: 2.88; N: 9.43%; N/P (atomic ratio): 7.25; 81.5% of total α-casein P) was prepared according to MELLANDER[6] (excluding the lead treatment).

Column chromatography on Dowex 1-X2 involved three purification steps (Fig. 1).

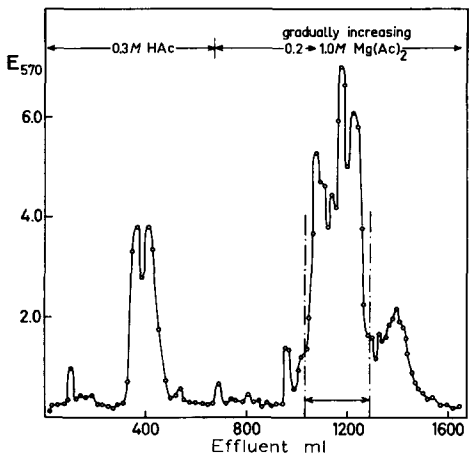

Fig. 1a.

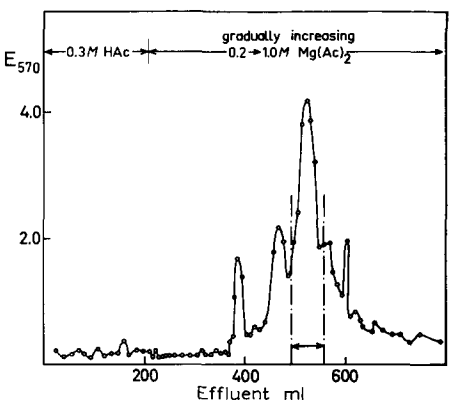

Fig. 1b.

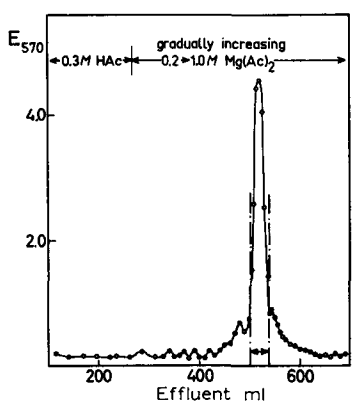

Fig. 1c.

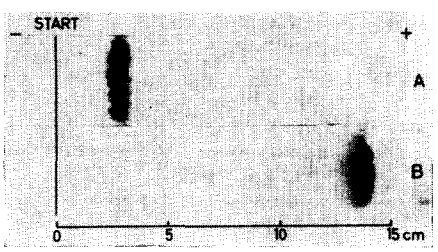

Fig. 1d.

Fig. 1. Chromatography on Dowex 1-X2 of phosphopeptides from a trypsin α-casein hydrolysate, and paper electrophoreses of a purified fraction. O—O, ninhydrin colour at 570 mμ, given by 0.1 (a) or 0.2 (b, c) ml aliquots of the effluent fractions after alkaline hydrolysis (100°, 2 h) according to HIRS *et al.*[14]. (a) Chromatography of 5.0 g crude barium phosphopeptide on a 2.8 × 40 cm column. (b) Re-chromatography of 300 mg Mg-phosphopeptide, obtained from (a) on a 1.5 × 55 cm column. (c) Re-chromatography of 115 mg Mg-phosphopeptide, obtained from (b) on a 0.9 × 115 cm column. (d) Paper electrophoreses at 23° of about 0.5 mg Mg-phosphopeptide from (c) (ninhydrin colour) in: (A) 0.4 *M* acetic acid (500 V, 5 h) (B) 0.2 *M* formic acid adjusted to pH 2.8 with triethylamine (400 V, 5 h).

The resin, 200–400 mesh, was used in the acetate form. The phosphopeptide was applied in 0.3 *M* acetic acid and the column was eluted with the same solvent and later with magnesium acetate solution of gradually increasing concentration. The mixing chamber (volume, 1 l), which initially contained 0.2 *M* magnesium acetate was connected to an upper chamber, filled with *M* magnesium acetate. Several experiments were carried out in respect of the re-chromatographic runs, and the main shape of the effluent curves was always the same. The peptide fractions were combined as indicated by the vertical lines in Fig. 1 and concentrated to small volume by lyophilisation. Magnesium phosphopeptide was then precipitated by adding 99.5 % ethanol. The precipitate was washed with 99.5 % ethanol and ether and then dried at 45° *in vacuo*. The following amounts of phosphopeptide were obtained from the separations shown in Fig. 1: Ia, 3.99 g (P: 3.24 %; N/P (atomic ratio) 6.1; 73.1 % of total α-casein P); Ib, 125 mg (P: 3.25 %; 30.6 % of total α-casein P); Ic, 58 mg (N: 8.66; P: 3.33; S: 1.0 %; N/P: 5.75, N/S (atomic ratio): 19.8; 15.8 % of total α-casein P).

Paper electrophoresis was done with the equipment previously described[3]. The results are shown in Fig. 1d.

N-terminal group analysis was done on about 2 mg Mg-phosphopeptide by SANGER's dinitrophenylation (DNP) method[7]. After hydrolysis of the DNP-peptide (5.7 *M* HCl, 110°, 8 h) only DNP-aspartic acid and ε-DNP-lysine were detected by paper chromatography[8] (*tert.*-amyl alcohol–0.1 *M* phthalate, pH 5.5) of the ether and the water phases respectively.

Quantitative amino acid analysis according to MOORE *et al.*[9] gave the results shown in Table I. Analytical data given by BENNICH *et al.*[1] are included in Table I for comparison.

TABLE I

AMINO ACID COMPOSITION OF THE PHOSPHOPEPTIDE

The analyses were carried out according to MOORE *et al.*[9]. The values are corrected for blank and are expressed as molar ratios, based on a minimum glutamic acid content taken as 10.00.

Duration of hydrolysis	*Asp*	*Thr*	*Ser*	*Glu*	*Pro*	*Gly*	*Ala*	*Val*	*Met*	*Ileu*	*Lys*	NH_3
22 h*	3.78	1.03	5.22	10.00	1.24	1.19	2.06	1.75	1.73	3.23	2.74	
22 h	4.05	0.91	4.56	10.00	0.97	1.06	2.63	1.84	1.72	3.47	1.95	3.92**
72 h	3.96	0.85	4.29	10.00	1.21	0.92	2.03	2.00	1.98	3.67	2.03	3.77**
144 h	3.75	0.94	2.90	10.00	1.06	1.09	2.13	2.06	1.64	3.70	2.34	3.14**
Probable minimum number of amino acid residues	4	1	6	10	1	1	2	2	2	4	2	3***

* Recalculated from the analysis by BENNICH *et al.*[1].

** Corrected for contamination by 0.4 μmole of NH_3 (*cf.* ref. 14) and for NH_3 formed by serine destruction.

*** Tentative value of amide groups.

DISCUSSION

Characterisation and analyses indicate that a homogenous phosphopeptide can easily be prepared from a tryptic α-casein hydrolysate by anion exchange chromatography, using a buffer with a sufficient concentration of magnesium ions. The presence of

these complexing ions seems to be necessary for the purification of this phosphopeptide, though phosphopeptides with less acidic properties are well separated in more conventional buffer systems (see *e.g.* STRID[10]).

The peptide isolated, probably has a minimum amino acid composition of Asp_4, Thr, Ser_6, Glu_{10}, Pro, Gly, Ala_2, Val_2, Met_2, $Ileu_4$, Lys_2, $(NH_3)_3$, $(PO_4H_3)_7$*, with aspartic acid in the N-terminal position. It is apparently the same peptide as that previously prepared[1] by zone electrophoresis. It was then reported to contain at least one and possibly two lysine residues. The present investigation establishes a minimum of two lysine residues.

In view of trypsin specificity, one of these lysine residues should occupy the C-terminal position. The occurrence of a second lysine residue may indicate the presence of a peptide bond involving a lysine carboxyl group that is resistant to hydrolysis. This resistance could be attributed to protection of the bond by an adjacent negatively charged group[11], *i.e.* in this case a phosphoryl-[12], glutamic acid-[13,14] or aspartic acid group[15], or the presence of the sequence Lys·Pro[14,16–18].

According to the analytical figures obtained during the course of purification, the present peptide contains 16 % of the α-casein phosphorus. This percentage may be somewhat too low, since losses in the precipitation and washing procedures may reach 10 %, and since only the peptide material in the combined fractions was taken into account. Further work is in progress on the phosphorus linkages and the amino acid sequence of the peptide.

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* This composition gives N/P and N/S atomic ratios of 5.72 and 20.0. The analytical data gave 5.75 and 19.8.