An Application of Diagonal Electrophoresis to the Selective Purification of Serine Phosphate Peptides

SERINE PHOSPHATE PEPTIDES FROM OVALBUMIN

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A diagonal-electrophoresis method for the selective purification of serine phosphate peptides was applied to tryptic, chymotryptic and peptic digests of oxidized ovalbumin. This method is based on the release of the phosphate group bound to serine by treatment with alkaline phosphatase on paper. The identified serine phosphate peptides were purified by paper electrophoresis at pH 6.5 and 2.0, dephosphorylation with bacterial alkaline phosphatase, and paper electrophoresis at pH 2.0 again, in that order. The presence of two groups of serine phosphate peptides was apparent from the amino acid composition. One group contained no lysine, cysteic acid, proline, leucine or isoleucine (sequence 1) and the other had all those amino acids (sequence 2). Further degradation with subtilisin of those peptides and 'dansyl'-Edman sequence analysis established their partial sequences. The proposed sequences are as follows (with 'SerP' representing serine phosphate): sequence 1, -Ala-Gly-Arg-Glu-Val-Val-Gly-SerP-Ala-Glu-Ala-Gly-Asp-Val-Ala-Ala-Ser-(Val, Glx₂, Ser, Phe)-Arg-; sequence 2, -Asp-Lys-Leu-Pro-Gly-Phe-Gly-Asp-SerP-Ile-Glx-Ala-Glx-CySO₃H-Gly-(Thr, Ser, Val)-(Asp, His, Val)-. The partial sequence of one of the phosphopeptides, Asp-(Glu, Ile, SerP), reported by Flavin (1954) was used to establish the proposed sequence 2.

The biological importance of phosphoproteins is indicated by the number of phosphoenzymes and their abundance in embryonic and rapidly growing tissue. Phosphoproteins are also present in large amounts in milk and eggs. Their biological function remains unknown. It has been suggested that they could be the suppliers of phosphorus and essential amino acids in the developing organism or that they may function as phosphotransferases (Perlmann, 1955).

The nature of the linkage between phosphorus and protein in phosphoprotein not associated with lipids or nucleic acids was first investigated by Lipmann & Levene (1932) and Lipmann (1933) who isolated serine phosphate from acid hydrolysates of vitellinic acid and casein. More recently serine phosphate has been isolated from partial acid hydrolysates of phosvitin (Williams & Sanger, 1959) and from acid hydrolysates of enzymes such as phosphoglucomutase (Anderson & Jollès, 1957).

During studies of the amino acid sequence around the active site of phosphoglucomutase a method for the selective purification of serine phosphate peptides by diagonal electrophoresis was developed

(Milstein, 1967). The application of this technique to ovalbumin, which contains two atoms of phosphorus/molecule (Perlmann, 1952), is reported in the present paper. It was shown by Perlmann (1952) that intestinal or potato phosphatase released one of the two phosphorus atoms of ovalbumin rapidly and the other more slowly, so that after 24 hr. 95% of the protein was transformed into phosphorus-free ovalbumin. Flavin (1954) identified two types of phosphopeptides in partial acid hydrolysates of ovalbumin, and suggested that one, which is resistant to hydrolysis by prostatic phosphatase, is -Asp-(Ala, Glu, Ile, SerP*)-, and the other, containing -SerP-(Ala, Glu)-, is susceptible to the action of the same enzyme. In the present paper the sequence of the two peptides containing serine phosphate are presented. The partial sequence of one of the phosphopeptides, Asp-(Glu, Ile, SerP), reported by Flavin (1954) was used to establish the proposed sequence 2.

*Abbreviations: in amino acid sequences: SerP, serine phosphate; Asx, aspartic acid or asparagine; Glx, glutamic acid or glutamine; CySO₃H, cysteic acid.

MATERIALS AND METHODS

Escherichia coli alkaline phosphatase was prepared by Pigretti & Milstein (1965); a commercial preparation of bacterial alkaline phosphatase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.; batches BAPSF 6FA and 6174A) was also used. Trypsin, chymotrypsin and pepsin were crystallized salt-free preparations from Worthington Biochemical Corp. (batches TR3F 6401 and PM 702 of trypsin and pepsin respectively; chymotrypsin was not numbered). Subtilisin B was from Novo Terapeutisk Laboratorium, Copenhagen, Denmark.

Two batches of ovalbumin were used: one was twice crystallized, freeze-dried and salt-free (L. Light and Co. Ltd., Colnbrook, Bucks.); the other was crystallized, freeze-dried and salt-free (grade V, lot 17B-8691; Sigma Chemical Co., St Louis, Mo., U.S.A.).

Performic acid oxidation. Ovalbumin was dissolved in cold 98% (w/w) formic acid (30mg./ml.) and twice its volume of performic acid was added. It was left at room temperature for 1.5 hr. and freeze-dried without dilution.

Proteolytic digestions of protein. Tryptic and chymotryptic digestions were carried out in 1% (w/v) NH_4HCO_3 , pH 7.8, at 37° for 17 hr. The enzyme/substrate ratio used was 1:100 (w/w), and the final protein concentration was 2.5 mg./ml. Peptic digests were carried out in 0.01N-HCl under the same conditions. The digests were freeze-dried two or three times.

Enzymic digestion of peptides. Peptides were digested with subtilisin B in 1% (w/v) $(\text{NH}_4)_2\text{CO}_3$, pH 9.0 (final concentration of peptide 1 nmole/ $\mu\text{l.}$), with 25 $\mu\text{g.}$ of enzyme/0.1 μmole of peptide; digestions were carried out at 37° for 4–6 hr.

Digestion of peptides with bacterial alkaline phosphatase was performed by dissolving the peptide (0.2–0.4 μmole) in 0.05 ml. of 0.2M- $(\text{NH}_4)_2\text{CO}_3$, pH 8.9. A freshly prepared aqueous solution (2 $\mu\text{l.}$ of 10 mg./ml.) of the purified *E. coli* alkaline phosphatase was added, and the mixture was incubated for 24 hr. at room temperature and dried twice in a desiccator over P_2O_5 .

High-voltage paper electrophoresis. This was carried out as described by Milstein & Feinstein (1968).

Purification and detection of phosphorylated peptides. Ovalbumin was oxidized with performic acid and digested with different proteolytic enzymes. One sample was digested with trypsin, one with chymotrypsin and one with pepsin, 100 mg. of ovalbumin being used in each digestion. The tryptic, chymotryptic and peptic digests were each separated by paper electrophoresis at pH 6.5 at 53 v/cm. for 1.5 hr.; Whatman 3MM paper was loaded with 1.3 mg. of digest/cm. A strip was cut from each electrophoretogram and was subjected to the diagonal technique to detect the phosphorylated peptides. The cut strip was carefully wetted with a 0.25 mg./ml. solution of bacterial alkaline phosphatase (Worthington Biochemical Corp.) in 0.2M- $(\text{NH}_4)_2\text{CO}_3$, pH 8.9. The strip was kept in a damp desiccator for 1 hr. at room temperature, dried and sewn to a new sheet of chromatography paper, which was run by electrophoresis under the same conditions as before at right angles to the first electrophoresis. The dephosphorylated peptides ran off the diagonal towards the cathode. The bands containing

the phosphorylated peptides were cut out of the pH 6.5 paper, sewn to a new sheet of paper and re-run by electrophoresis at pH 2.0 at 70 v/cm. for 30–60 min. The phosphorylated peptides were again located by the diagonal technique. They were eluted with 1% acetic acid, the eluates were dried and the peptides were dephosphorylated with bacterial alkaline phosphatase. These dephosphorylated peptides were then further purified by paper electrophoresis at pH 2.0. A mixture containing lysine, arginine, histidine, alanyl-glycine, glycine, valine, DNP-lysine, glutamic acid, aspartic acid, taurine, cysteic acid, Cyanol FF and a spot of red Pentel-pen ink was used as a marker in every run (Milstein, 1966). Peptides were located by dipping the paper in cadmium-ninhydrin reagent (Heilmann, Barrolier & Watzke, 1957) and leaving it in the dark for at least 12 hr. or heating the paper at 80–100° for 5 min. after dipping.

The mobilities of peptides at pH 6.5 were measured relative to aspartic acid (Offord, 1966). The mobilities of peptides at pH 3.5 were expressed relative to the mobility of alanyl-glycine in the same run, unless otherwise stated, taking as mobility zero the midpoint of the taurine spot. The acidic peptides were indicated by a negative sign. The mobilities of peptides at pH 2.0 were expressed relative to lysine, unless otherwise stated.

Total acid hydrolysis. Peptide samples (15–30 nmol.) were hydrolysed in 0.2 ml. of constant-boiling HCl in evacuated sealed tubes at 105° for 18–20 hr.

Amino acid composition. The amino acid compositions of very simple peptides and those of peptides obtained in very small amounts were determined by paper electrophoresis at pH 2.0 on Whatman no. 1 paper at 105 v/cm. for 15–20 min.

Quantitative amino acid analyses were carried out either in a Beckman Spinco model 120 amino acid analyser fitted with a high-sensitivity unit, in the Medical Research Council Laboratory of Molecular Biology, Cambridge, by the method of Spackman, Stein & Moore (1958), or in a Technicon amino acid analyser fitted with a micro column (0.3 cm. $\times$ 140 cm.) of Technicon 17 μ type B resin, by using a nine-chamber Variograd gradient of sodium citrate buffer (D. Beale & C. M. Kent, unpublished work). Serine and threonine were not corrected for decomposition during hydrolysis.

Edman degradations and N-terminal analyses. They were performed as described by Milstein & Feinstein (1968). In some cases the 1-dimethylaminonaphthalene-5-sulphonylamino acids were identified by paper electrophoresis, as described by Gray (1967).

Peptide nomenclature. Peptides were designated by a capital letter to indicate the enzyme used either for the original hydrolysis of the protein or for the further hydrolysis of the peptides. The capital letters used were as follows: T, trypsin; C, chymotrypsin; P, pepsin; Ph, alkaline phosphatase; S, subtilisin. Small letters, when used, indicated the relative mobility of the peptide on electrophoresis at pH 6.5, peptide a being the most basic peptide of the digest. When the peptide from the initial enzymic digest was degraded further by enzymes, the products were similarly designated by a capital letter to indicate the enzyme used and a number for each of the products. Scheme 1 shows the preparative procedure for all the peptides described in the text.

Scheme 1. Preparative procedure for all the peptides described in the text. pH values refer to the pH at which paper electrophoresis was carried out. For the nomenclature of peptides see the text.

Fig. 1. Detection of the phosphorylated peptides of ovalbumin. The oxidized protein was digested with (a) chymotrypsin, (b) trypsin and (c) pepsin. The proteolytic digest was fractionated by paper electrophoresis at pH 6.5 and a marker strip cut from the paper was treated with a solution of bacterial alkaline phosphatase. The strip was then sewn on to a new sheet of paper, as shown in the Figure, and run again at pH 6.5 at right angles to the original direction. A mixture of amino acids and coloured markers was run at the side of the paper; the arrow indicates the position of a blue marker, Cyanol FF. Peptides dephosphorylated by the alkaline phosphatase treatment lie off the diagonal.

RESULTS

Fig. 1 shows the results obtained after the diagonal technique had been applied to proteolytic digests of oxidized ovalbumin. Two main and one weakly stained phosphorylated peptides were revealed in the tryptic digest, whereas two main peptides were shown in the chymotryptic digest and four in the peptic digest (Fig. 1). These peptides were purified as described in the Materials and Methods section and their quantitative amino acid compositions are shown in Table 1. The peptides that were impure, or were obtained in such a low yield that no further analysis of them was possible, are not included in the Table. Peptides TPha and PPha, for instance, were difficult to purify since they ran too close to the diagonal at pH 6.5. Further purification of peptide TPha was attempted by paper electrophoresis at pH 3.5 with no success. Table 1 shows that there were two groups of peptides: one group (peptides TPhc, PPhb and PPhd) contained no significant amounts of lysine, cysteic acid, proline, isoleucine and leucine; the other group (peptides CPha, CPhb and PPhc) contained all these amino acids. It was thus apparent that there were two different serine phosphate sequences; these were called sequence 1 and sequence 2 respectively.

Sequence 1. The *N*-terminal sequences of peptides PPhb and PPhd determined by the 'dansyl'-Edman method are shown in Fig. 2. The *N*-terminal sequence of peptide PPhd contained the dipeptide

Val-Val, which is extremely stable and is not hydrolysed under the conditions of hydrolysis used (18–20 hr. at 105°). Therefore a quantitative amino acid-composition analysis of the neutral and acidic amino acids of this peptide was performed after 90 hr. hydrolysis. This is shown in Table 1.

The other peptide that had a common part with peptides PPhb and PPhd was peptide TPhc (Table 1 and Fig. 2). It had glutamic acid at the *N*-terminus. From its mobility at pH 6.5 (Table 1) and its size all the glutamic acid residues and the aspartic acid residue must have been present in the acid form. This was confirmed by the mobilities of peptides PPhb and PPhd. Peptide TPhc contained three serine residues, one of which should have overlapped with the serine of the peptic peptides mentioned above. To position this serine residue, TPhc was subjected to subtilisin digestion. The digest was separated by paper electrophoresis at pH 6.5 and the neutral peptides were purified by paper electrophoresis at pH 3.5. Table 2 shows the mobilities and amino acid compositions of the peptides thus obtained. Peptide PPhb was also digested with subtilisin and the digest run at pH 6.5; among the peptides obtained were two that were very acidic at pH 6.5, which appeared to be identical with the two acidic subtilisin peptides obtained from peptide TPhc. The most acidic (peptide PPhbS1) had a mobility of 0.84 and the other (peptide PPhbS2) a mobility of 0.77. These values were the same as those of peptides TPhcS5 and TPhcS4 respectively (Table 2). Peptides PPhbS1 and PPhbS2 had

Table 1. *Phosphorylated peptides obtained by proteolysis of oxidized ovalbumin*

Peptides were purified by paper electrophoresis at pH 6.5 and 2.0, dephosphorylated and run again on paper electrophoresis at pH 2.0. Peptide TPhc was further purified by paper electrophoresis at pH 6.5. Mobilities at pH 2.0 were measured by taking the mobility of lysine as 1 in the same run. The amino acid composition of peptide CPhb is the average of two analyses. Experimental details are given in the text.

Peptide	Mobility		Amino acid composition (moles/mole of peptide)														N-Terminus		
	pH 2.0	pH 6.5	Lys	His	Arg	CySO ₃ H	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Phe		
TPhc	0.28	0.62	—	—	0.98	—	1.07	—	2.91	3.95	—	2.12	3.98	3.23	—	—	0.84	Glu	
CPha	0.37	0.32	1.13	0.79	—	0.84	3.21	1.14	2.06	2.14	0.92	3.12	1.30	1.75	1.05	1.20	1.00	Asp	
CPhb	0.13	0.48	1.10	—	—	1.01	2.75	1.00	1.89	2.13	1.02	2.88	1.05	1.00	0.90	1.03	0.86	Asp	
PPhb	0.98	0.40	0.20*	—	1.16	—	1.18	0.20*	1.09	2.13	—	3.02	2.84	2.12	0.16*	0.23*	—	Ala	
PPhb (90hr. hydrolysis)			Not determined				1.06	—	0.77	2.15	—	3.21	3.20	3.20	—	—	—		
PPhc	0.39	0.51	0.97	—	—	0.92	2.15	1.03	1.74	1.89	0.89	2.79	1.08	1.25	1.00	1.05	0.83	Asp	
PPhd	0.57	0.55	0.20*	—	—	—	1.32	0.19*	1.00	1.34	<0.10*	2.04	2.00	2.22	0.17*	0.24*	0.12*	Val	

*Amino acid present as an impurity.

at pH 3.5, the same mobilities as peptides TPhcS5 and TPhcS4 respectively. Peptides PPhbS1 and TPhcS5 were pooled and the *N*-terminal sequence shown in Fig. 2 was determined by the 'dansyl'-Edman method. Peptides PPhbS2 and TPhcS4 were also pooled. Their amino acid composition (Table 2), determined by paper electrophoresis, and their mobility suggested that they were similar to peptides TPhcS5 and PPhbS1 except for the *N*-terminal residue (see Fig. 2), which should be alanine. Two Edman degradations were done and the rest of the sequence was characterized by the 'dansyl'-Edman method (see Fig. 2). Peptide TPhcS1 should occupy the *C*-terminus of peptide TPhc, since it contains the basic residue, and this left two alanine residues to be accounted for by peptide TPhcSN1.

Fig. 2 shows the proposed sequence around one of the serine phosphate groups, deduced from the results presented above.

Sequence 2. The peptides used to study this sequence were peptides CPha, CPhb and PPhc. The 'dansyl' method showed that all three peptides had aspartic acid as *N*-terminal group. The 'dansyl'-Edman method applied to the three peptides showed that they had the same *N*-terminal part, and that the difference was situated in the *C*-terminus (Fig. 3).

Peptide PPhc was digested with subtilisin. The digest was run on paper electrophoresis at pH 6.5, and the strips containing the neutral amino acids as well as those containing peptides PPhcS1 and PPhcS3 were run at pH 3.5. Table 3 shows the mobilities and amino acid compositions of the peptides thus obtained. Peptide PPhcSN (which came from the neutral band at pH 6.5) was subjected to two Edman degradations, and the third and fourth residues were determined by the 'dansyl'-Edman method (see Fig. 3). This information and the amino acid composition (Table 3) were indications that the peptide came from the *N*-terminal portion. Its mobility at pH 6.5 was zero, its charge was zero and therefore the aspartic acid was present in the acid form. Peptide PPhcS3 gave two peptides when run at pH 3.5, peptides PPhcS3' and PPhcS3'' (Table 3). Peptide PPhcS3'' was a dipeptide. Its sequence was determined by the 'dansyl'-Edman method, and the charge corresponding to a peptide of that size and mobility was 1. This indicated that the aspartic acid was in the acid form. Peptide PPhc contained the sequence -Gly-Phe-Gly-; peptide PPhcSN had only one glycine residue and contained phenylalanine. The dipeptide Gly-Asp should occupy a position next to the phenylalanine residue since it was the only subtilisin derivative with *N*-terminal glycine (see Fig. 3). The sequence of peptide PPhcS1 was determined (Fig. 3). The charge corresponding to a peptide of that size and

Table 2. *Peptides obtained by subtilisin digestion of peptide TPhc*

Amino acid compositions were determined by paper electrophoresis at pH2.0. Purification was by electrophoresis at the pH values shown. For experimental details see the text.

Peptide	Purification method	Mobility		Amino acid composition								N-Terminus
		pH 6.5	pH 3.5	Arg	Asp	Ser	Glu	Gly	Ala	Val	Phe	
TPhcS1	6.5	0.31		+	—	+	+	—	—	+	+	Ser
TPhcS2	6.5	0.49		—	—	+	+	+	—	+	—	
TPhcS4	6.5 and 3.5	0.77	0.04	—	+	—	+	+	++	+	—	
TPhcS5	6.5 and 3.5	0.84	—0.14	—	+	—	+	+	+	+	—	Glu
TPhcSN1	6.5 and 3.5	0.00	1.00	—	—	—	—	—	++	—	—

Fig. 2. Proposed sequence of peptides TPhc, PPhb and PPhd, containing sequence 1 of ovalbumin. * Position of the phosphate group before dephosphorylation. — — —, *N*-Terminal analyses by the 'dansyl'-Edman procedure. Peptide nomenclature is defined in the text. Sites of enzyme action: T, trypsin; P, pepsin; S, subtilisin B.

Fig. 3. Proposed sequence of peptides CPha, CPhb and PPhc, containing sequence 2 of ovalbumin. * Position of the phosphate group before dephosphorylation. C, Site of action of chymotrypsin; other symbols are defined in Fig. 2. Peptide nomenclature is defined in the text.

mobility was 1, indicating that only one of the glutamic acid residues was charged; the other was present as glutamine. This peptide must follow the aspartic acid residue, since Flavin (1954) showed that the sequence around this serine

phosphate is Asp-(SerP,Ile,Glu). Peptide PPhcS2 had, by the 'dansyl'-Edman method, the *N*-terminal sequence $\text{CySO}_3\text{H-Gly-}$. All this information led to the proposed sequence shown in Fig. 3.

Table 3. Peptides obtained by subtilisin digestion of peptide PPhc

All the peptides were purified by paper electrophoresis at pH 6.5 and 3.5. Peptide PPhcSN was further purified at pH 2.0; at this pH its mobility was 0.61, relative to histidine in the same run. Mobilities of peptides PPhcS2 and PPhcS3 at pH 3.5 were measured relative to the mobility of aspartic acid in the same run.

Peptide	Mobility		Amino acid composition (moles/mole of peptide)														N-Terminus
	pH 6.5	pH 3.5	Lys	CySO ₃ H	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Leu	Ile	Phe		
PPhcSN	0.00	0.59	0.85	—	1.04	—	0.19*	0.16*	1.00	1.14	<0.10*	—	0.96	—	0.91	—	—
PPhcS1	0.44	0.34	—	<0.10*	<0.10*	—	0.93	2.01	—	0.15*	1.02	—	—	0.96	—	Ser	—
PPhcS2	0.62	—0.71	—	1.00	—	0.98	1.00	<0.10*	—	1.07	—	1.00	<0.10*	—	—	CySO ₃ H	—
PPhcS3'	0.81	—1.05	—	0.85	0.30*	1.16	0.55*	0.40*	—	1.40	0.21*	—	—	—	—	CySO ₃ H	—
PPhcS3"	0.81	0.08	—	—	1.09	—	<0.10*	<0.10*	—	0.99	—	—	—	—	—	Gly	—

*Amino acid present as an impurity.

DISCUSSION

Two serine phosphate peptides of about 20 residues each have been isolated and their partial sequences established. To establish the proposed sequence 2, the composition of the phosphopeptide Asp-(Glu,Ile,SerP) reported by Flavin (1954) was used. The method used to isolate these peptides, diagonal electrophoresis, has proved to work successfully when applied to ovalbumin. The method has some advantages over the Dowex 50 method used by Flavin (1954) in that it is specific and can be applied to tryptic peptides. These generally contain a basic residue because of the specificity of trypsin and for this reason are not retained by Dowex 50. The diagonal procedure has thus proved successful on a protein of molecular weight 45 000, by using paper techniques exclusively, with comparatively small amounts of starting material.

Flavin (1954) identified two types of phosphopeptides in partial acid hydrolysates of ovalbumin, suggesting that the one that is resistant to hydrolysis by prostatic phosphatase is sequence 2 (-Asp-SerP-Ile-) and sequence 1 (-Gly-SerP-Ala-) is susceptible to the action of the same enzyme. Flavin (1954) subjected an oxidized sample of ovalbumin to partial acid hydrolysis and ran it through a Dowex 50 column. He found the acidic peptides, such as Asp-SerP or Asp-(Glu,Ile,SerP); less acidic peptides, such as Val-Gly-SerP or Gly-SerP-Ala were, however, not detected in spite of the large amount of ovalbumin used (1.5g.) and of the hydrolysis conditions used (11N-hydrochloric acid at 37° for 3-4 days), which would not be expected to cause inversion of dipeptide sequences.

Shaw (1962) studied the serine phosphate sequences of ovalbumin by using a very elegant technique developed by Sanger & Hocquard (1962). The incubated minced hen oviduct was incubated with [³²P]phosphate and ³²P-labelled ovalbumin was synthesized *in vitro* (Sanger & Hocquard, 1962).

Ottesen (1958) reported that when ovalbumin was digested with purified subtilisin A at pH 6.4 the heptapeptide Glu-Ala-Gly-Val-Asp-Ala-Ala was released. The latter peptide is very similar to peptide TPhcS5 or peptide PPhbS1, obtained by subtilisin B digestion of peptide TPhc or peptide PPhb respectively (see Fig. 2). The difference lies in the positions of valine and aspartic acid, which are inverted; otherwise the similarity of these two sequences is striking. It is not known whether they come from the same part of the molecule.

The radioactivity technique, in spite of all its undeniable advantages, has been successfully applied only in the study of short sequences. The method is unsuitable for peptides longer than five or

six residues, and the occurrence of several artifacts complicates the interpretation of the results. It is noteworthy that 300mg. of ovalbumin was enough for the characterization and the determination of the sequences of peptic, tryptic and chymotryptic peptides of about 20 residues of ovalbumin around each phosphorylated site.

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