ISOLATION FROM EMBRYONIC BOVINE DENTAL ENAMEL OF A POLYPEPTIDE (E_3) CONTAINING AS ITS ONLY PHOSPHORYLATED SEQUENCE, GLU–*O*-PHOSPHOSERINE–LEU

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1. Introduction

The tripeptides Glu–*O*-phosphoserine–Tyr and Glu–*O*-phosphoserine–Leu have been identified in embryonic bovine enamel [1,2]. Because of the difficulties encountered in isolating the individual peptides, however [3–5], it has not been clear whether both sequences occur in all of the phosphopeptides or certain sequences occur only in specific peptides. We have isolated homogeneous samples of E_3, a phosphorylated peptide of mol. wt 5000–6000 containing 46 amino acids. All three of the serine residues are phosphorylated and all three are in the sequence, Glu–*O*-phosphoserine–Leu.

2. Materials and methods

2.1. *Isolation of* E_3

Soluble proteins of embryonic bovine enamel [6] in 0.01 M ammonium bicarbonate, 6 M urea, pH 8.3, were fractionated on a 5.0 × 100 cm column of Bio-Gel P-10, 50–100 mesh at 20°C using ascending chromatography [7]. Fractions enriched in E_3 were then subjected to ion-exchange column chromatography using DE-52 resin, at pH 8.3, in 6 M urea at 20°C [7].

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2.2. *Analytical methods*

Analytical polyacrylamide disc-gel electrophoresis was performed in 5% polymerized gel, at pH 8.3, using 6 M urea in all solutions [7]. Aliquot samples of the E_3 peptide were also electrophoresed in 3%, 5% and 7.5% gels and in 10%, 15% and 20% gels using SDS as the dissociative solvent. The N-terminal amino acid was determined by the cyanate procedure of Stark et al. [8] and by phenylisocyanate degradation manually [9] as well as by custom built [10,11] and commercial automatic protein sequenators (Beckman sequencer, Model 890B, Beckman Instruments, Inc.). The carboxy-terminal amino acid was determined after digestion of the peptide with DFP treated carboxypeptidase A (EC 3.4.2.1) (Worthington Biochemical, Freehold, NJ) and by hydrazinolysis [12]. Quantitative amino acid analysis was performed on a commercial automatic amino acid analyzer (Phoenix Precision Instrument Co., New York, NY) on samples hydrolyzed in constant boiling triple distilled 6 N HCl at 105°C in vacuo, correcting for hydrolytic destruction by timed hydrolyses (24 h, 48 h and 96 h). Serine was determined directly on the amino acid analyzer, correcting for phosphoserine destruction by timed hydrolyses in 4 N HCl at 105 °C (4 h, 8 h, 12 h and 24 h). Tryptophan was determined by in vacuo hydrolysis in 6 N HCl, 24 h at 105°C in the presence of 0.01% phenol. Phosphorus was determined by the methods of Dryer

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et al. [13] and Lowry et al. [14]. Molecular weight of the peptide was determined by analytical sedimentation equilibrium [15], gel filtration in 5 M guanidine HCl (pH 8.0) on an analytical Bio-Gel P-10 column [16] and SDS polyacrylamide gel electrophoresis [17], the latter two methods employing standard proteins for calibration. Sedimentation equilibrium runs were made on a Spinco Model E ultracentrifuge in a 12 mm cell at 60 000 rev/min at room temperature in either 6 M urea or 6 M guanidine HCl, in 0.01 M Tris–HCl, pH 8.1 containing 0.2 M NaCl, at protein concentrations varying from 0.1–2.0 mg/ml. Raleigh Interference optics were used. The Roark-Yphantis Program [15] was used to analyze the data. The molecular weight was also calculated using a least square analysis of five of the amino acids [18].

2.3. *Isolation of small peptides containing O-phosphoserine*

Aliquot samples of freeze-dried enamel proteins were hydrolyzed in vacuo with 11 N HCl at 37°C for 44 h and peptides separated by ion-exchange chromatography [1]. Other aliquot samples of the E_3 peptide were suspended in 5 ml 1 M urea solution pH 7.7, 0.01 M ammonium bicarbonate and reacted with chymotrypsin (EC 3.4.5, Worthington) for 16 h at 37°C [19]. The freeze-dried peptides were dissolved in a minimum amount of pyridine formate buffer, pH 3.1 and chromatographed on a peptide analyzer similar to that of Hill and Delaney [20] equipped with a split stream system.

3. Results

Isolation of homogeneous samples of the phosphorylated polypeptide E_3 was accomplished using the two step procedure of gel filtration and ion-exchange chromatography (fig.1A, B) as evidenced by the fact that the peptide migrated as a single band when run in 3%, 5%, 7.5% and 10% gels containing 6 M urea at pH 8.3 and in 10%, 15% and 20% gels using SDS as the dissociating agent. Quantitative total amino acid phosphorus analyses revealed a very close correspondence between the contents of serine and phosphorus. The degradation curve for *O*-phosphoserine in the timed hydrolysis experiments confirmed that all three of the serine residues were phosphorylated.

Methionine (sulfoxide) was identified as the N-terminal amino acid by the cyanate procedure of Stark [8], by phenylisocyanate degradation and by automated amino acid sequencing which also showed that the N-terminal sequence was Met–Pro–Leu with a 96% repetitive yield. The only additional amino acid detected after Leu was Glu, recovered in very reduced yields (10–15%). A single ninhydrin-positive peak eluting in the position of serine was found after carboxypeptidase A digestion. Since all the serine residues are present as *O*-phosphoserine, and the enzyme has no activity with this residue, glutamine, which co-elutes with serine, is considered to be the C-terminal amino acid. This was confirmed by hydrazinolysis.

3.1. *Molecular weight determination*

A single symmetrical peak was obtained using Schlieren optics by sedimentation velocity ultracentrifugation in a double sector synthetic boundary cell at 26 000 rev/min and 56 000 rev/min. Equilibrium ultracentrifugation also demonstrated that E_3 was homogeneous since the $\log_n C$ versus $R^2/2$ plot gave a straight line with a correlation coefficient of 0.99. The average mol. wt was calculated to be 5400 ± 10%. The molecular weight of E_3 determined by analytical Bio-Gel P-10 chromatography in 6 M guanidine HCl was 5000 ± 10% with K_d 0.69, compared with insulin (mol. wt 6000, K_d 0.58) and insulin B chain (mol. wt 3800, K_d 0.78). The molecular weight by SDS–polyacrylamide gel electrophoresis was 5000 ± 10%. E_3 had a mobility of 0.906 compared with 0.896 for insulin and 0.916 for insulin B chain.

The minimum number of residues per molecule was calculated to be 46, and the minimum molecular weight based on the amino acid composition, 4950 ± 10% [18]. The corrected amino acid composition of the molecule is Asp_2, Thr_1, $SerP_3$, Glu_4, Pro_{12}, Gly_3, Ala_1, Val_1, Met_2, Ile_1, Leu_6, Tyr_3, Phe_1, Lys_1, His_3, Arg_1, Trp_1.

3.2. *Isolation of peptides after chymotrypsin digestion of the whole peptide*

At least thirteen fractions could be isolated from E_3 by ion-exchange chromatography after digestion with chymotrypsin. Phosphorus and amino acid analyses of the peptides revealed that ∿90% of the total phosphorus content of the peptide was recovered in a tripeptide containing only glutamic acid, *O*-phosphoserine and

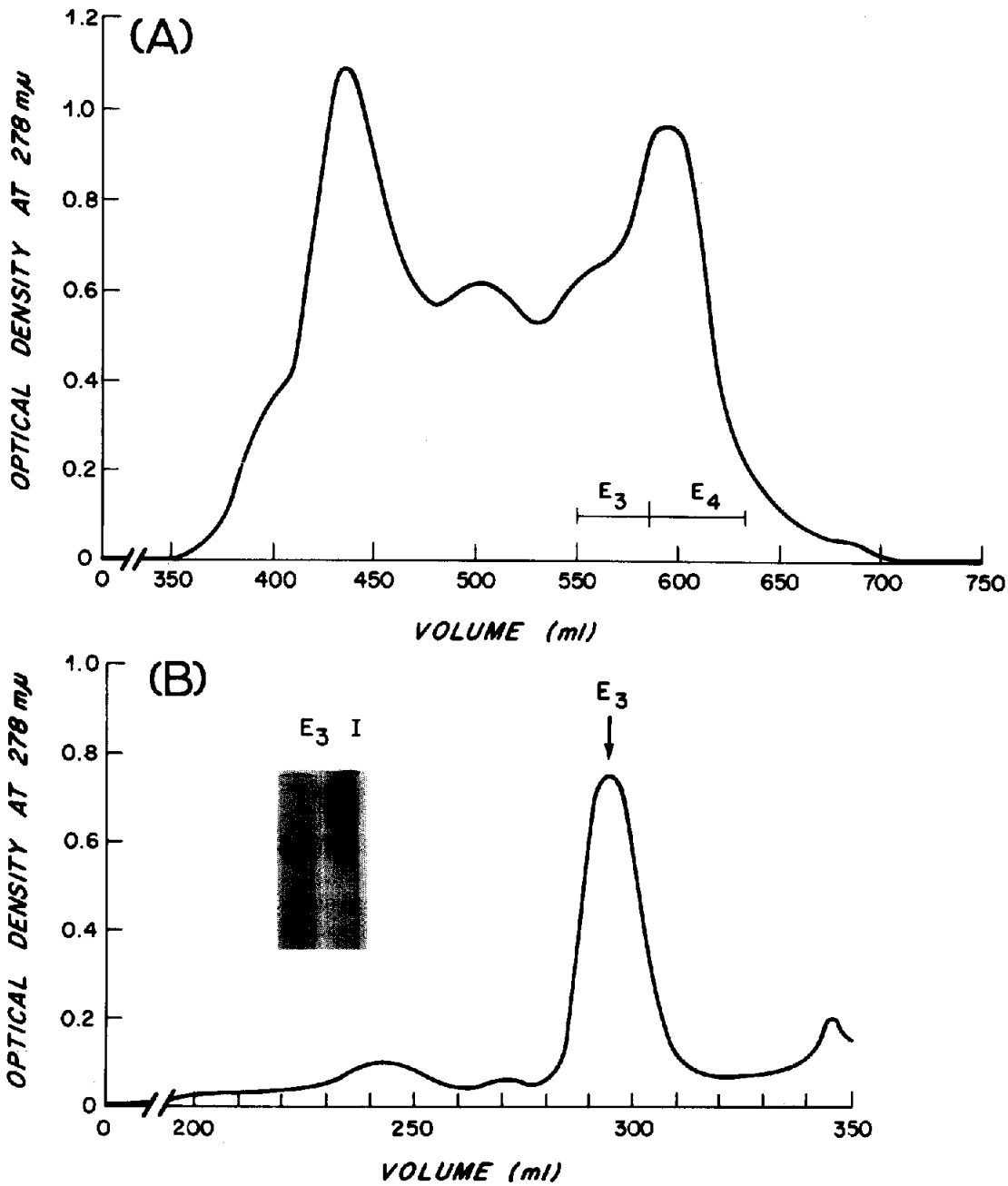

Fig.1. (A) Bio-Gel P-10 gel filtration of the neutral soluble proteins of organic matrix of embryonic bovine enamel, at pH 8.3, in 0.01 M NH_4HCO_3, 6 M urea. A 150 mg sample in 20 ml of the above buffer was applied to a 5.0 × 100 cm column at 20°C and the flow rate maintained at one ml/min. The protein was monitored by its 278 nm absorption and by analytical polyacrylamide gel electrophoresis, at pH 8.8, in 6 M urea. (B) DE-52 Ion exchange chromatography of the fraction enriched in the E_3 phosphorylated peptide obtained by Bio-Gel P-10 filtration. Samples from the Bio-Gel P-10 column were applied directly to a 0.9 × 20 cm column of DE-52 previously equilibrated with 0.01 M Tris acetate in 6 M urea, at pH 8.1. A 0–0.01 M NaCl gradient at pH 8.3, at 20°C was used with a column elution rate of 30 ml/h. A homogeneous sample of E_3 phosphorylated polypeptide was obtained as indicated by analytical polyacrylamide gel electrophoresis. (I initial sample of soluble proteins, E_3 purified peptide.)

leucine. Sequential analysis by manual Edman degradation indicated that the sequence of this tripeptide is Glu–*O*-phosphoserine–Leu [1]. This was confirmed by amino acid analysis and sequencing of the tripeptides isolated by ion exchange chromatography from partial acid hydrolysates.

4. Discussion

The recent finding that all of the *O*-phosphoserine residues in the E_4 enamel peptide are in the sequence, Glu–*O*-phosphoserine–Tyr [7] suggested that other enamel peptides may contain phosphoserine only in the sequence Glu–*O*-phosphoserine–Leu. This is experimentally confirmed for the E_3 peptide. These data also establish that the two major phosphorylated peptides in embryonic bovine enamel, E_3 and E_4, have different primary structures. These data do not distinguish whether the two peptides are initially synthesized as a single larger polypeptide [21–23] which is later cleaved in the tissue in vivo or possibly by proteolysis during the preparation of the enamel proteins, or whether the two peptides are synthesized as two distinct chains. Proteolysis of a single large peptide during preparation seems most unlikely since the two peptides can be isolated from the teeth of embryos dissected immediately after decapitation in solutions containing enzyme inhibitors [24].

Since small amounts of Glu were found on automatic peptide sequencing as the fourth amino acid in the N-terminal sequence, and all three Glu residues are adjacent to *O*-phosphoserine, one may presume that the sequence of the first 6 amino acids in the E_3 peptide is Met–Pro–Leu–Glu–*O*-phosphoserine–Leu. The presence of Leu and glutamic acids in close juxtaposition to the *O*-phosphoserine residues may be very important in the binding of calcium ions by the peptide and is clearly relevant to the postulated role of phosphopeptides in enamel calcification [1,2]. A complex bond between calcium, carboxyl and phosphate groups would have a higher binding constant than a calcium–carboxyl or a calcium–phosphate bond alone. It is also likely that bound calcium ions would be stereochemically fixed in a unique position as a result of the multiple ligands involved. Juxtaposition of leucine to the presumed site of calcium binding is interesting in light of electron spin resonance studies of enamel, which revealed an interaction between the mineral and leucine [25].

The findings may also be relevant to the role of phosphorylated components in mineralization of bone and dentin [26–32], since soluble and collagen-bound phosphorylated peptides of dentin and bone are rich in aspartic and glutamic acids [32–35] and calcium binding studies have indicated that some of the calcium ions are probably bound to collagen as a complex between carboxyl and phosphate groups [36].

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