A Simple Procedure for the Isolation of a Major Amelogenin Polypeptide Component

By Alan G. FINCHAM

*Department of Biochemistry, University of the West Indies,
Mona, Kingston 7, Jamaica, West Indies*

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A simple, reproducible gel-filtration procedure for the isolation of one of the major 'J-Group' polypeptides of the bovine foetal dental-enamel matrix is described. The purified polypeptide was characterized by amino acid analysis, electrophoresis, cleavage with CNBr and *N*-terminal analyses. The isolated component is shown to be closely similar to an amelogenin component described by other workers.

Since Eastoe (1963) reported on the curious amino acid composition of the protein matrix of developing dental enamel, there has been increasing interest in the nature and function of these 'amelogenin' proteins (Burgess & Maclaren, 1965; Nikiforuk & Sognaes, 1966; Eggert *et al.*, 1973; Smales, 1975; Seyer & Glimcher, 1977), it being postulated that these proline-rich proteins are concerned with the mechanisms of calcification and ultrastructural organization of the maturing dental enamel.

Although several studies have been directed to the isolation and characterization of discrete amelogenin proteins (Fincham, 1971; Eggert *et al.*, 1973; Seyer & Glimcher, 1971), reproducible procedures have been elusive (Mechanic, 1971), and the interpretation of the various data confusing (Fincham, 1978).

Studies have centred on the isolation and partial sequence determination of the lower-molecular-weight matrix components ('J-Group' of Eggert *et al.*, 1973). The present study reports a simple isolation procedure and partial characterization of one of these components from the bovine dental-enamel matrix.

A preliminary account of this work was presented at the Third International Symposium on Dental Enamel, Washington, in 1978.

Experimental

Isolation of bovine dental-enamel-matrix protein

The demineralized protein from 5-7-month-old foetuses *in utero* was prepared as previously described (Fincham, 1968).

Isolation and purification of a J-Group polypeptide

Samples (500-800 mg) of freeze-dried enamel-matrix protein were chromatographed on a column of Bio-Gel P6 (Bio-Rad Laboratories, Richmond,

CA 94804, U.S.A.) at 10°C by using upward-flow elution with 0.05 M-formic acid. The fractionation was monitored at 280 nm and selected fractions were bulked and directly freeze-dried. Fractions from this separation, or their products on cleavage by CNBr, were further chromatographed by using a column of Bio-Gel P4 under similar conditions (see Figs. 1, 2 and 3 below).

Electrophoresis

Polyacrylamide-gel electrophoresis was carried out in 6 M-urea-loaded 7.5% (w/v) polyacrylamide gels as previously described (Fincham, 1978). Analytical gel isoelectric focusing was conducted by using similar urea-loaded gels containing pH 3-10 Ampholine (LKB-Produkter A.B., Stockholm, Sweden) by using the Multiphor (LKB) horizontal gel-electrophoresis system. The gel slabs were stained with Coomassie Brilliant Blue R250 as described by Vesterberg (1972). pH measurements on the gels were made by equilibrating 5 mm discs (punched from the gel) with 5 ml of water.

Cleavage with CNBr

Samples of the purified polypeptide (10-20 mg) were treated with CNBr (Serva Feinbiochemica, Heidelberg, Germany) in 70% (v/v) formic acid by the method of Epstein *et al.* (1971). The reaction product was diluted 10-fold with water and freeze-dried.

Amino acid analysis

Samples were hydrolysed in triple-distilled constant-boiling HCl containing 0.01% phenol for 22 h at 110°C in sealed, N₂-flushed ampoules. Analyses were made with a commercial amino acid analyser (Beckman Instruments, model 119, fitted with on-line integration).

N-Terminal analyses and partial Edman degradations

The procedures of Gray (1967) were used. In all cases the identity of derivatives was confirmed by co-chromatography with the authentic compound.

Results

The elution profile of the enamel matrix from the Bio-Gel P6 column is shown in Fig. 1. It was found that, although the earliest fractions (25–35) contained mixtures of the 'C-Group' (Eggert *et al.*, 1973) components, and intermediate fractions both C- and J-Group components, a distinct trailing peak (fractions 70–85) appeared to contain a single

Fig. 1. Gel-exclusion chromatography of bovine dental enamel matrix (800mg) on a column of Bio-Gel P6 (102cm $\times$ 2.5cm), eluted by upward-flow with 0.05M-formic acid (20ml/h) at 10°C

Recoveries were: fractions 26–54, 397mg (50%); 55–65, 80mg (10%); and 66–86, 114mg (14%).

Fig. 2. Chromatography of combined fractions 66–86 from the chromatogram of Fig. 1 on Bio-Gel P4

The column was 1.5cm $\times$ 50cm in size and was eluted with 0.05M-formic acid by using upward flow at 10°C. Fractions 29–40 yielded 32mg of purified amelogenin component.

J-Group polypeptide. This observation was considered important in view of the numerous previous failures to isolate purified amelogenins by gel-filtration procedures (Fincham, 1968; Mechanic, 1971). Recoveries of protein from this column procedure were usually some 75% by weight, of which this trailing component accounted for some 14%. In repeated (four) chromatographic experiments, the elution profiles were found to be closely similar.

In re-chromatography of fractions 66–86 from the separation shown in Fig. 1 using Bio-Gel P4 (Fig. 2), it was found that fractions 22–28 contained principally a single J-Group component, but with some contamination from other J-Group components. The fractions 29–40 yielded some 32mg of an electrophoretically homogeneous polypeptide (Fig. 2).

Examination of the polyacrylamide-gel-electrophoresis patterns of the various fractions (Fig. 1) showed that the mobility of the J-Group isolate from the retarded peak appeared similar to that of a component seen in the earlier column fractions (55–64). It was thought possible that this resulted from aggregation effects, which have created serious difficulties in previous attempts at isolations (Mechanic *et al.*, 1967; Mechanic, 1971). However, gel isoelectric focusing of these fractions showed that the purified component had a pI value in the range 6.1–6.3, as compared with values in the range 5.7–6.1 for the other J-Group-containing fractions.

Thus the fractions 55–64 (Fig. 1) contained a mixture of J-Group components that were different from that isolated from the trailing peak. It is believed that this mixture contains the 'E1', 'E2' and 'E3' polypeptides described by Seyer & Glimcher (1971). These workers used Bio-Gel P10 chromatography in 6M-urea-loaded buffers at pH8.3, in which system the 'E4' polypeptide was found to be retarded with respect to the other J-Group components.

The results of multiple amino acid analyses of the isolated polypeptide are shown in Table 1, the data being calculated assuming one residue of valine per molecule. Hydrolytic-loss corrections of 5 and 10% were applied to threonine and serine respectively; tryptophan and phosphate were not determined.

Recoveries from the CNBr-cleavage experiments were found to be some 90–95% by weight, and polyacrylamide-gel electrophoresis showed a major component in the reaction product, with a mobility slightly lower than that of the parent polypeptide.

Fractionation of the CNBr cleavage product on Bio-Gel P4 (Fig. 3) showed four peaks of absorption at 280nm. Samples of these fractions were hydrolysed and subjected to amino acid analysis. It was found that peak B had a composition identical with that of peak C (Table 2). In a second chromatogram, in which the concentration of the formic acid eluent

Table 1. Amino acid analyses of purified J-Group component
Content (residues/molecule) (molar ratios based on valine = 1.00)

Analysis no. ...	1	2	3	4	5	Mean	Residues/molecule
Amino acid							
Proline	9.70	8.20	8.88	7.75	7.98	8.50	8-9
Aspartic acid	1.63	1.16	1.54	1.35	1.43	1.42	1-2
Glutamic acid	4.16	3.39	3.82	3.60	3.74	3.74	4
Threonine	1.04	0.86	1.01	0.81	0.84	0.91	1
Serine	2.80	2.38	2.73	2.27	2.48	2.53	2-3
Glycine	5.66	4.95	5.16	5.03	5.18	5.20	5
Alanine	0	0	0	0	0	0	0
Valine	1	1	1	1	1	1	1
Methionine	2.92	2.31	2.42	2.72	2.70	2.61	3
Isoleucine	2.32	1.74	1.79	1.75	1.81	1.88	2
Leucine	3.80	3.18	3.22	3.32	3.45	3.39	3
Tyrosine	6.90	5.82	5.89	5.65	5.76	6.00	6
Phenylalanine	1.20	1.36	1.40	1.37	1.10	1.23	1
Lysine	1.06	1.03	1.02	0.93	1.01	1.01	1
Histidine	2.68	2.82	2.84	2.72	2.81	2.77	3
Arginine	1.51	1.97	1.55	1.72	2.27	1.80	2
Tryptophan			Not determined				
						Total:	43-46

Fig. 3. Chromatography of product of cleavage with CNBr of the purified amelogenin from the column fractionation shown in Fig. 2

A column (1.5 cm $\times$ 51 cm) of Bio-Gel P4 was eluted with dilute formic acid (12.5 ml/h) at 10°C. (a) 0.05M-formic acid; (b) 0.10M-formic acid. Note the loss of the CN-B peak in the second chromatogram, with a corresponding increase in the area of peak CN-C.

was increased to 0.1 M (Fig. 3), peak B was eliminated with a proportional increase in peak C. A plot of elution volume against molecular weight (calculated from amino acid composition) suggested that the peak B component was a dimer of the peak C compo-

nent (peptide CN-C). Polyacrylamide-gel electrophoresis of the isolated peptides produced by cleavage with CNBr showed a single band in the case of peptide CN-A, which corresponded to the band seen in the gel-electrophoretic experiment on the unfractionated cleavage product. No bands were seen in gels on which peak-CN-C or peak-CN-D components were run.

The amino acid compositions found for the peptides produced by cleavage with CNBr are shown in Table 2. Only glycine was detected in the hydrolysate of peptide CN-D, but the observed A_{280} was deduced to be derived from tryptophan, since spectrophotometry of this peptide showed a characteristic tryptophan spectrum. Some discrepancies were noted for glutamic acid, arginine and methionine between the sum of the data from the peptides produced by cleavage with CNBr and the parent polypeptide. The presence of *N*-terminal methionine in the parent polypeptide explained the difference in this case, since dansylation showed this as the *N*-terminal residue.

Dansylation of the fragments produced by cleavage with CNBr showed peptide CN-C to have isoleucine as its *N*-terminal residue and peptide CN-D to have a glycine residue at its *N*-terminus. The results of the dansylation of peptide CN-A were unclear, however; a single cycle of dansyl-Edman degradation showed leucine as the second residue, and amino acid analysis of the degraded peptide showed a decrease in the number of proline residues from six to five per molecule, indicating this amino acid as the *N*-terminal residue of peptide CN-A.

Table 2. Amino acid compositions of the peptides produced by cleavage with CNBr, isolated from the J-Group component of the present study, compared with the data from the polypeptide 'E4' of Seyer & Glimcher (1977)

Peptide ...	Content (residues/molecule)								
	Present study					Polypeptide 'E4'			
	CN-A	CN-B	CN-C	CN-D	Sum*	CNBr-2	CNBr-3	CNBr-4	Sum
Amino acid									
Proline	6	3	3	0	9	5	3	0	8
Aspartic acid	1	0	0	0	1	1	0	0	1
Glutamic acid	2	1	1	0	3	2	1	0	3
Threonine	1	0	0	0	1	1	0	0	1
Serine	1-2	1	1	0	2-3	2	1	0	3
Glycine	2	1-2	1-2	1	4-5	2	2	1	5
Alanine	0	0	0	0	0	0	0	0	0
Valine	1	0	0	0	1	1	0	0	1
Methionine†	1	1	1	0	2	1	1	0	2
Isoleucine	1	1	1	0	2	1	1	0	2
Leucine	3	0	0	0	3	3	0	0	3
Tyrosine	3	2	2-3	0	5-6	4	2	0	6
Phenylalanine	1	0	0	0	1	1	0	0	1
Lysine	1	0	0	0	1	1	0	0	1
Histidine	2	1	1	0	3	2	1	0	3
Arginine	0	1	1	0	1	0	1	0	1
Tryptophan				‡		0	0	1	1
Total residues	26-27	12-13	12-14	2‡	39-45	27	13	2	41

* Sum of CN-A+CN-C+CN-D (see the text).

† As homoserine plus homoserine lactone.

‡ Qualitatively determined by spectrophotometry.

Discussion

Gel-exclusion chromatography in acid-pH eluents has previously been shown to provide useful crude separations of amelogenins (Fincham, 1968). In the present case, the use of Bio-Gel P6 appears to provide a simple and rapid fractionation of the matrix to yield this major J-Group component.

It is instructive to compare the data of the present study with those of other workers (Eggert *et al.*, 1973; Seyer & Glimcher, 1971, 1977). Table 3 shows that, apart from one residue difference in the case of proline, there is close agreement between the compositions of the isolate 'E4' (Glimcher & Seyer, 1977), 'j' (Eggert *et al.*, 1973) and the polypeptide described in the present study. Further, Seyer & Glimcher (1977) used cleavage with CNBr in their study of the partial sequence of the E4 polypeptide, and it is noteworthy that the amino acid compositions of the fragments isolated are closely similar in the two studies (Table 2).

CNBr cleavage will generate a homoserine residue at the C-terminus of all of the derived peptides, except for the C-terminal section of the parent polypeptide (unless the C-terminal residue of the parent polypeptide is methionine). Thus the fragment CN-D of the present study was identified as being the C-terminal section of the polypeptide.

The peptide CN-C of the present study has a composition similar to that of peptide 'CNBr-3' of Seyer & Glimcher (1977), and was found to have isoleucine as the *N*-terminal residue, thus positioning a further residue in the published partial sequence. Peptide CN-A is closely similar to peptide 'CNBr-2', but with a difference of one residue of proline that is not resolved by careful study of the quantitative data of Seyer & Glimcher (1977). Such a discrepancy in proline content is not unexpected in view of the low sensitivity of the determination of this compound as compared with other amino acids.

It is concluded that the polypeptide isolated in the present study is almost certainly identical with the 'E4' polypeptide and with the component 'j' described by Eggert *et al.*, (1973). Such a position of accord is the more welcome in an area of dental research so long plagued with ambiguities (Fincham, 1978).

The polypeptide E4 is a major low-molecular-weight component of the bovine enamel matrix, and components of similar electrophoretic characteristics have been demonstrated in the foetal enamel of other mammalian species (Katz *et al.*, 1969) and in humans (A. G. Fincham, unpublished work).

The components 'E4' (Seyer & Glimcher, 1971, 1977) and 'j' (Eggert *et al.*, 1973) were obtained in low yield by procedures involving either preparative

Table 3. Comparison of data on the amino acid compositions of J-Group amelogenin isolates from bovine dental-enamel matrix. Data of the present study are from analyses of peptides produced by cleavage with CNBr. References: E3 and E4, Seyer & Glimcher (1971); h and j, Eggert *et al.* (1973).

Amino acid	Content (residues/molecule)				
	Present study	E4	j	E3	h
Proline	9	8	9	12	12
Aspartic acid	1	1	1	2	2
Glutamic acid	3	3	3	4	3-4
Threonine	1	1	1	1	1
Serine	2-3	3	3	3	2
Glycine	5	5	5	3	2
Alanine	0	0	0	1	1
Valine	1	1	1	1	1
Methionine	3	3	2	2	2
Isoleucine	2	2	2	1	1-2
Leucine	3	3	3	6	4-5
Tyrosine	5-6	6	5	3	2-3
Phenylalanine	1	1	1	1	1
Lysine	1	1	1	1	1
Histidine	3	3	3	3	2-3
Arginine	1	1	1	1	1
Tryptophan	†	1	1	1	1

† Qualitatively determined by spectrophotometry

electrophoresis, or gel and ion-exchange chromatography using eluents loaded with disaggregating reagents (6M-urea). Such procedures are complex in operation and frequently difficult to reproduce. The simple two-stage gel-filtration procedure here described is believed to provide a rapid and reproducible procedure for the preparation of this important amelogenin component.

In view of recent proposals regarding the origin of the multiple amelogenin components (Chrispens *et al.*, 1978), which postulate derivation (possibly by selective proteolysis) from a parent 'pro-amelogenin' protein, the establishment of the integrity and sequences of the amelogenins had assumed greater importance than hitherto. The observations of Fukae *et al.* (1978) on the presence of homologies of sequence between C- and J-Group components in the developing enamel of the pig are noteworthy in this context.

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