Isolation and characterization of pig enamelines

Hardy LIMEBACK

Faculty of Dentistry, University of Toronto, Toronto, Ontario M5G 1G6, Canada

Enamel proteins were extracted from pig developing enamel by sequential extraction procedures. Two proteins identified as enamelines by slab-gel electrophoresis (M_r 67000 and 63000) were separated from amelogenins by gel sieving and ion-exchange chromatography. Their enamelin characteristic was confirmed by hydroxyapatite-binding studies and amino acid analysis. Degradation of extracted enamel proteins was also studied *in vitro*. The larger of the two enamelines appeared to be resistant to degradation by endogenous enamel proteinases. Hydroxyapatite showed strong binding with the enamelines, but did not prevent the degradation of the M_r -63000 enamelin. These results indicate that at least one high- M_r enamelin in pig developing enamel is a source of enamelin breakdown products.

INTRODUCTION

Two distinct classes of enamel proteins have been shown to be the major organic constituents of developing enamel matrix. Although their function in controlling crystal growth during enamel maturation is still unknown, their biochemical properties suggest that they have separate roles. For example, amelogenins are low- M_r proteins that are degraded rapidly during maturation of enamel and do not bind strongly to hydroxyapatite (for a review, see Fincham & Belcourt, 1985). One study has suggested that portions of the amelogenin molecule demonstrate crystal-growth-inhibition activity (Doi *et al.*, 1984). Enamelines, on the other hand, are larger proteins (M_r 50000–70000), which are more strongly bound to enamel crystals and appear to be preferentially retained during enamel maturation (Termine *et al.*, 1980).

Because they are so abundant in developing enamel, amelogenins have been extensively studied. The complete amino acid sequence for the major bovine amelogenin protein is known (Takagi *et al.*, 1984). The enamelines, however, are more difficult to obtain in quantities sufficient for extensive biochemical studies. The purpose of the present study was therefore to isolate preparative quantities of purified enamelines in order to investigate their biochemical nature and determine their fate during enamel maturation.

EXPERIMENTAL

Tissue extractions

Unerupted second-mandibular molars were dissected from pig mandibles obtained from a local abattoir. All cellular debris was scraped from the surface, and a 5 mm band of developing enamel was dissected from each tooth coronal to the cemento-enamel junction. The pooled scrapings (15 g) were sequentially extracted with equal volumes (200 ml) of 4.0 M-guanidinium chloride (Sigma, St. Louis, MO, U.S.A.)/0.05 M-Tris/HCl, pH 7.4 (G-extract), and then with guanidinium chloride/EDTA (E-extract), a solution identical with that for the G-extract except that it also contained 0.5 M-EDTA (Fisher, Fairlawn, NJ, U.S.A.), essentially by the methods of Termine *et al.* (1980). After two extractions by each of the G- and E-extraction solutions, the

resulting supernatants were dialysed exhaustively against 1% (v/v) acetic acid by using 3500 Da-cut-off Spectrapor dialysis tubing, and freeze-dried before further purification. In an alternative extraction procedure, by the methods of Belcourt *et al.* (1982), 3.0 g of pig developing enamel scrapings were suspended in 10% (v/v) acetic acid and dialysed for 6 days at 4 °C against the same concentration of acetic acid, by using 12000 Da-cut-off tubing, until all the mineral was dissolved. The resulting protein solution, called A-extract, was dialysed further against 1% acetic acid, centrifuged for 20 min at 10000 *g* to remove undissolved protein, and then freeze-dried before further purification.

Gel-filtration chromatography

In the first purification step, the enamel proteins in the A-, G- and E-extracts were eluted on a precalibrated Bio-Gel (Pharmacia, Dorval, PQ, Canada) P100 gel-sieving column (2.5 cm × 100 cm) with 0.1 M-formic acid. The flow rate was 10 ml/h, and 4.2 ml fractions were collected. The A_{280} of each fraction was measured. Fractions were also analysed by SDS/PAGE (see below), and those peaks containing enamelines were pooled and freeze-dried before further separation by ion-exchange chromatography.

Ion-exchange chromatography

E-extract proteins (4 mg) from Bio-Gel P100 peak B were further separated in 0.05 M-Tris/HCl buffer, pH 7.4, containing 7 M-urea, by fast protein liquid chromatography (f.p.l.c.; Pharmacia), by using a Poly-anion preparatory column. A linear salt gradient was applied (0–1.0 M-NaCl) to elute the proteins, at a flow rate of 1.0 ml/min. Fractions (1.0 ml) were collected and the appropriate fractions pooled, diluted by the addition of an equal volume of salt-free buffer, re-applied and re-eluted under the same conditions. Samples from selected fractions were analysed by SDS/PAGE and immunodot assay.

Degradation assay

For each incubation mixture, 0.1–0.2 mg of enamel protein was dissolved in 50 μ l of 50 mM-sodium acetate buffer, pH 6.0, and incubated at 37 °C for 48–72 h. The reaction was stopped by adding slab-gel sample buffer to

Fig. 1. Gel-filtration chromatography of pig enamel proteins

Bio-Gel-P100 elution profiles of enamel proteins extracted from pig developing enamel are shown in (a), and corresponding Coomassie Blue-stained SDS/PAGE patterns are shown in (b). The elution positions of Blue Dextran (V_0), albumin (Alb), ovalbumin (Ova) and chymotrypsinogen (Chy) are indicated. The proteins contained in the A-extract (a1) Bio-Gel-P100 peaks are revealed in the electrophoretic patterns in (b1): lanes 1 and 2 contained proteins obtained from Bio-Gel-P100 peak A, lanes 3 and 4 from peak B, and lanes 5–10 from peaks C–H respectively. The proteins in two of the E-extract Bio-Gel P100 peaks (a2) are shown in (b2), where lane 2 represents proteins in peak B, lane 3 represents proteins from peak C and lane 1 shows the proteins contained in the E-extract before separation on the Bio-Gel P100 column. For comparison purposes, the G-extract elution profile is shown in (a3). The positions of two M_r ($\times 10^{-3}$) markers on the slab gels are indicated.

the mixture and heating to 60 °C for 15 min before analysis by SDS/PAGE. Control samples were pre-incubated mixtures. In some experiments 25 mg of commercial hydroxyapatite (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was added to the sample and then removed by centrifugation at 12000 g for 5 min in a Microfuge. Proteins adsorbed to the hydroxyapatite were dissolved by using the acetic acid extraction method above.

SDS/polyacrylamide-slab-gel electrophoresis (SDS/PAGE)

Enamel proteins were analysed on 12.5%-polyacrylamide gels by using essentially the same methods as

originally described by Laemmli (1970). Samples were loaded on a 5%-polyacrylamide stacking gel and run in Tris buffers under constant voltage (65 V) for 16–18 h. The slab gels were stained for 1 h with 2.5 g of Coomassie Brilliant Blue R-250 (Bio-Rad)/l in 25% (v/v) propan-2-ol/10% (v/v) acetic acid, then destained overnight in 10% propan-2-ol/10% acetic acid, or they were stained by the silver stain technique (Bio-Rad).

Amino acid analysis

Enamelin (0.02 mg) was hydrolysed in 6 M-HCl at 110 °C under vacuum. The hydrolysate was analysed on a Beckman 121 amino acid analyser, with norleucine as an internal standard.

Fig. 2. Purification of enamelin by f.p.l.c. on a Polyanion column

Partially purified enamelines from the Bio-Gel P100 separation of the E-extract of pig enamel were purified further on a Polyanion column by f.p.l.c. Elution was performed with a Tris/urea buffer and a salt gradient, which was recorded (the diagonal straight line). The A_{230} of the eluted material was continuously monitored and recorded (lower tracings, continuous lines). The positions of the 1.0 ml fractions were also recorded (upper tracings, dotted lines). (a) E-extract proteins (4 mg) from peak B of the Bio-Gel P100 column (Fig. 1a2); (b) fractions 32–38 from f.p.l.c.-1 (a, above), diluted to lower the salt concentration and re-run (f.p.l.c.-2) on the f.p.l.c. column. The arrows indicate those fractions that were analysed by PASGE, and the bars indicate the fractions pooled for further analysis.

Immunodot assay

Affinity-purified antibodies directed against a low- M_r pig amelogenin (Limeback *et al.*, 1986) were used to identify amelogenins in an immunodot assay (Bio-Rad). Samples (1.0 μ l) from the f.p.l.c. columns were dotted manually on nitrocellulose paper, treated with the affinity-purified antibody and developed with Protein A-gold.

RESULTS

Gel-filtration chromatography

Three different profiles of the extracts of enamel proteins were produced by the Bio-Gel P100 separations (Fig. 1). The A-extract, or acetic acid extract, produced a profile very similar to that previously published by Fincham *et al.* (1982). The G-extract profile was similar, except that peaks A and B were smaller. (Peak H was increased in size, owing to the lower- M_r -cut-off tubing used during dialysis.) The E-extract profile showed an increase in the relative size of peak B. Analysis of these extracts by SDS/PAGE is also shown in Fig. 1. Enamelins were eluted in peak B, the same elution position as bovine serum albumin, immediately after the void-volume peak. The initial identity of each Bio-Gel P100 peak was determined by SDS/PAGE (Fig. 1b). Peak B obtained from the A-extract separation (Fig. 1a1) contained two enamelin proteins. The M_r values of these enamelines were estimated by SDS/PAGE to be 67000 and 63000 (Fig. 1b1). A minor M_r -35000 component was also found in this peak. In contrast, peak B from the separation of the E-extract proteins (Fig. 1a2) contained only the M_r -67000 enamelin (Fig. 1b2).

Ion-exchange chromatography

F.p.l.c. fractionation of the Bio-Gel-P100 peak-B proteins from the E-extract, which appeared to contain

Table 1. Comparison of the amino acid compositions of pig M_r -67000 enamelin (this study) and rabbit M_r -70000 enamelin (Slavkin *et al.*, 1982)

Abbreviations: Tr, trace amounts detected; ND, not determined.

	Composition (residues/1000)	
	Pig	Rabbit
Hyp	0	
Asp	99	97
Thr	54	59
Ser	55	76
Glu	177	164
Pro	83	57
Gly	75	81
Ala	87	97
Cys*	Tr	32
Val	17	59
Met	Tr	Tr
Ile	35	35
Leu	95	96
Tyr	7	15
Phe	57	40
His	32	18
Lys	85	55
Arg	42	30
Trp	ND	ND

* Special precautions to minimize losses during hydrolysis were not taken.

a single enamelin protein, resulted in several bound peaks (Fig. 2a). The material eluted in fractions 32–38 was re-applied to the column and re-eluted (Fig. 2b). Analysis of the f.p.l.c. peaks by SDS/PAGE indicated

Fig. 3. Analysis of f.p.l.c.-purified enamelin by PASGE and immunodot staining

Enamel proteins from the f.p.l.c. column separation in Fig. 2 were analysed on a 12.5% polyacrylamide slab gel; the silver stain was used to detect the protein bands (a). The positions of low- M_r standards (Std.) are indicated. The numbers above each track refer to the fraction numbers from the f.p.l.c. separations. A sample of E-extract proteins (Mix) was included for comparison. Immunodot analysis of the fractions from f.p.l.c.-1 is shown in (b). Positions a1-a6 represent the six f.p.l.c.-1 fractions (e.g. fraction 35, which contained the M_r -67 enamelin, was placed in position a5). Positions b1, b3 and b5 contained peak-B proteins from the Bio-Gel P100 separation of the E-extract, and positions c1, c3 and c5 contained G-extract amelogenins. The test strip was incubated with affinity-purified anti-amelogenin antibodies, and the control was treated with non-immune serum during the first antibody-incubation step. Colour was developed with the Protein A-gold technique.

that a M_r -67000 enamelin and a minor component of M_r 33000 appeared to be eluted together in the same salt concentrations, indicating that both bound strongly to the ion-exchange resin (Fig. 3a). The re-applied material appeared to be eluted earlier in the salt gradient, since salt from the previous run was not removed by dialysis. Immunodot analysis of the f.p.l.c. peaks showed that only the material that failed to bind to the resin reacted positively to the affinity-purified anti-amelogenin antibody (Fig. 3b). Furthermore, the assay showed that E-extract Bio-Gel-P100 peak-B proteins contained amelogenins, which were successfully removed by the f.p.l.c. purification.

Amino acid analysis

The amino acid composition of the E-extract M_r -67000 enamelin purified by ion-exchange chromatography is shown in Table 1. The results demonstrate the enamelin nature of this protein. The amino acid composition published for the rabbit M_r -70000 enamelin (Slavkin *et al.*, 1982) is included for comparison.

Enamel-protein degradation

The degradation of enamelin was studied by PASGE. Enamel-protein preparations that had undergone column chromatography followed by $(\text{NH}_4)_2\text{SO}_4$ precipitation

Fig. 4. Enamel-protein degradation

Enamel-protein extracts were incubated for 72 h at 37 °C under different conditions and analysed along with control samples by SDS/PAGE and Coomassie Blue staining. Lanes 1 and 2: Bio-Gel-P100 peak-B enamellins from A-extract before (1) and after (2) incubation; samples were overloaded to demonstrate degradation products. Lanes 3–7: A-extract enamel proteins before (3) and after (4) incubation, after removal of hydroxyapatite-bound proteins (5), after incubation with hydroxyapatite (6), and released after solubilization of the incubated hydroxyapatite (7). Abbreviations: E, enamellins; A, amelogenins.

and extraction still demonstrated considerable degradation relative to unincubated samples, indicating that either (a) not all of the enzyme activity in developing enamel was removed by these procedures, or (b) some enamel proteins have autolytic activity. Nevertheless, the degradation pattern produced in these experiments showed that the M_r -63000 enamelin was susceptible to degradation (Fig. 4). Synthetic hydroxyapatite added to the incubation mixture of enamel proteins removed both enamellins from solution, but did not prevent the degradation of the M_r -63000 enamelin (Fig. 4, lanes 3–7).

DISCUSSION

Two major enamelin proteins were found in the acetic acid extracts of developing pig enamel, enamellins of M_r 67000 and 63000. The SDS/PAGE pattern of these enamellins agrees with published data (Zeichner-David *et al.*, 1985). In contrast, the E-extract lacked the M_r -63000 enamelin. The enamel-protein incubation results suggest that the M_r -63000 enamelin is susceptible

to degradation by endogenous enzyme activity. Since proteinase inhibitors were omitted from the extraction solutions, to allow for isolation and testing of enamel proteinases, it is likely that the M_r -63000 enamelin was degraded during extraction even in the presence of 4 M-guanidinium chloride. Other studies have shown that proteinases found in developing enamel have optimal activity at pH 5.0–6.0 and pH 8.0–9.0, but lower activity at low pH values (Shimizu *et al.*, 1979; Moe & Birkedal-Hansen, 1979). Acetic acid extraction at pH < 4 would therefore promote the isolation of two undigested enamelin proteins instead of just one.

Despite efforts to separate the observed endogenous enzyme activity from the enamel-protein substrates by using column chromatography and $(\text{NH}_4)_2\text{SO}_4$ fractionation, degradation of enamellins could still be observed. Although self-degradation cannot be entirely ruled out as an explanation of these results, it is more plausible that the degradation of enamellins under these conditions was due to specific enamel proteinases, which were closely associated with the substrate during its preparation, even after special precautions were taken to remove these enzymes. A similar relationship has been described for collagenase and its substrate, collagen (Pardo *et al.*, 1980).

That the M_r -63000 and -67000 proteins were indeed enamellins was confirmed by a number of observations. First, the mineral-bound protein fractions were enriched with these high- M_r enamel proteins, both during the sequential extraction procedures and during the synthetic-hydroxyapatite-binding studies. Second, their behaviour on ion-exchange columns and their migration positions on SDS/PAGE were similar to those described for bovine enamellins (Belcourt *et al.*, 1982). The M_r -63000 enamelin was co-eluted on ion-exchange columns with the M_r -67000 enamelin. Third, the amino acid analysis of the M_r -67000 enamelin showed a low content of proline and a lack of methionine, indicating that contaminating amelogenin proteins were absent. A similar result was obtained by Slavkin *et al.* (1982), who isolated the rabbit M_r -70000 enamelin directly from slab gels. Both enamellins were resistant to CNBr digestion, whereas amelogenins were readily digested, confirming the lack of methionine (results not shown).

These experiments have demonstrated, for the first time, the degradation of enamelin *in vitro* by endogenous enzymes and suggest that the source of pig enamellins intermediate in size is an enamelin of M_r 63000. The M_r -67000 enamelin appears to be resistant to degradation by endogenous enamel proteinases, and together these findings support the concept of the presence of both high- and low- M_r enamelin proteins in mature enamel (Termine *et al.*, 1980).

The functions of enamellins during enamel formation are still unknown. Some workers even challenge the existence of enamellins (Strawich & Glimcher, 1985). Nevertheless, high- M_r proteins with typical enamelin characteristics have been described in the enamel of several other mammals (reviewed by Fincham & Belcourt, 1985). In the light of the above findings, the hypothesis that enamellins are protected from degradation because of their high affinity to enamel crystals (Belcourt *et al.*, 1982) applies only to the largest of the pig enamellins; the remainder of the enamellins do not appear to be protected from degradation even after binding to hydroxyapatite. Additional biochemical

studies should now be directed towards the purification and characterization of the other high- M_r enamel proteins found in pig developing enamel.

Special thanks are due to Mary MacKinnon and Carmelo Domenicucci for their expert technical assistance, to Sharon Armstrong for typing this manuscript and to Dr. Jaro Sodek for providing f.p.l.c. facilities. This work was supported by grant MA-9121 from the Medical Research Council of Canada.

REFERENCES

- Belcourt, A. B., Fincham, A. G. & Termine, J. D. (1982) *J. Dent. Res.* **61**, 1031–1032
- Doi, Y., Eanes, E. D., Shimokawa, H. & Termine, J. D. (1984) *J. Dent. Res.* **63**, 98–105
- Fincham, A. G. & Belcourt, A. B. (1985) in *The Chemistry and Biology of Mineralized Tissues* (Butler, W. T., ed.), pp. 240–247, Ebsco Media, Birmingham
- Fincham, A. G., Belcourt, A. B., Lyaruu, D. M. & Termine, J. D. (1982) *Calcif. Tissue Int.* **34**, 182–189
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Limeback, H., Tung, P. S., MacKinnon, M. & Sodek, J. (1986) in *Progress in Developmental Biology*, part B (Slavkin, H. C., ed.), pp. 405–408, Alan R. Liss, New York
- Moe, D. & Birkedal-Hansen, H. (1979) *J. Dent. Res.* **58(B)**, 1012–1013
- Pardo, A., Soto, H., Montfort, J. & Perez-Tamayo, R. (1980) *Connect. Tissue Res.* **7**, 253–261
- Shimizu, M., Tanabe, T. & Fukae, M. (1979) *J. Dent. Res.* **58(B)**, 782–789
- Slavkin, H. C., Zeichner-David, M. & Siddiqui, M. A. Q. (1982) in *Current Advances in Skeletogenesis* (Silbermann, M. & Slavkin, H. C., eds.), pp. 24–33, Excerpta Medica, Amsterdam
- Strawich, E. & Glimcher, M. J. (1985) *Biochem. J.* **230**, 423–433
- Takagi, T., Suzuki, M., Baba, T., Minegishi, K. & Sasaki, S. (1984) *Biochem. Biophys. Res. Commun.* **121**, 592–597
- Termine, J. D., Belcourt, A. B., Christner, P. J., Conn, K. M. & Nylén, M. U. (1980) *J. Biol. Chem.* **255**, 9760–9768
- Zeichner-David, M., Vides, J., Snead, M. & Slavkin, H. (1985) in *The Chemistry and Biology of Mineralized Tissues* (Butler, W. T., ed.), pp. 264–269, Ebsco Media, Birmingham

Received 4 August 1986/10 November 1986; accepted 11 December 1986