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Identification and characterization of enamel proteinases isolated from developing enamel

Amelogeninolytic serine proteinases are associated with enamel maturation in pig

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During tooth formation nearly all of the protein matrix of enamel is removed before final mineralization. To study this process, enamel proteins and proteinases were extracted from pig enamel at different stages of tooth development. In the enamel maturation zones, the major enamel matrix proteins, the amelogenins, were rapidly processed and removed. Possibly associated with this process *in vivo* are two groups of proteinases which were identified in the enamel extracts by enzymography using amelogenin-substrate and gelatin-substrate polyacrylamide gels and by the degradation *in vitro* of guanidinium chloride-extracted amelogenins. One group of proteinases with gelatinolytic activity consisted of several neutral metalloendoproteinases having M_r values from 62000 to 130000. These proteinases were inactive against amelogenins, casein and albumin, and were present in approximately equal proportions in enamel at all developmental stages. In the other group, two serine proteinases, with apparent non-reduced M_r of 31000 and 36000, exhibited amelogeninolytic activity. The substrate preference of the enamel serine proteinases was indicated by their limited degradation of casein and their inability to degrade gelatin and albumin. Contrasting with the distribution of the metalloendoproteinase enzymes, the serine proteinases were found only in the enamel scrapings taken from late-maturing enamel. The amelogenin degradation patterns *in vivo*, observed in the enamel scrapings, were similar to those produced in assays *in vitro* using partially purified fractions of enamel proteinases and amelogenin substrate. Together, these data strongly indicate an important role for the serine proteinases, and possibly the gelatinolytic proteinases, in the organized processing of the enamel protein matrix during enamel formation.

INTRODUCTION

Tooth enamel is formed by the mineralization of a protein matrix containing two or more groups of enamel proteins. Amelogenins form the major group and predominate in the matrix of early developmental enamel, and are subsequently degraded and removed as the mineral content of maturing enamel increases (Robinson & Kirkham, 1985). Enamelins are a minor component of the enamel matrix, but seem to be partially retained in mature enamel (reviewed by Fincham & Belcourt, 1985). Some enamelins appear to be processed during enamel development (Slavkin *et al.*, 1979; Termine *et al.*, 1980; Robinson *et al.*, 1982), whereas others appear to be resistant to degradation (Menanteau *et al.*, 1986; Limeback, 1987). The specific functions of these two groups of enamel proteins are yet to be established, as are the proteolytic pathways involved in their degradation.

Progressive degradation of the enamel protein matrix and replacement of the protein degradation products by mineral occurs extracellularly in maturing enamel and gives rise to the fully mineralized product, mature enamel (discussed by Fincham & Belcourt, 1985). Relatively few reports have described proteolytic activity in enamel protein preparations (Shimizu *et al.*, 1979; Moe & Birkedal-Hansen, 1979; Carter *et al.*, 1984; Crenshaw & Bawden, 1984; Menanteau *et al.*, 1986; Limeback, 1987). Although Robinson *et al.* (1984) identified plasminogen activator in mineralizing enamel, in general past reports conflict on the number, nature and M_r of the enamel proteinases. Indeed, the enzymes extracted with the major amelogenins have been reported to have characteristics of serine, cysteine and neutral metalloendoproteinases.

To investigate enamel proteinases and their potential role in the processing of enamel matrix proteins, we developed a simple enzymogram technique utilizing amelogenin-substrate polyacrylamide gels. In this paper, we report experiments that establish the presence of two groups of enamel proteinases which have different temporal distributions during enamel development. Two serine proteinases (M_r 31000 and 36000) with amelogeninolytic activity were found only in maturing enamel associated with the degradation of amelogenins. A second group of several proteinases, with gelatinolytic activity and characterized as neutral metalloendoproteinases, were found in relatively constant proportions throughout developing enamel.

Abbreviations used: APMA, *p*-aminophenylmercuric acetate; G-extract, guanidinium chloride-extracted enamel proteins; NEM, *N*-ethylmaleimide; PASGE, polyacrylamide-slab-gel electrophoresis; pESP, pig enamel serine proteinase; PMSF, phenylmethanesulphonyl fluoride; TIMP, tissue inhibitor of metalloendoproteinases.

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EXPERIMENTAL

Enamel sampling at different stages of development

Unerupted mandibular molars were dissected from fresh pig mandibles, carefully cleared of soft tissue and wiped with tissue paper to remove surface debris (Limeback, 1985). The molars were paired with respect to size and stage of crown development (Robinson *et al.*, 1987). Transitional enamel was sometimes visualized as a distinct band separating developing enamel from maturation enamel. Only molars in which the transitional enamel occurred midway between the amelo-cemento junction and the cusp tip on the mesio-lingual aspects of the crowns were selected for the study.

Enamel protein processing and proteolytic activity were investigated by analysis of enamel slices. With a razor blade, bands of enamel scrapings were collected in 1 mm increments from the amelo-cemento junction to the cusp tip. Ten strips of enamel were collected, and these included all the developing enamel from the cell-free enamel surface to, but not including, the amelo-dentinal junction. Enamel scrapings were air dried at 24 °C for 2 h and weighed. Small portions were removed for calcium analysis, and the protein in the remainder was extracted as described below.

Extraction of enamel proteins and proteinases

Enamel proteins and proteinases were extracted from enamel scrapings by exhaustive dialysis in M_r-3500-cut-off Spectrapor dialysis tubing against 10% (v/v) acetic acid at 4 °C, and the demineralized material was freeze-dried and weighed (Limeback, 1987). Amelogenins ($M_r < 26000$), for use as substrate in proteinase assays *in vitro*, were prepared by separation of a 4 M-guanidinium chloride extract (G-extract) of pooled enamel scrapings from developing pig teeth on a Bio-Gel P100 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) column equilibrated in 0.1 M-formic acid as previously described (Limeback, 1987).

A second portion (1 g) of the same G-extract preparation was dissolved in 250 ml of assay buffer [50 mM-Tris/HCl (pH 7.4)/0.2 M-NaCl/5 mM-$CaCl_2$/Brij 35 (0.5 mg/ml)/NaN_3 (0.2 mg/ml)] and processed for the isolation of enamel proteinases by modified methods of Shimizu *et al.* (1979). Briefly, the amelogenins were removed by precipitation at 45% satn. of $(NH_4)_2SO_4$ and centrifugation. The proteinases in the 45% satd.-$(NH_4)_2SO_4$ supernatant were then precipitated by increasing the $(NH_4)_2SO_4$ saturation to 65%. The resulting precipitate was reconstituted at 100-fold concentration relative to the original G-extract in 2.5 ml of assay buffer.

Calcium analysis

To locate biochemically the position of the transitional enamel, enamel scrapings, collected for calcium analysis, were dried to completion at 70 °C for 48 h, weighed and dissolved in 1.0 ml of 6.0 M-HCl. These samples, and similarly prepared calcium standards, were diluted to 50 ml with 1.0% (w/v) lanthanum oxide and read against a reagent blank at 422.5 nm in a Perkin–Elmer model 2380 atomic-absorption spectrophotometer. The calcium content of the samples was calculated on a percentage-weight basis by using standard curves (0–10.0 μg of calcium/ml).

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

Enamel proteins were analysed by silver staining (Bio-Rad) after electrophoresis on 12.5% (w/v) cross-linked polyacrylamide gels in the presence of SDS (0.1 mg/ml), in the buffer system of Laemmli (1970). For enzyme assays and enzymography (see below), 0.75 mm-thick mini-slab gels were used (Matsudaria & Burgess, 1978). For all gels a 4% (w/v) cross-linked polyacrylamide stacking gel was used (Laemmli, 1970). Protein bands resolved after electrophoresis were quantified by scanning of the gels on an E-C spectrophotometer (Mandel) and determining the areas of the peaks.

Enzymography

Proteolytic activity in enamel extracts was assayed by a highly sensitive enzymography technique. Discontinuous 10% (w/v) cross-linked polyacrylamide mini-slab gels, 0.75 mm thick, were cast with partially purified amelogenins prepared as described, heat-denatured (60 °C, 20 min) type I collagen (gelatin), casein or albumin (all from Sigma, St. Louis, MO, U.S.A.), all at a concentration of 40 μg/ml. Samples were prepared for electrophoresis at either 4 or 6 μg/μl concentrations in Laemmli (1970) sample buffer, and 10 μl portions were electrophoresed under non-reducing conditions without prior heating. After electrophoresis at 150 V for 40 min, the substrate gels were processed by the methods of Heussen & Dowdle (1980), modified as follows. The gels were rinsed twice in 2.5% (v/v) Triton X-100 for 10 min each to remove the SDS, then incubated in 50 mM-Tris/HCl assay buffer, pH 7.4, or in 50 mM-sodium acetate assay buffer, pH 5.5, at 37 °C for 47 h. The gels were then fixed for 5 min in 15% (v/v) acetic acid, and the undigested substrate and sample protein were made visible by a highly sensitive staining technique utilizing 1.0 mg of Coomassie Brilliant Blue G250/ml in 0.2 M-H_3PO_4/$(NH_4)_2SO_4$ (50 mg/ml) (Neuhoff *et al.*, 1985). Proteolytic activity was detected as cleared bands against the aqua-blue background stain of the undigested substrate in the gel. The M_r of the non-reduced enzymes were calculated by using standard M_r marker proteins (Pharmacia Fine Chemicals, Uppsala, Sweden) or pre-stained M_r marker proteins (Bio-Rad Laboratories) electrophoresed on the same gels but under reduced conditions (65 mM-dithiothreitol, and heated at 65 °C for 20 min before electrophoresis).

Proteinase activity was assayed in the presence of 10 mM-EDTA, 5 mM-PMSF or 5 mM-NEM (all from Sigma) added to the assay buffers and compared with the appropriate solvent controls. Human gingival fibroblast gelatinase present in 0.25 μl of serum-free 24 h-conditioned cell culture medium (1.25 ml/10^6 cells) was used as a positive control. Tissue inhibitor of metalloendoproteinases (TIMP), partially purified from ROS 17/2.8 rat osteosarcoma cells (Overall & Sodek, 1987), was also tested at a concentration of 20000 units/ml [in our assay 1 unit of TIMP inhibits 2 units of collagenase (EC 3.4.24.7) by 50%; 1 unit of collagenase degrades 1 pg of soluble collagen/h at 27 °C]. To activate any latent neutral metalloendoproteinases, samples were incubated with 1 mM-*p*-aminophenylmercuric acetate (APMA) for 60 min at 22 °C before electrophoresis.

The cleared bands on the enzymograms were quantified by laser densitometry at 633 nm with an LKB 2202

Ultrascan laser densitometer, with integration of the generated peaks performed by a customized software program ('Curves', written by Dr P. N. Lewis, Department of Biochemistry, University of Toronto, Toronto, Ont., Canada) on a MacIntosh Plus computer (Apple Computers Inc., Cupertino, CA, U.S.A.).

Amelogenin degradation *in vitro* and pH-dependency of enamel proteinases

To analyse the degradation of amelogenins by the enamel proteinases, 40 μg of partially purified pig amelogenin in 10 μl of assay buffer was added as substrate to 10 μl of the 45–65 %-satd.-$(NH_4)_2SO_4$ G-extract enamel proteinase fraction, then adjusted to 60 μl (final volume) in assay buffer at pH 5.5 or 7.4 and incubated at 37 °C for 60 h. For enzyme characterization EDTA (10 mM), PMSF (1 mM) or TIMP (20000 units/ml) was included in the incubation mixtures. Digestion was stopped by the addition of Laemmli sample buffer and heating at 90 °C for 15 min before electrophoretic analysis on 12.5 % (w/v) cross-linked polyacrylamide mini-slab gels. Controls contained either incubated substrate alone or unincubated substrate and enzyme preparation.

The pH-dependency of the G-extract proteinase activity against amelogenins was determined by incubation in assay buffers at various pH values. The buffers used were 0.05 M-sodium acetate adjusted to pH 4.0, 5.0 and 6.0 with acetic acid, and 0.05 M-Tris/HCl, pH 7.0, 8.0 and 9.0. Densitometric scanning of the protein bands resolved after SDS/PASGE separation of the amelogenin degradation products was used to quantify amelogenin degradation. The percentage degradation was determined by calculating the ratio of scanned peaks produced by the amelogenin degradation products (< 20 kDa) to the areas of the scanned peaks of the undigested amelogenins (> 20 kDa). The control samples (0 % degradation), containing equal proportions of degraded and undegraded amelogenins added as substrate, and a sample exhibiting maximal activity (100 % degradation), containing 100-fold excess of amelogenin degradation products relative to the undigested amelogenins, were used to estimate percentage degradation.

RESULTS

A typical SDS/PASGE analysis of the enamel proteins in the developing enamel and the corresponding calcium and protein analyses of a pair of unerupted pig molars are shown in Fig. 1. Abrupt changes in the calcium and protein contents in these teeth occurred in the fifth and sixth enamel slices. The transitional enamel was observed usually between the sixth and seventh enamel slice. SDS/PASGE analysis of enamel extracts prepared from consecutive slices taken from discrete regions (mesial marginal ridge, mesial–lingual cusp) of other teeth showed that the major amelogenins (M_r 20000, 24000 and 26000) occurred in relatively constant proportions, except in the three most coronal slices at the cusp tip, where processing of these amelogenins was evident. However, there was a significant loss of protein per weight of enamel from the enamel slices occurring before this in the transitional enamel, starting with the fifth enamel slice. Protein yields in tooth slices prepared for enzymography were typically in the range of 300–500 μg per slice in developing enamel and ~80 μg per slice in mature enamel.

Acetic acid extracts of developing pig enamel contained two classes of proteinases, as shown by enzymography using substrate/polyacrylamide gels (Fig. 2). Multiple forms of gelatinolytic enzymes were present, with the predominant M_r-62000 and -68000 species showing electrophoretic mobilities identical with human gingival fibroblast gelatinase (Fig. 2*c*). In addition, M_r-92000 and -130000 gelatinases were also present, the M_r values of the non-reduced gelatinases being calculated by using reduced protein M_r standards. The gelatinolytic enzymes were not active against amelogenins, casein or albumin, even after prolonged incubation. They were characterized as neutral metalloendoproteinases since they were absolutely inhibited by 10 mM-EDTA but were unaffected by 1.0 mM-PMSF, 5 mM-NEM (results not shown), or 1.0 mM-APMA. The organomercurial APMA did not appear to cause further activation of the enamel gelatinases, unlike the fibroblast M_r-68000 enzyme, which was partially converted into a M_r-64000 species after reaction with APMA before electrophoresis (Figs. 2*c* and 3*a*, lane F+). Gelatinolytic activity was also abolished by incubation at pH 5.5. Nonetheless, the enzymes appeared to be relatively stable at acidic pH, since enzymic activity could be recovered after the acetic acid demineralization of enamel. Such stabilization may be a consequence of the association between the enzyme and substrate, as previously shown for collagenase (Overall *et al.*, 1987).

The enamel extracts also contained two serine proteinases, with apparent M_r values of 31000 and 36000 when electrophoresed under non-reducing conditions, that were active on amelogenins (Fig. 2*a*), but to a lesser extent on casein (Fig. 2*b*) and gelatin (Fig. 2*c*), and not at all on albumin (results not shown). Indeed, only under some conditions, where large amounts of enamel extract were electrophoresed, or prolonged incubation times were used, was the gelatinolytic activity of these enzymes apparent (e.g. Fig. 3*a*, lane P). In addition to the local degradation of amelogenin substrate incorporated into the gels, the degradation of the amelogenin bands (M_r 33000 and 40000) adjacent to the enzyme bands in the enamel extracts could also be observed. The M_r-31000 and -36000 proteinases were inhibited by 1.0 mM-PMSF, but not by 1.0 mM-APMA or 5.0 mM-NEM (Fig. 2). EDTA (10 mM) caused a slight increase in enzymic activity. At pH 5.5 enzymic activity was decreased by ~60 %, possibly owing to incomplete renaturation of the enzymes at this pH compared with pH 7.4. For convenience, we have designated the pig M_r-31000 and -36000 enamel serine proteinases as pESP-31 and pESP-36, respectively, until their characterization is complete.

Analysis of the same samples of developing enamel shown in Fig. 1 by gelatin and amelogenin enzymography revealed differences in the distribution of the two enzyme activities throughout the developing enamel (Fig. 3). Relative to total protein, gelatinase activity was present in similar amounts in all enamel slices, but with a slight increase in the higher-M_r gelatinases apparent late in development (Fig. 3*a*, lane 4; 7–8 mm enamel slice). In contrast, pESP activity was only present in the late-maturation enamel in the 7–8 mm and 9–10 mm enamel slices taken from near the cusp tip (Fig. 3*b*). These slices also demonstrated increased amelogenin degradation *in vivo* (see also Fig. 1, lanes 8–10), indicating that pESP activity and the degree of amelogenin degradation were correlated in pig developing enamel.

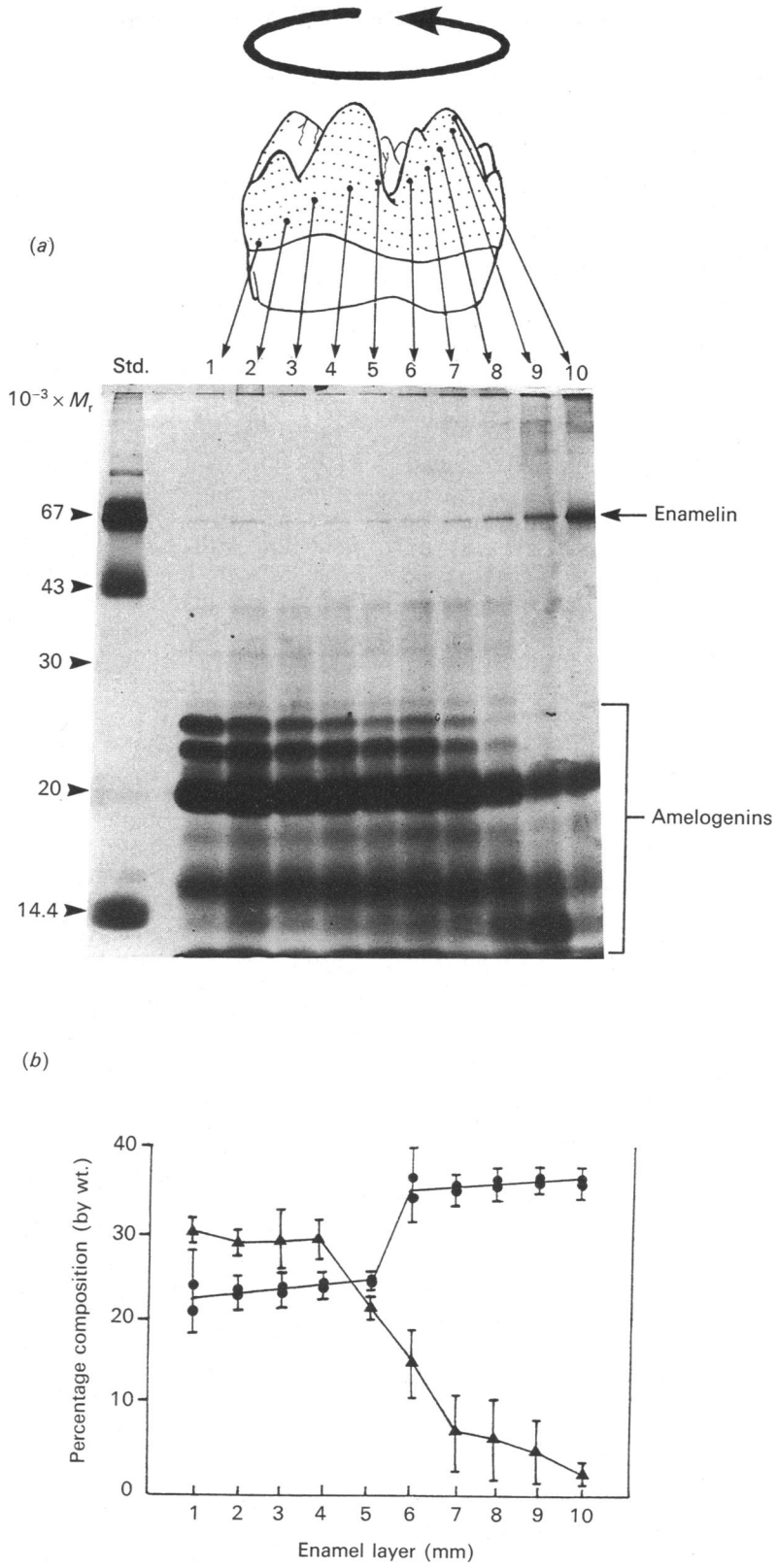

Fig. 1. Analysis of pig enamel during development

Circumferential enamel scrapings were taken in 1 mm strips and dissolved in 10% (v/v) acetic acid, dialysed and freeze-dried as described in the Experimental section. Panel (*a*), equal portions (50 μg) of proteins from each enamel strip were analysed by SDS/PASGE on a 12.5% cross-linked polyacrylamide slab gel and stained with silver (Std., standard proteins). Panel (*b*), the calcium content (●) of the 1 mm enamel scrapings taken from the tooth shown in (*a*) and from a tooth at a similar stage of development are compared with the protein content (▲), as estimated by weighing the material recovered after exhaustive dialysis and freeze-drying. Error bars denote the range in the measurements obtained from the two teeth. Percentage compositions were calculated from the dry weights.

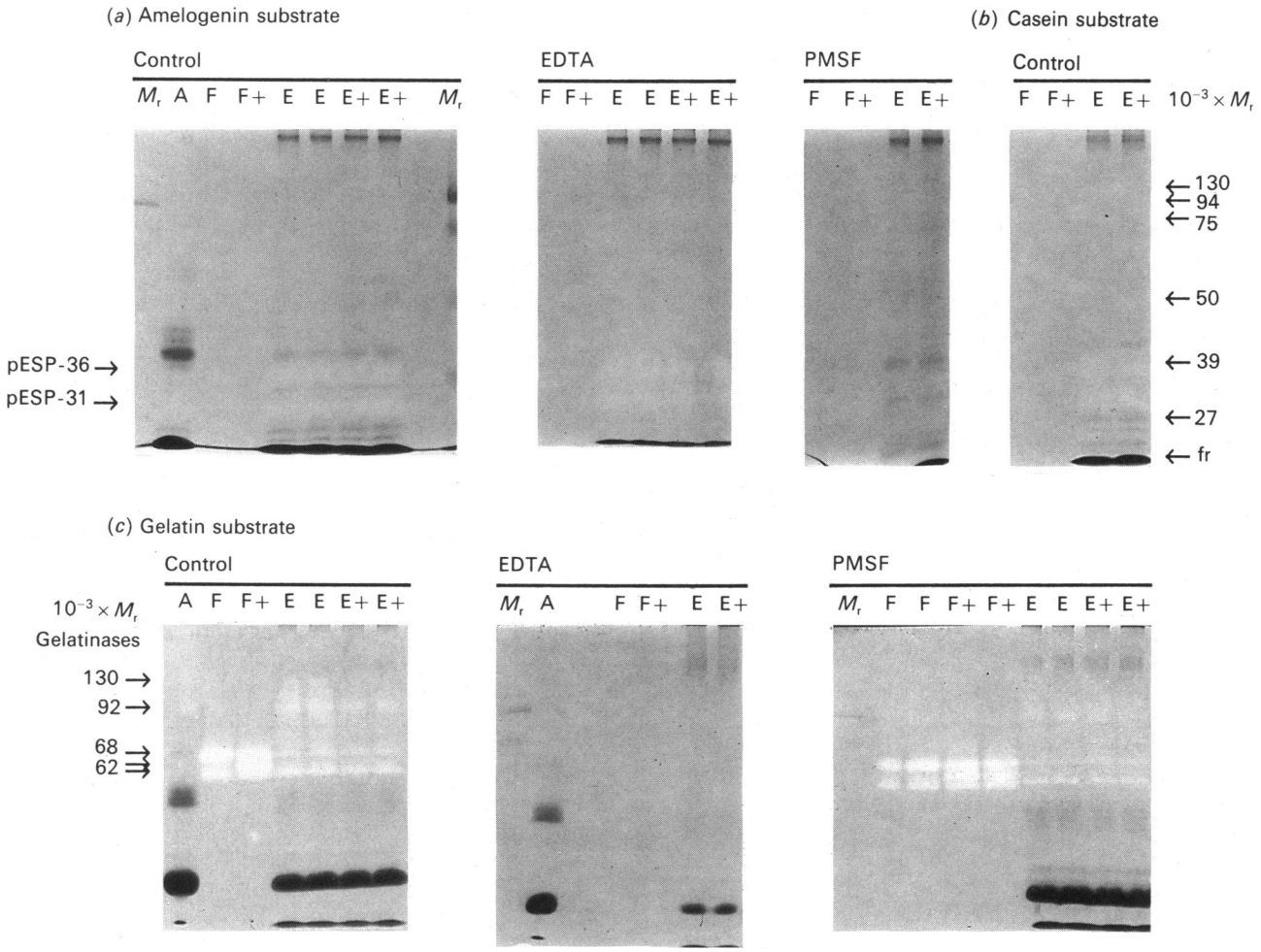

Fig. 2. Characterization of pig enamel proteinases by enzymography

Equal portions (60 μg) of acetic acid-extracted enamel proteins (E) and extracts treated with 1 mM-APMA (E+) were electrophoresed, and the gels were processed for enzymography using (*a*) amelogenins, (*b*) casein and (*c*) gelatin incorporated as substrate in 10% (w/v) cross-linked polyacrylamide mini-slab gels as described in the Experimental section. Incubations were in assay buffer (plus solvent) alone, pH 7.4 (control), or assay buffer in the presence of 10 mM-EDTA, or 1 mM-PMSF as indicated. Undigested substrate was stained with Coomassie Brilliant Blue G250, the cleared zones indicating the presence of proteinases. A, amelogenin (45 μg) used as the substrate in the amelogenin-enzymogram gels. F, F+, fibroblast-secreted gelatinase incubated in the absence or the presence of 1 mM-APMA respectively before electrophoresis. M_r markers visible are phosphorylase *b* (94000) and albumin (67000). The M_r values ($\times 10^{-3}$) of prestained reduced M_r markers are also indicated. Two enamel serine proteinases of M_r 31000 and 36000 (pESP-31 and pESP-36) were resolved that degraded amelogenins and casein. The gelatinases migrated with non-reduced apparent M_r values of 130000, 92000, 68000 and 62000 as indicated.

The degradation patterns obtained from the digestion of amelogenins by the 45–65%-satd.-$(NH_4)_2SO_4$ enamel enzyme fraction of enamel G-extract are presented in Fig. 4. The pH 5.5 test (t_1) samples (substrate plus enzyme, no inhibitors, incubation for 72 h at 37 °C) showed specific amelogenin degradation compared with controls (c_1, c_2). The t_1 samples produced a slight decrease in the amount of the substrate at pH 7.4, but the specific degradation pattern was not as obvious. Use of inhibitors showed that the specific amelogenin degradation was inhibited by PMSF (~80% inhibition), EDTA (~60% inhibition), or PMSF and EDTA added together (~80% inhibition), but not by TIMP.

The c_1 and t_1 samples (pH 5.5) were densitometrically scanned (Fig. 4*bi*) and compared with scans of the samples *in vivo* (Fig. 1) showing the least (slice 1–2 mm) and most (slice 9–10 mm) amelogenin degradation (Fig. 4*bii*). This comparison showed that the samples obtained from both experiments contained similar amelogenin degradation products (M_r 10000 and 16000). The pH optimum for the enamel proteinase extract was around pH 5 (Fig. 4*c*).

DISCUSSION

In this study we have developed a sensitive amelogenin-enzymogram technique to investigate proteinase activity present in as little as 1–2 mg of enamel slices taken from developing enamel. Enzymes belonging to the serine and neutral metallo-proteinase classes were identified in the organic matrix of developing pig enamel. Two serine proteinases, pESP-31 and pESP-36, which electro-

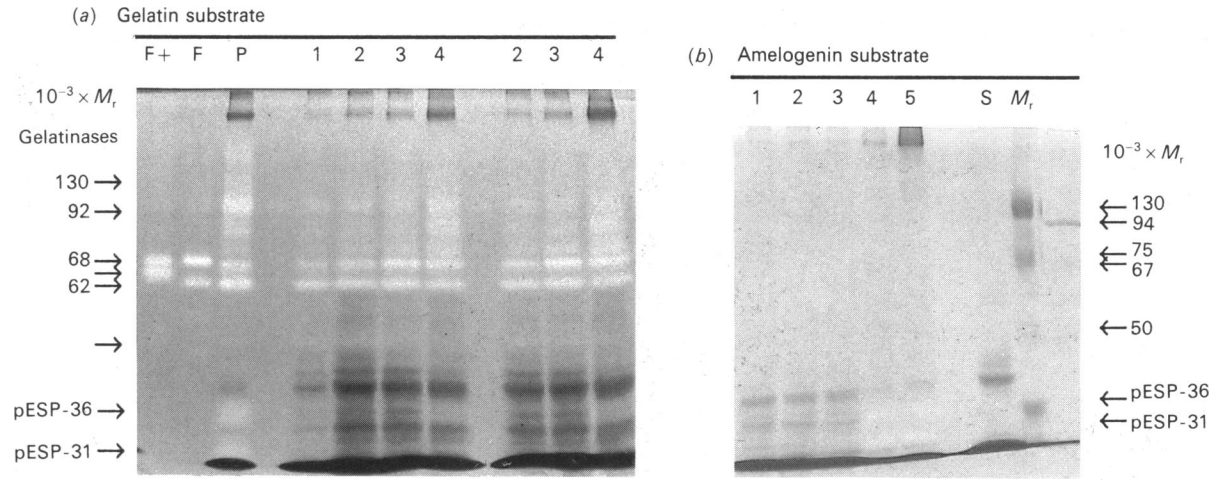

Fig 3. Enamel proteinase activity during enamel development

Acetic acid extracts from enamel slices prepared from the tooth shown in Fig. 1 were analysed by (*a*) gelatin and (*b*) amelogenin enzymography as described in the Experimental section. Lane 1, 1–2 mm enamel slice; lane 2, 3–4 mm enamel slice; lane 3, 5–6 mm enamel slice; lane 4, 7–8 mm enamel slice; lane 5, 9–10 mm enamel slice. The low yields of protein in the 9–10 mm enamel slices did not allow analysis by gelatin enzymography. F, F+, fibroblast-secreted gelatinase incubated in the absence or presence of 1 mM-APMA respectively before electrophoresis. P, pooled enamel extracts from a second tooth used as a positive control. S, amelogenin used as substrate in the amelogenin enzymogram, showing no degradation in the pESP region. M_r, molecular-mass markers as described for Fig. 2.

phoresed with apparent M_r values of 31000 and 36000 respectively under non-reducing conditions, were shown to degrade amelogenins, indicating that these enzymes may be important in the degradation of enamel matrix proteins during enamel maturation. In support of this, we further showed that pESP-31 and pESP-36 were only present in the matrix at stages of enamel maturation where the amelogenins were being maximally degraded *in vivo*. The substrate preference of pESP-31 and pESP-36 for amelogenins was shown by the degradation of amelogenins incorporated as substrate in polyacrylamide gels, by the specific degradation of amelogenins electrophoresing adjacent to the pESP bands, and by the lack of enzymic activity against gelatin and albumin. Some enamelins appear resistant to degradation (Menanteau *et al.*, 1986; Limeback, 1987), so it is of interest that none of the enamel proteinases degraded albumin, a major component of enamelin fractions (Limeback & Chu, 1988).

Gelatinolytic enzymes, characterized as neutral metalloendoproteinases, were also present in the enamel extracts. The major forms of the gelatinolytic enzymes electrophoresed with apparent M_r values of 68000 and 62000 under non-reducing conditions and co-migrated with the M_r -68000 and -62000 gelatinases synthesized by human gingival fibroblasts. However, their possible identity with fibroblast gelatinase is unknown. Minor forms of gelatinases were also present with M_r values of 92000 and 130000, the latter possibly representing dimers of the M_r-62000 or -68000 forms. Unlike the pESPs, the gelatinases were present in approximately equal abundance, relative to total protein, throughout all stages of enamel development, the amounts of the higher-M_r forms possibly increasing proportionately late in enamel maturation. Since the protein content of the enamel slices decreased markedly in the 5–10 mm enamel slices, the actual amounts of the gelatinases present late in enamel development were very low.

Similarly to the fibroblast gelatinase, the M_r-68000 form of the major enamel gelatinase may be the zymogen form of the enzyme. Notably, the fibroblast gelatinase failed to degrade [^{14}C]glycine-labelled gelatin unless activated by APMA (results not shown). That the zymogen form was active after SDS/PASGE in enzymography assays is not surprising, since related proteinases, collagenase and stromelysin, are activated by SDS or electrophoresis, or both (Birkedal-Hansen & Taylor, 1982; Chin *et al.*, 1985; Herron *et al.*, 1986). Thus the enzymography assays did not clearly establish whether the enamel proteinases were present in the precursor latent or active forms in the enamel extracts. However, the results of the amelogenin assays *in vitro* show that amelogenins added to the enamel proteinase fractions were degraded without the addition of known activators (APMA) of metalloendoproteinases, but such degradation was decreased 60% by EDTA, an inhibitor of metalloproteinases. Moreover, since APMA did not cause a decrease in M_r and any further activation of any enamel pro-gelatinase, contrasting with that observed for some of the fibroblast gelatinase, this indicates the presence of a significant amount of active gelatinase in the enamel extracts, possibly present as the M_r-62000 form.

Characterization of the enamel proteinases by the two enzyme assays was in general agreement. That is, maximal inhibition of amelogenin degradation was achieved by serine proteinase inhibitors, but no inhibition was seen with cysteine proteinase inhibitors (NEM, APMA). Although the gelatinases appeared incapable of amelogenin cleavage in enzymograms, their activity nonetheless appears essential for amelogenin degradation in the assays *in vitro*, as indicated by the 60% inhibition of amelogenin degradation by EDTA. Interestingly, the EDTA inhibition of amelogenin degradation occurred despite the increased amelogeninolytic activity of pESPs in the enzymograms. The partial inhibition of amelogenin de-

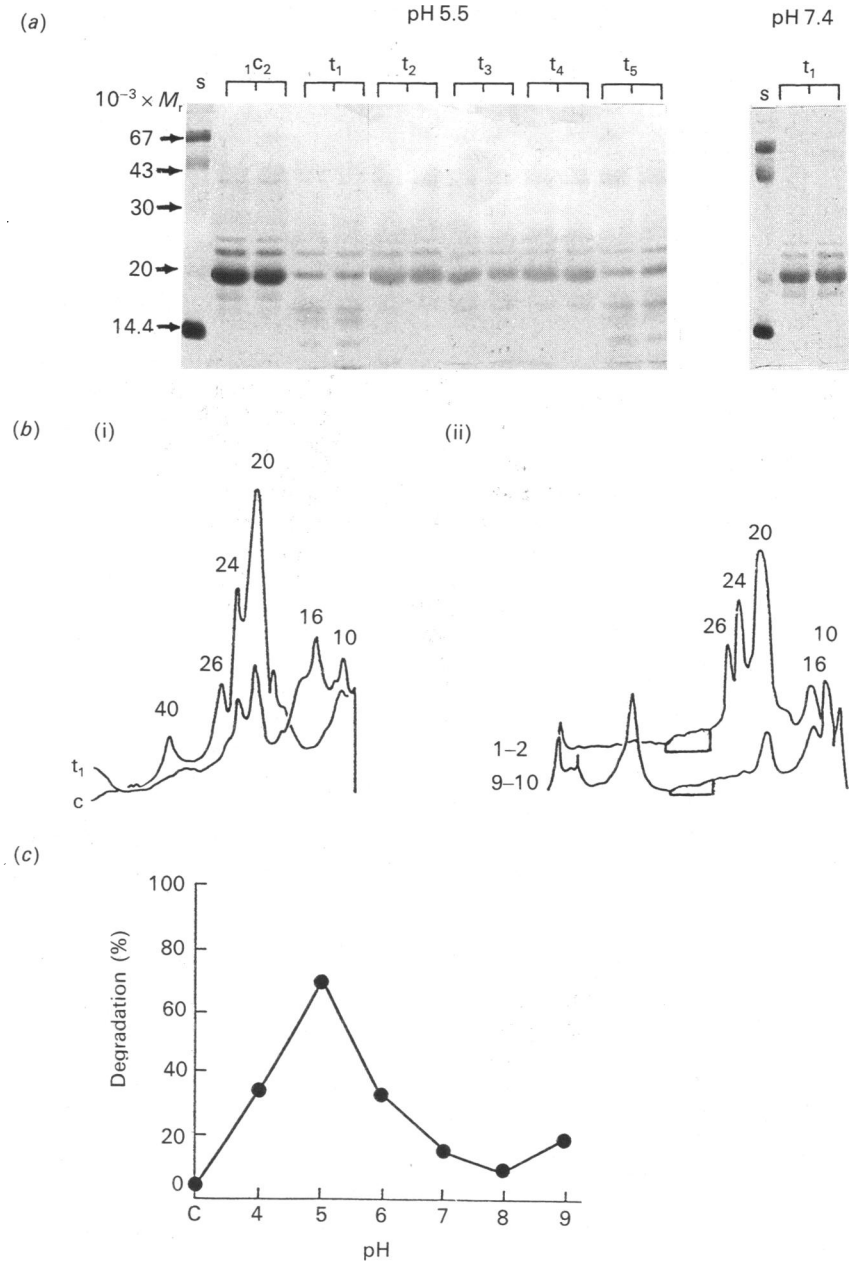

Fig. 4. Characterization of guanidinium chloride-extracted enamel proteinase activity *in vitro*

(*a*) SDS/PASGE patterns obtained from the degradation *in vitro* of pig amelogenin by enamel proteinases present in the 45–65%-satd.-$(NH_4)_2SO_4$ precipitate of G-extract enamel proteins, assayed as described in the Experimental section. Key: c_1, unincubated substrate control; c_2, incubated substrate control; t_1, extract, no inhibitors; t_2, extract plus 1 mM-PMSF; t_3, extract plus 10 mM-EDTA; t_4, extract plus 1 mM-PMSF and 10 mM-EDTA; t_5, extract plus TIMP; s, M_r standards. (*b*) Comparison of the densitometric-scan patterns of the assay reaction products from (i) the assays of amelogenin degradation *in vitro* and (ii) those obtained from two enamel slices (1–2 mm; 9–10 mm). Characteristic M_r-10000 and -16000 amelogenin degradation products are demonstrated in the scans from both experiments. M_r values ($\times 10^{-3}$) are shown beside the corresponding peaks. (*c*) The pH optimum of the G-extract enamel proteinases under the conditions of the assay *in vitro* and with buffers as described in the Experimental section.

gradation by EDTA has also been reported by others (Moe & Birkedal-Hansen, 1979). Gelatinase may act synergistically with pESP to complete the degradation of amelogenin cleavage products first generated by pESP activity on intact amelogenins. Alternatively, gelatinase may act as an activator of a latent pro-pESP secreted by the ameloblasts later in the maturation of enamel. Indeed, stromelysin, a related metalloendoproteinase, can activate procollagenase (Murphy *et al.*, 1987). Thus the extent of the activity of pESP may be dependent on the presence and activity of the enamel gelatinases.

It is likely that the enzymes identified in the enamel extracts are secreted enzymes, and not a result of cell contamination during the preparation of the enamel before collection of the enamel scrapings. After removal of the soft tissue, the enamel surface was wiped sufficiently with tissue paper to remove all cellular debris, including any torn Tome's processes remaining attached to the

surface (Shimizu, 1979). In any case, the distribution of pESP activity in the tooth slices was opposite to that which would be expected resulting from contamination of cell-associated enzymes. That is, if it were to occur, the greatest cellular contamination would be in slices taken from early-developmental enamel (1 and 2 mm enamel slices), where the Tome's processes occur in association with the matrix. Moreover, it is likely that any cellular contamination would also result in the presence of cysteine lysosomal enzymes, but these were not found in the enamel samples.

Robinson *et al.* (1984) demonstrated two plasminogen activators, of M_r 66000 and 62000, present in enamel. Although plasminogen activator was not specifically assayed for here, it is unlikely that the M_r-68000 and -62000 neutral metalloendoproteinases demonstrated by enzymography in our study are the urokinase-type or tissue-type plasminogen activators, since these are serine proteinases. Further, plasminogen activators are incapable of degrading gelatin (Heussen & Dowdle, 1980).

Previous studies have partially characterized the proteolytic activity in enamel extracts (Shimizu *et al.*, 1979; Moe & Birkedal-Hansen, 1979; Carter *et al.*, 1984; Crenshaw & Bawden, 1984). Although conflicting results were reported, the enzymes characterized appear to be of the serine proteinase class, but often were reported to have characteristics of neutral metalloendoproteinases. The unequivocal demonstration of both neutral metalloendoproteinases and serine proteinases in the same enamel extracts reported here now enables rational interpretation of these reports. Overall, our data indicate that it is the serine proteinases which play the most important role in the degradation of amelogenins. First, pESPs degraded the amelogenin substrate in the enzymograms as well as adjacent amelogenin bands, whereas the gelatinases appeared inactive against the intact amelogenin substrate. Second, amelogenin degradation in the enzymograms was inhibited by PMSF, but not by EDTA, APMA, NEM or TIMP. Third, only those tooth slices which showed the most amelogenin breakdown products contained the pESP activity, whereas the gelatinase activity remained relatively unchanged. Moreover, the amelogenin degradation pattern resulting from the digestion of amelogenin *in vitro* by the enamel extracts containing pESP was very similar to the pattern observed *in vivo*.

Collectively, it appears that progelatinases are synthesized, deposited and then activated in the developing matrix. Limited amelogenin processing appears to occur after the transitional enamel stage in a precise temporal manner corresponding to the synthesis and activity of the enamel serine proteinases. Since a large proportion of the matrix proteins have already been removed from enamel before the complete degradation of the major amelogenins by these enzymes, we propose that the function of the pESPs is to degrade amelogenins which do not diffuse readily towards the resorbing ameloblasts of mature enamel. The actual identity of pESP-31 and pESP-36 and their homology with other serine proteinases is currently unknown. However, the purification of the enamel serine proteinases is likely to be a difficult undertaking, given the extremely small amounts of these enzymes that were present in the maturation enamel, which, even by using a highly sensitive enzymography technique, were only readily detected by using long incubation times.

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