Non-specific esterases in partly mineralized bovine enamel

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Activity for non-specific esterase was demonstrated in the matrix of developing bovine enamel with α-naphthyl acetate and 5-bromoindoxyl acetate as the esterase substrates. By use of high-performance liquid chromatography gel filtration, ion-exchange chromatography, and electrophoresis three esterases were shown to be present in the enamel matrix. The enzymes showed highest activity at pH 6.5–7.5. In sections a strong reaction was observed in the secretory ameloblasts. The esterases may be proteolytic enzymes that participate in the degradation of the matrix proteins. □ *Electrophoresis; enamel matrix; high-performance liquid chromatography; histochemistry; proteolytic enzymes*

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Developing enamel matrix consists mainly of large amounts of low-molecular amelogenins and a small group of higher molecular enamelins. During mineralization the amelogenins are degraded and removed, whereas the enamelins seem to be partially retained in mature enamel (1).

Several histochemical studies of the enamel organ indicate that proteolytic enzymes might play a part during formation of dental hard tissues (2–7). Partly mineralized enamel matrix contains serine proteases that have been suggested to cause reductions in the molecular weights of the matrix proteins (8). Matthiessen (9) observed a high activity for non-specific esterases in secretory ameloblasts, and recently serine esterase inhibitors have been shown to inhibit bone resorption (10). To study further proteolytic enzymes that could participate in the maturing of the dental enamel by degradation of the enamel proteins, this paper describes the non-specific esterases in partly mineralized bovine enamel.

Materials and methods

Spectrophotometric and electrophoretic procedures

Unerupted teeth taken from 1- to 2-year-old calves freshly obtained from the slaughterhouse were dissected free, and the soft tissues were carefully removed. The partly mineralized enamel was isolated by scraping. Light microscope studies confirmed that no ameloblast remnants were present on the surface of the cleaned immature teeth.

To evaluate the influence of the extraction solution on enzyme activity, different solutions for demineralization of the partly mineralized enamel were tested: 1 M NaCl in 0.03 M Tris buffer, pH 7.4, or 4 M guanidine-HCl in 0.07 M Sørensen phosphate buffer, pH 7.4. Other extractions with ethylenediaminetetraacetic acid (EDTA), Tris buffer, and acetic acid were carried out as previously described (11). The extraction step was repeated three times, and the supernatant dialyzed three times for 24 h each against cold-distilled water and lyophilized. The resulting enamel powder was used for enzyme activity determination. α-Naphthyl acetate (αNA) and 5-bromoindoxyl acetate (IAc) served as substrates for the spectrophotometric measurements. For demonstration of enzymatic bands after gel electrophoresis IAc was used as substrate.

IAc esterase activity was determined in a sample solution with 1 ml 0.2 M Tris-maleate buffer, pH 8.5, 100 µl substrate solution (1 mg IAc/ml), 25 µl nitroblue-tetrazolium

(NBT, 5 mg/ml), and 100 µl enamel solution (1 mg/ml). The absorption of the NB-diformazan produced during the reaction was read at 575 nm (12). αNA esterase activity was determined after incubation with 600 µl 0.07 M phosphate buffer, pH 7.4, 300 µl substrate (0.5 mg αNA/ml), and 100 µl enamel solution (1 mg/ml). The absorbance of the released α-naphthol was measured spectrophotometrically at 328 nm. To determine the pH optimum of the reaction, Tris–maleate buffer and phosphate buffer in the pH range 5.5–8.5 were used. The enzyme was characterized by adding various inhibitors to the reaction solution: phenylmethylsulfonylfluoride (PMSF), mersalyl, triorthocresylphosphate (TOCP), and EDTA.

Samples of tooth enamel extract were analyzed by high-performance liquid chromatography (HPLC) on an ion-exchange column (DEAE 5 PW). The individual fractions were examined for protein in accordance with Lowry et al. (13) and for esterase activity as above. The column was loaded with 100-µl samples. The flow was 0.5 ml/min, and 0.5-ml fractions were obtained. For HPLC gel filtration an SW 300 column was used. The flow was 0.5 ml/min, and 1-ml fractions were obtained.

The acrylamide gel for the zymogram analyses was a native 7.5% polyacrylamide slab gel. The electrophoresis was performed at 10°C for 3 h with constant 74-mA current. The lanes were loaded with 40 µg enamel protein. After electrophoresis the gel was incubated in an esterase medium containing 25 ml 0.1 M Tris–maleate buffer, pH 7.2, 8 mg IAc in 3 ml dimethylformamide, and 12 mg NBT.

Table 1. The influence of various extraction media on the preservation of enamel esterase activity

Extraction medium	Enzyme activity
Guanidine-HCl	100%
NaCl	96%
EDTA	51%
Tris	48%
HAc	31%

Histochemical studies

Tooth germs were fixed for 1 h with 2% methanol-free formaldehyde, generated from paraformaldehyde, in 0.1 M Tris–maleate buffer, pH 7.2, with 1% $CaCl_2$ and 4% sucrose. After fixation the tissue was demineralized in Tris–maleate buffer with 10% magnesium–EDTA (14) for 2 weeks at 4°C. The specimens were then frozen in isopentane cooled to −150°C with liquid nitrogen and cut in 6-µm sections. The hexazonium pararosanilin (HPR) method (15) was used to demonstrate esterase activity with αNA as substrate.

Results

The solution in which the proteins were extracted from the partly mineralized enamel influenced the preservation of the enzyme activities. As can be seen in Table 1, the highest esterase activity was found after extraction with guanidine-HCl.

The chromatogram obtained with HPLC is shown in Fig. 1. The separation of the gel filtration column (SW 300) yielded only one protein peak with absorption at 280 nm. When the isolated fractions from the column were tested for enzyme activity with IAc as substrate, three peaks showing esterase activity were obtained. The first peak had a higher molecular weight than the protein fraction. The two other peaks came out with high fraction numbers, indicating that these two esterases both are of low molecular weights.

On the ion-exchange column (DEAE 5PW) the proteins were eluted in fractions 18–25, with an incomplete separation of the different peaks (Fig. 2). Three separate enzyme peaks were obtained. The first merged with the tailing of the protein elution, whereas the two other esterase peaks came out with high fraction numbers.

When the enamel proteins were incubated at different pH values, the esterases showed maximal activity at pH 7.5 when IAc was used as the substrate, whereas a pH maximum at 6.5 was recorded with αNA in the incubation medium (Fig. 3). PMSF, TOCP,

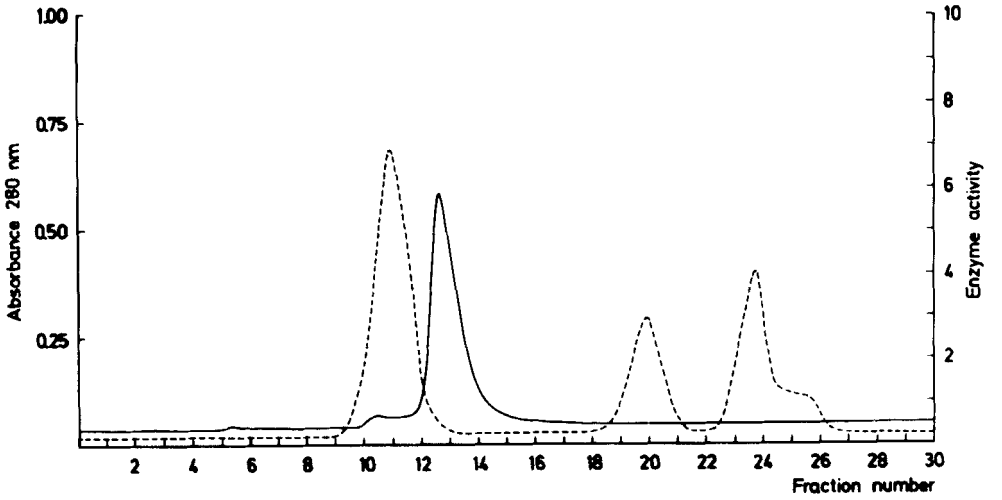

Fig. 1. High-performance liquid chromatography of a bovine enamel extract by use of a gel filtration column (SW 300). Protein concentration was measured at 280 nm (——). Esterase activity was measured at 575 nm with 5-bromoindoxyl acetate as substrate (– –).

EDTA, and mersalyl concentrations of 1 mM inhibited the enzyme activity 40–50% whether αNA or IAc was used as substrate.

Incubation of the electrophoretically separated enamel proteins in IAc medium (Fig. 4) showed three distinct, slowly migrating enzyme bands.

In the sections of the tooth germs stained for αNA activity a strong cytoplasmic reaction was observed apical to the nucleus in the secretory ameloblasts. The enamel matrix showed no staining. Whereas all the secretory odontoblasts were evenly stained, the reaction in the secretory odontoblasts was non-homogeneous; this is, some odontoblasts showed strong activity, whereas others were not stained for αNA esterase (Fig. 5).

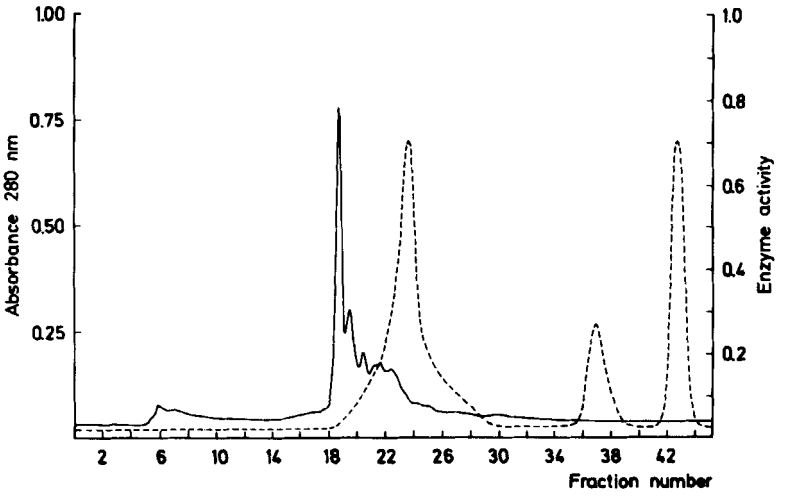

Fig. 2. High-performance liquid chromatography of a bovine enamel extract by use of an ion-exchange column (DEAE 5 PW). Protein concentration was measured at 280 nm (——). Esterase activity was measured at 575 nm with 5-bromoindoxyl acetate as substrate (– –).

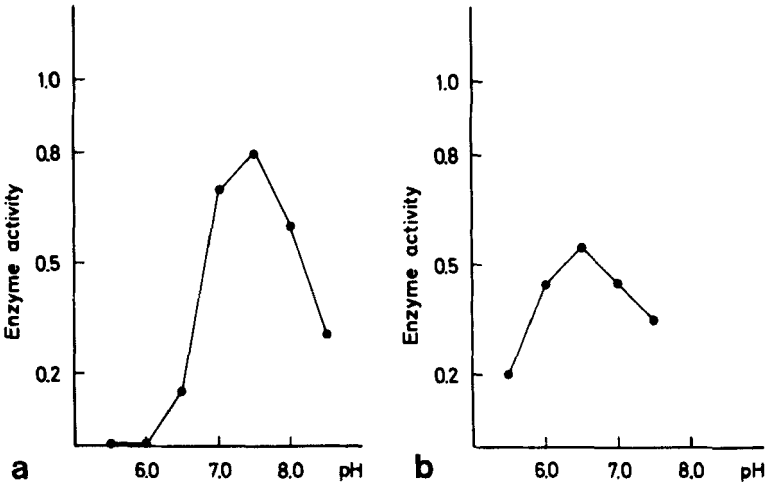

Fig. 3. The influence of pH on esterase activity. 3a. 5-Bromoindoxyl acetate esterase. 3b. α-Naphthyl acetate esterase.

Discussion

Amelogenins are low molecular weight proteins that are degraded during maturation of the enamel. Their function might be to control crystal growth activity (16).

Several studies have suggested the presence of one or more proteases in the matrix of developing enamel (5–7, 17–19). Thus, the degradation of amelogenins during development of the enamel might be due to proteolytic activity.

Recently, we observed some correlation between esterase and esterprotease isoenzymes in kidney, liver, and lung (20). Esterase activity in fetal bovine enamel matrix has been reported by McMahon et al. (21), who found low levels of glycerol ester hydrolase activity. Hydrolysis of benzoyltyrosine ethyl ester and *p*-nitrophenyl acetate by enzymes in embryonic enamel matrix has been described by Crenshaw & Bawden (8). The chromatographic and electrophoretic results of the present study indicate the presence of three esterase isoenzymes in the matrix of partly mineralized bovine enamel.

The present results obtained by letting enzyme inhibitors influence the enamel esterase showed that organophosphates (TOCP and PMSF), organomercury (mersalyl), and a divalent cation chelator (EDTA) caused a partial inhibition. Overall & Limeback (7) found both serine proteases and metalloendoproteinases in developing

Fig. 4. Zymogram showing three slowly migrating bands with esterase activity. 5-Bromoindoxyl acetate was used as substrate. The cathode is at the left.

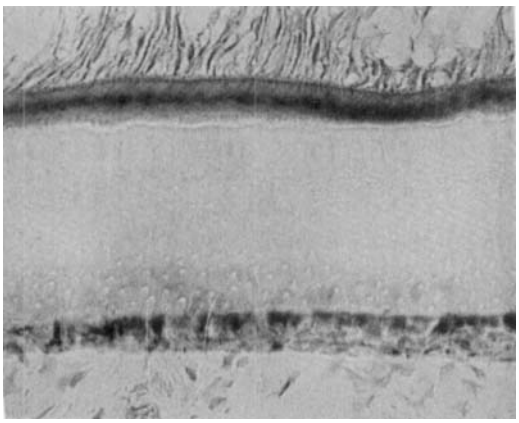

Fig. 5. Micrograph showing α-naphthyl acetate esterase activity in secreting odontoblasts and ameloblasts. In the ameloblasts (top) a homogeneous enzyme reaction can be seen in all cells, whereas in the odontoblasts (bottom) the reaction is much less homogeneous. (Magnification, ×300.)

pig enamel matrix. Strong inhibition of the proteolytic enzyme activity in developing porcine enamel by PMSF has been observed by Crenshaw & Bawden (8) and Menanteau et al. (5). Previous reports on EDTA inhibition of enamel protease activity are somehow conflicting. Inhibition was observed by Moe & Birkedal-Hansen (17) and Menanteau et al. (5), whereas Shimizu et al. (18) found no such effect. This and the partial inhibition observed in the present study could be explained by suggesting that some of the matrix isoenzymes are metalloenzymes, whereas others do not require divalent cations. Our results with mersalyl indicate that sulfhydryl groups may be involved in the regulation of matrix esterase activities. Complete inhibition of enamel protease with mercuric chloride and *p*-aminobenzoic acid was found in developing porcine enamel by Carter et al. (19).

Different pH optima have been observed for the proteases in developing porcine enamel. Shimizu et al. (18) detected acid proteinases with maximal enzyme activity between pH 5.5 and 6.5, whereas Carter et al. (19) found a broad pH optimum around neutral (pH 6.0–8.0) for proteolytic enzyme activity. Our measurements indicate that the enamel esterases had a neutral or slightly acid pH optimum.

Robinson et al. (22) have provided evidence of the presence of a plasmin-dependent proteolytic system in the mineralizing matrices of human enamel. They suggest that the source of plasminogen activators is either a transport from the capillary system or a product from secreting ameloblasts. Enamel esterases could also be either from plasma or of cellular origin. We found a high esterase activity in the secretory ameloblasts. Further, Matthiessen (9) and Luo & Li (4) have observed high non-specific esterase activity in Tome's processes of human ameloblasts. It thus seems likely that the esterases in the enamel matrix are a product of the secretory ameloblasts.

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