Identification of a novel proteinase (ameloprotease-I) responsible for the complete degradation of amelogenin during enamel maturation

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During enamel formation the proteins of the extracellular matrix, particularly amelogenins, are removed prior to maturation. In order to investigate this process and to improve our understanding of the function of proteinases during enamel maturation, proteinase fractions were isolated from developing pig enamel and assayed for proteolytic activity *in vitro*. A recombinant murine amelogenin, M179, was used as a substrate. Two major groups of enamel proteinases were defined as high-molecular-mass [‘high-molecular-weight’ in Moradian-Oldak, Simmer, Sarte, Zeichner-David and Fincham (1994) Arch. Oral Biol. 39, 647–656] and low-molecular-mass proteinases. Here we report the characterization of one of the proteinases present in the low-molecular-mass group. We demonstrate that this proteinase is a serine proteinase capable of degradation of M179 following cleavage of the tyrosine-rich amelogenin polypeptide from the N-

terminal region. A partial N-terminal sequence of the proteinase was obtained (LPHVPHRIPPYGRPXTXNEEGXNPYFXXF-FXXHG). An anti-peptide antibody directed against a synthetic peptide corresponding to the first 14 amino acids of the above sequence was produced. The presence of the proteinase in the acetic acid extract was confirmed by Western blotting. Searching using the amino acid sequence determined in this study showed it to be also present in the 32 kDa and 89 kDa enamel proteins reported by Fukae, Tanabe, Murakami and Tohi [(1996) Adv. Dent. Res., in the press]. We therefore identify the 32 kDa enamel proteinase as an enamel proteinase (‘ameloprotease-I’) which is responsible for amelogenin degradation in maturing enamel. We propose that the 89 kDa enamel protein is a precursor of ameloprotease-I, the first enamel protein for which a function has been defined.

INTRODUCTION

Dental enamel is one of the most profound examples of extracellular matrix biomineralization in vertebrates [1]. It is a unique mineralizing system in which the unusually thick and elongated carbonated hydroxyapatite crystals are oriented to construct a highly ordered microarchitecture [2]. In the secretory phase of enamel development, extremely long and thin mineral ‘ribbons’ are formed; later, in the maturation stage, these thin carbonated apatite structures grow in both thickness and width, concomitant with apparent proteinase processing and removal of the organic matrix. The major proteins, constituting more than 90 % of the extracellular matrix at the secretion stage, are amelogenins [3,4]. The remaining 10 % of the proteins of the enamel extracellular matrix are composed of the acidic enamelines [4], tuftelins [5], proline-rich non-amelogenins also classed as enamelines [6], ameloblastins [7] and enamel proteinases [8–13]. Although considerable advances have been made recently in terms of their primary structures, the specific functions of enamel proteins have yet to be established.

Amelogenins are biochemically distinguishable from other proteins by their unusual proline-rich hydrophobic amino acid composition, and they are believed to play fundamental roles in the highly controlled processes of enamel biomineralization. The observed heterogeneity of amelogenins present in enamel protein preparations from developing teeth is now known to arise both from alternative splicing of the primary amelogenin transcript and from multiple proteolytic cleavages of the secreted proteins (for a review on amelogenin biochemistry, see [14]). Studies on proteolytic activities present in developing enamel from porcine

[8], bovine [9] and rat [13] teeth have indicated that there are two main classes of proteinases: serine proteinases and metalloproteinases. Data are also available indicating that the proteinases studied are specifically active against amelogenins [15–17]. Degradation of the protein components, particularly amelogenins, in the enamel extracellular matrix during crystal growth and maturation appears to be the fundamental function of enamel proteinases. Proteolytic cleavage may also result in modification of the structural properties of the amelogenins and their intermolecular interactions [17]. An understanding of the mechanisms by which amelogenins are proteolytically processed and finally removed during enamel maturation is essential for investigating the function of these tissue-specific proteins in enamel biomineralization.

We have previously demonstrated that the C-terminal region of the recombinant murine amelogenin M179 was cleaved by a calcium-dependent metalloproteinase, isolated from developing bovine enamel, with apparent molecular mass 60–68 kDa [17]. Furthermore, we showed that the rest of the molecule remained intact during incubation with the 60–68 kDa metalloproteinase. In the present paper, we report the isolation and purification of an approx. 30 kDa proteinase from developing pig enamel which is capable of hydrolysis of amelogenin *in vitro*. We used the zymogram technique for detection of proteolytic activity [18]. Using the recombinant amelogenin M179 expressed in an *Escherichia coli* system as a substrate [19], we characterized the novel proteinase as a serine proteinase. We report a partial N-terminal amino acid sequence for this proteinase, which we found to be identical to a sequence present in the 32 kDa enamel protein reported by Tanabe et al. [20]. An antibody directed against a synthetic

Abbreviations used: APMSF, 4-(amidino)phenylmethanesulphonyl fluoride; CBB, Coomassie Brilliant Blue; HMM, high molecular mass; LMM, low molecular mass; PVDF, poly(vinylidene difluoride); TRAP, tyrosine-rich amelogenin polypeptide.

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peptide corresponding to the first 14 amino acids of the N-terminal sequence was produced, and the presence of the deduced ~ 30 kDa proteinase in the acetic acid extract was confirmed by Western blotting. We therefore identify the 32 kDa enamel as being an amelogeninase [21] which we name 'ameloprotease-I'. Uchida et al. [22] have previously shown that the 89 kDa enamel, for which the complete primary sequence has recently been reported [23], is the precursor of the 32 kDa protein, identified here as an enamel proteinase. A possible activation mechanism for ameloprotease-I during enamel biomineralization is discussed.

EXPERIMENTAL

Amelogenin M179 expression and purification

The recombinant murine amelogenin protein M179 was expressed, purified and characterized as previously described [19]. The protein contains 179 residues, lacking only the N-terminal methionine present in the native murine amelogenin M180 [24] and a phosphorylated serine at position 16 [25].

Isolation of the proteinase fractions

Enamel matrix proteins were isolated from developing pig molars by acetic acid extraction, desalting and lyophilization as previously described [26]. The acetic acid extract was fractionated into two major fractions, fraction A [rich in the high-molecular-mass (HMM) proteinases] and fraction B [rich in the low-molecular-mass (LMM) proteinases] using a size-exclusion HPLC system [17,27]. Fraction B was used for further purification by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Approx. 10 mg of fraction B was dissolved in 8.5 ml of water, and 1.5 ml of 100 % satd. $(\text{NH}_4)_2\text{SO}_4$ was then added to remove the bulk amelogenins which co-chromatographed with the proteinases on the size-exclusion column, by precipitation at 15 % $(\text{NH}_4)_2\text{SO}_4$. After a 20 min incubation at room temperature, the mixture was centrifuged in an Eppendorf 5415C centrifuge for 15 min at 14000 *g*. The supernatant was used for further precipitation by increasing the $(\text{NH}_4)_2\text{SO}_4$ saturation to 20 %, 45 %, 65 % and 80 %. The 65 % fraction showed the highest content of LMM proteinases, as judged by the zymogram assay. The pellet was dissolved in 0.1 % trifluoroacetic acid, desalted against double-distilled water after a 20 min centrifugation in a Microcon microconcentrator (Amicon, Beverly, MA, U.S.A.), lyophilized, divided into 50 μl aliquots and stored at -20°C . The protein concentration in these fractions (estimated by UV absorbance at 220 nm) was 1.2 mg/ml.

SDS/PAGE, Western blotting and zymography

SDS/PAGE of the incubation mixture was carried out using the discontinuous buffer system of Laemmli [28] in the presence of 0.1 % SDS, using a 15 % acrylamide gel. The gels were stained with Coomassie Brilliant Blue (CBB). Proteolytic activity was visualized by zymography using an SDS/12 %-acrylamide gel containing 0.1 % gelatin according to the method of Heussen and Dowdle [18]. Samples for electrophoresis were prepared as described elsewhere [17]. Amelogenin-containing gels were prepared in the same manner by replacing the gelatin with an amelogenin fraction extracted from the pig as previously described [25]. Two-dimensional gel electrophoresis was conducted according to the method of O'Farrell [29]. For the isoelectric focusing, Pharmalyte 4–6.5 IEF (Biotech Pharmacia) was used and electrophoresis was conducted at 300 V for 16 h at room temperature. The second-dimension SDS/PAGE was conducted

using a 12 % acrylamide gel at 100 mA for 4 h. For immunoblots against anti-M179 antibody, one second-dimension gel was transferred to a poly(vinylidene difluoride) (PVDF) membrane (Immobilon P; Millipore) using a Semi-phor TE77 apparatus (Hoefer, San Francisco, CA, U.S.A.) and then immunostained using a 1:1000 dilution of the anti-M179 antibody. Immunoblots for detection of ameloprotease-I were performed similarly by transferring a regular SDS/PAGE gel on to a PVDF membrane using a dilution of 1:250 of an anti-peptide antibody. The anti-peptide antibody was produced (Bio-Synthesis Inc., Lewisville, TX, U.S.A.) against a synthetic peptide of 14 amino acid residues (LPHVPHRIPPGYGR) representing the N-terminal sequence of ameloprotease-I.

M179 proteolysis experiments

The recombinant protein (100–150 $\mu\text{g/ml}$) was incubated with the 65 %-satd. $(\text{NH}_4)_2\text{SO}_4$ proteinase fractions in 20 mM sodium acetate, pH 6, containing 2 mM Ca^{2+} at 37°C for 48–72 h. Proteinase additions were at a ratio of 1:10 (w/w; proteinase fraction/M179). The effect of inhibitors was examined by their addition to the incubation mixtures, and proteolysis products were analysed by reverse-phase HPLC as previously described [17]. Mass analyses were performed by electrospray MS (M-Scan Inc., West Chester, PA, U.S.A.) employing a VG Biotech BIO-Q instrument with a quadrupole detector. Computation of the mass of the product was carried out using Mac-BioSpec software (PE SCIEX, Thornhill, Ontario, Canada). For the time-staged incubation experiments with ameloprotease-I, M179 (180 $\mu\text{g/ml}$) was incubated with approx. 150 $\mu\text{g/ml}$ proteinase isolated from two-dimensional SDS/PAGE gels.

N-terminal amino acid sequence determination

A two-dimensional gel separation was transferred to a PVDF membrane at 150 mA for 1.5 h and then stained with CBB. The spots corresponding to where enzymic activity was detected by zymograms were cut out and taken for amino acid sequence analysis. Edman sequence determinations were carried out by the Microchemical Core Facility at the University of California at San Diego. Samples were subjected to N-terminal protein sequencing using an Applied Biosystems Procise Model 494 sequenator equipped with an on-line HPLC for phenylthiohydantoin-amino acid detection. Data were quantified using the 610 software package from Applied Biosystems and by measuring absorbance at 270 nm.

Purification of ameloprotease-I

In a similar way to that described above, second-dimension gels were stained with CBB and the spots corresponding to proteolytic activity were cut from five gels. The proteinase was eluted overnight in a Tris/glycine buffer containing 1 % SDS, desalted against water using Micropure-EZ and Microcon (Amicon), washed with 50 % methanol to remove CBB, and then lyophilized.

RESULTS

Isolation of the 65 %-satd. $(\text{NH}_4)_2\text{SO}_4$ proteinase fraction

In order to examine the presence of proteolytic activities in the isolated fractions, zymograms of those fractions using amelogenin and gelatin as substrates were performed. Zymograms of the acetic acid extract of developing pig enamel showing both gelatinolytic and amelogeninase activities of this fraction are

Figure 1 Analysis of proteinases from pig enamel extracellular matrix by zymography

Zymograms of fractions isolated from developing pig enamel showing the gelatinolytic (**A**) and amelogeninase (**B**) activities of the proteinases in these fractions at pH 8 and pH 6. (**A**) Lane 1, gelatin-containing zymogram of the acetic acid extract at pH 6; lane 2, as lane 1 but at pH 8; lane 3, gelatin-containing zymogram of the 65% $(\text{NH}_4)_2\text{SO}_4$ fraction showing mainly the presence of the LMM proteinases. (**B**) Lane 1, amelogenin-containing zymogram of the acetic acid fraction at pH 6; lane 2, as lane 1 but at pH 8; lane 3, Gibco-BRL HMM standard proteins (molecular masses indicated on the left, in kDa).

shown in Figures 1(A) and 1(B) respectively. The acetic acid extract contained two main groups of proteinases: the HMM proteinases (60–68 kDa), which were previously described in developing bovine enamel by Moradian-Oldak et al. [17] (in that paper the term ‘high-molecular-weight proteinases’ was used), and the LMM proteinases (25–30 kDa) which are described in this paper. As seen in Figure 1(A) the LMM proteinases appeared to show higher activity against gelatin at pH 8 (lane 2) than at pH 6 (lane 1). However, the amelogeninase activity of these proteinases appeared to be slightly greater at pH 6 (Figure 1B, lane 1). Note also the amelogeninase activities near 43, 40 and 90 kDa. Figure 1(A), lane 3, shows the gelatinolytic activity of

Figure 2 Analysis of proteolysis experiments by SDS/PAGE

CBB-stained SDS/PAGE of M179 incubated with and without the 65%-satd. $(\text{NH}_4)_2\text{SO}_4$ proteinase fraction, showing the changes in the 20 kDa M179 substrate during incubation. Lane 1, Gibco-BRL HMM standard proteins (molecular masses indicated on the left, in kDa); lanes 2 and 3, M179 after 48 h of incubation without and with the proteinase fraction respectively; lane 4, 65% $(\text{NH}_4)_2\text{SO}_4$ fraction before incubation; lane 5, M179 after 48 h of incubation with the proteinase fraction in the presence of 10 mM EDTA in the incubation buffer.

Figure 3 Analysis of proteolysis experiments by HPLC

Reverse-phase HPLC profiles of M179 recombinant amelogenin incubated at 37 °C for 48 h in 20 mM sodium acetate, pH 6, containing 2 mM Ca^{2+} and the 65%-satd. $(\text{NH}_4)_2\text{SO}_4$ proteinase fraction, showing the effects of different inhibitors: trace A, proteinase fraction only; B, M179 only; C, M179 plus proteinase fraction in the absence of inhibitors; D–H, as C, but in the presence of 10 mM EDTA (D), 50 μM APMSF (E), 1 mM aprotinin (F), 600 μM phosphoramidon (G) or 100 μM chymostatin (H). All inhibitors were added to the incubation buffer as described in the Experimental section.

the 65%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction enriched in the LMM proteinases. The 65% $(\text{NH}_4)_2\text{SO}_4$ fraction shown in Figure 1(B) was used for incubation experiments with the recombinant M179 amelogenin. This fraction contained two main bands corresponding to proteolytic activity around 25–30 kDa (Figure 1B, lane 2). These two bands may well be isoforms of the same proteinase.

Characterization of the 65%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction

In order to examine the activity against amelogenin of the LMM proteinase present in the 65%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction, the proteinase fractions were incubated with M179 as substrate. The enzymic activity against amelogenin M179 of the 65% $(\text{NH}_4)_2\text{SO}_4$ fraction as a source of the LMM proteinase was analysed by SDS/PAGE and reverse-phase HPLC. This activity was found to be greater at pH 6 (results not shown). Figure 2 presents the patterns of the CBB-stained SDS/PAGE gels of the incubation mixture, showing the changes in the 20 kDa M179 substrate (Figure 2, lane 2) during incubation with the 65% $(\text{NH}_4)_2\text{SO}_4$ fraction. M179 is shown in lane 2. At least seven bands corresponding to proteolytic products are apparent in lane 3, and lane 4 shows the same amount of the 65% $(\text{NH}_4)_2\text{SO}_4$ fraction as was used in lane 3 for the incubation experiment. The 65% $(\text{NH}_4)_2\text{SO}_4$ fraction itself contained few protein bands (Figure 2, lane 4), and these did not match the bands corresponding to the products in lane 3. Note the difference in intensity of the substrate M179 band between lane 2 (before incubation) and

Figure 4 Analysis of M179 degradation products by MS
(A) MS data (experimental data) compared with values computed from the amelogenin sequence. (B) Amino acid sequence of the recombinant amelogenin M179. Vertical lines indicate proteolytic cleavage sites determined on the basis of mass numbers. Two major components (1 and 2) are shown.

Figure 5 Two-dimensional Western blot analysis of pig acetic acid extract using an anti-M179 antibody
Immunoblot of pig acetic acid extract using an anti-M179 antibody, demonstrating that the three constituents in Figure 3A of [27] are not amelogenins. The arrow indicates the position of the three constituents relating to ameloprotease-I. The proteins in this fraction were first separated by isoelectric focusing (IEF) using the pH range 6.5–4.0, and then by SDS/polyacrylamide slab gel electrophoresis.

lane 3 (after incubation). The broad doublet around 15 kDa (Figure 2, lanes 3 and 4) appears to be related to protein traces that have accumulated in the proteinase fraction during the purification steps and is not related to the products. Figure 2, lane 5, shows the inhibitory effect of EDTA on the activity of the 65 % (NH₄)₂SO₄ fraction, suggesting a calcium requirement for this action of the proteinase.

Incubation of M179 with the 65 % (NH₄)₂SO₄ fraction resulted in the formation of major products eluting before M179, at about 35 % (v/v) acetonitrile in the reverse-phase HPLC system (Figure 3, trace C). Generation of products as shown in Figure 3 (trace C) was inhibited by EDTA (Figure 3, trace D), as also shown by SDS/PAGE (Figure 2, lane 5). Serine-proteinase inhibitors such as 4-(amidinophenyl)methanesulphonyl fluoride (APMSF) (Figure 3, trace E), aprotinin (Figure 3, trace F) and chymostatin (Figure 3, trace H), and a metalloproteinase inhibitor, phosphoramidon (Figure 3, trace G), also inhibited proteinase activity. Collectively, these results indicate that the enzyme that degrades amelogenin is a serine proteinase whose activity may be dependent on calcium.

Amino acid analysis of the products isolated by reverse-phase

HPLC showed a high proportion of proline (27 %), 7.8 % histidine, 7.4 % leucine and a high proportion of glutamic acid and/or glutamine residues (23 %). However, no tyrosine was detected. This profile suggests that the products were the results of amelogenin proteolysis after the N-terminal tyrosine-rich amelogenin polypeptide (TRAP) [30] segment had been removed.

Electrospray MS analysis of the products isolated by reverse-phase HPLC indicated the presence of multiple components, of which two major constituents were identified to fit unique amelogenin sequences. Figure 4(A) shows the experimental and computed data for these two sequences. Product 1, having a molecular mass of 11 569.50 Da, matched the M179 sequence between Trp⁴⁴ and Pro¹⁴⁸ (Figure 4B). This sequence, with a computed molecular mass of 11 569.84 Da, is the result of TRAP cleavage from the N-terminus. Product 2, with a molecular mass of 11 532.00 Da, matched the M179 sequence between Pro⁵¹ and Pro¹⁵⁴, with a computed molecular mass of 11 532.81 Da (Figure 4B). An analogous fragment was also identified in amelogenin molecules isolated from acetic acid fractions of human, porcine and bovine enamel extracellular matrix [31].

Identification of an individual proteinase (ameloprotease-I) from two-dimensional SDS/PAGE, and amino acid sequence analysis

In an attempt to identify the serine proteinase responsible for the degradation of amelogenin, we developed two-dimensional gelatin gel zymograms [27]. A two-dimensional CBB-stained SDS/PAGE gel of a pig enamel acetic acid extract containing the ~ 30 kDa proteinase and the corresponding two-dimensional zymogram have been shown previously (see Figure 3 in [27]). We have shown that the positions of the proteinases were revealed by negatively stained areas, while the spots corresponding to their gelatinase activities were smeared. In addition, in the zymogram (Figure 3 in [27]) the activity of the ~ 30 kDa proteinase appeared in two rows, suggesting the presence of minor isoforms. In the CBB-stained gel only one row of spots (at least three spots) was seen. The spots corresponding to the ~ 30 kDa proteinase which we call ameloprotease-I had an apparent pI of 4.3–5.0 in the two-dimensional gel system. Western blot analysis using an anti-M179 antibody clearly demonstrated that the three constituents are not amelogenins (Figure 5, arrow). Amino acid sequence analyses of the three constituents gave identical N-terminal sequences: LPHVPHRIPPGYGRPTXNEEGXNPYFXFF-XXHG.

These results suggest that the three spots correspond to the same proteinase, but at different levels of post-translational modification. This partial amino acid sequence matches the sequence of a 32 kDa non-amelogenin protein isolated from porcine secretory enamel [20]. In addition, Fukae et al. [23] have reported the complete primary structure of a 89 kDa enamelin found in porcine secretory enamel. It was shown that the 32 kDa enamelin was derived from the 89 kDa enamelin. We therefore conclude that ameloprotease-I is the 32 kDa enamelin reported by Tanabe et al. [20], and we suggest that the 89 kDa enamelin is a precursor of ameloprotease-I [22].

Western blot using an anti-peptide antibody raised against the N-terminal sequence of ameloprotease-I

An antibody against a synthetic peptide comprising the first 14 amino acids of the N-terminus of ameloprotease-I (LPHVPHRIPPGYGR) was produced. In order to confirm the identity of ameloprotease-I, an immunoblot of a pig enamel acetic acid extract was performed using this antibody (Figure 6). The immunoblot in Figure 6 shows three bands corresponding to part of the sequence of the 30 kDa proteinase. The activities of these

Figure 6 Detection of ameloprotease-I and its precursors by immunoblotting

Western blot analysis of an acetic acid fraction isolated from developing pig enamel. The antibody was produced against a synthetic peptide deduced from the N-terminal 14 amino acid residues of ameloprotease-I. Positions of molecular mass markers are indicated on the left, in kDa.

Figure 7 HPLC analysis of M179 degraded by ameloprotease-I

Shown is the decrease in the area of the substrate peak, M179 (amelogeninase activity), eluted from HPLC (see inset) after incubation with ameloprotease-I for 5, 10, 20, 30, 45 and 130 min. Ameloprotease-I was isolated and purified as described in the Experimental section. Inset: trace 1, 5 min; 2, 10 min; 3, 20 min; 4, 30 min; 5, 45 min; 6, 130 min.

bands (at least two) are clearly seen in the zymograms in Figure 1. The presence of two additional bands, one near 90 kDa (between 97 and 68 kDa) and the second at the top of the gel, support the above notion that the 89 kDa and 142 kDa enamelin reported by Fukae et al. [23] and Tanabe et al. [20] are indeed precursors of ameloprotease-I.

Activity of ameloprotease-I against M179 substrate

In order to confirm the activity against amelogenin of protein spots on the two-dimensional gels (Figure 3B in [27]), these were assayed in proteolysis experiments with M179. The protein spots (ameloprotease-I) isolated from two-dimensional gels appeared to be highly active when incubated with M179. Figure 7 is a profile of amelogenin degradation by this enzyme at different time intervals (5, 10, 20, 30, 45 and 130 min), assayed by reverse-

phase HPLC. Note that after 2.2 h more than 90% of the substrate had been broken down. These data clearly demonstrate the ability of ameloprotease-I (with the N-terminal sequence LPHVPHRIPPYGR) to degrade amelogenin molecules.

DISCUSSION

The presence of serine proteinases in developing enamel has been described by several investigators. Crenshaw and Bawden [12] described the calcium-dependence of proteolytic activity (not against amelogenin) attributed to the serine proteinase class in unfractionated bovine enamel preparations. Overall and Limeback [11] reported two serine proteinases, pESP-31 and pESP-36, that degraded amelogenin and casein but not gelatin or albumin. Carter et al. [8] used [3 H]acetylcasein or [3 H]-labelled enamel proteins to detect the presence of a 29 kDa serine proteinase (PemB) in developing porcine enamel. Smith et al. [13] described a trypsin-like serine proteinase ($\sim$ 29 kDa) having highest activity against amelogenin during the maturation stage of amelogenesis. While there is a consensus that the LMM proteinase is a serine proteinase that degrades amelogenin in the inner layer of enamel, there has been uncertainty concerning the pH optimum and the calcium requirement of the proteinase. Furthermore, the actual identities of the proteinases studied remained unknown.

In the present study, an individual proteinase with an apparent molecular mass of $\sim$ 30 kDa has been identified, purified and characterized, and shown to have specific activity against the recombinant amelogenin M179. A partial N-terminal amino acid sequence is also reported. This is the first time that the function and identity of an enamel protein have been fully defined. We have previously demonstrated that the C-terminal region of M179 is cleaved by a 'calcium-dependent metalloproteinase', with an apparent molecular mass of 60–68 kDa, isolated from developing bovine enamel. Furthermore, we showed that the remainder of the molecule remained intact during incubation with the 60–68 kDa metalloproteinase, which appeared to be most active at pH 8 [17]. Here we demonstrate that M179 is completely degraded, following removal of the TRAP segment, by a proteinase with an apparent molecular mass of 30 kDa; we call this proteinase ameloprotease-I.

Characterization of ameloprotease-I showing amelogeninase activity

In vitro incubation experiments (at least $n = 15$) using a 65%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction as a source of amelogeninase and the recombinant amelogenin M179 as substrate resulted in the accumulation of certain products. MS analysis indicated a protein with an amino acid sequence that started at Leu⁴⁴ and terminated at Pro¹⁴⁸, demonstrating that the TRAP segment was cleaved from the complete molecule (Figure 4). Failure to detect tyrosine residues in an amino acid composition analysis of the products also confirmed TRAP cleavage, since all six tyrosine residues are within this segment. Difficulties in isolating individual major products from repeated incubation experiments by either HPLC techniques or SDS/PAGE suggested that, after TRAP cleavage, complete degradation of amelogenin was taking place. We therefore speculate that TRAP cleavage and complete degradation were the result of the same proteinase activity. TRAP cleavage by a purified enzyme of 30 kDa from developing bovine enamel was also reported by Sasaki et al. [15]. The activity of that enzyme was found to be optimal at pH 5.5–6.0. However, data on the characterization of the enzyme were limited.

Systematic incubation experiments (at least $n = 6$) showed that the activity of the proteinase in the 65%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction was blocked by the inhibitors EDTA, APMSF, apro-

tinin, antipain and chymostatin (a chymotrypsin-like serine-proteinase inhibitor). We therefore conclude that this proteinase is a serine proteinase with an activity similar to that of chymotrypsin.

Chymotrypsin (molecular mass 25 kDa), like thermolysin and subtilisin, hydrolyses bonds adjacent to a number of different side chains (mainly aromatic, hydrophobic and bulky amino acids) and is capable of extensive fragmentation of the substrate [32]. In contrast, trypsin-like enzymes are relatively restricted in the bonds that they will hydrolyse and, in general, will produce larger fragments. It is important to note that, although the proteinase reported in this study shows a substrate specificity similar to that of chymotrypsin action, its amino acid sequence does not match that of chymotrypsin. These data suggest the presence of a novel proteinase in the developing enamel extracellular matrix which is highly specific in its action to degrade amelogenin. Preliminary results have indicated that the proteinase is not active against albumin, supporting the notion that ameloprotease-I is specific to amelogenin (J. Moradian-Oldak, W. Leung, J. P. Simmer, M. Zeichner-David and A. G. Fincham, unpublished work).

The effect of EDTA on the enzymic activity of ameloprotease-I could be attributed to the calcium-dependence of the enzyme; however, we cannot rule out the possibility that the interaction with EDTA may destabilize the enzyme. It is known that some proteins interact with EDTA in such a way as to affect enzymic activity by substrate or enzyme-complex binding [32]. Preliminary proteolysis experiments in the absence of calcium ions in the buffer solution indicated that the proteinase still has activity (J. Moradian-Oldak, W. Leung, J. P. Simmer, M. Zeichner-David and A. G. Fincham, unpublished work). However, this experiment cannot be conclusive because there is a possibility that traces of calcium are present in the proteinase fraction itself or in the reagent buffers. Future investigations are required to study the calcium-dependency on the activity of the enzyme. Ameloprotease-I showed its highest activity against the M179 substrate at pH 6. However, the activity against gelatin was found to be optimal at pH 8 (Figure 1). A similar effect has been observed in the case of the 60–68 kDa proteinases, and this observation has been related to the changes in M179 structure and its susceptibility to enzymic activity resulting from changes in pH [17,33].

Ameloprotease-I activity always appeared as a doublet band in the zymograms (see Figure 3B in [27]). In the Western blot in Figure 6 of the present paper there are three bands in the ~30 kDa region corresponding to this proteinase. These could be isoforms of the same enzyme, or the smaller bands may be proteolytic products of the larger band.

Ameloprotease-I eluted from two-dimensional gels showed marked activity against M179, as demonstrated in a series of time-staged incubation experiments which showed that more than 90% of the substrate had been degraded by the proteinase after 2.2 h (Figure 7). Whether amelogenin is degraded into small polypeptide fragments or into individual amino acids is a subject of future investigation. The fact that detection of a product was difficult in this case may well be a result of the high enzyme/substrate ratio. Preliminary experiments showed that incubation of a crude acetic acid extract of porcine enamel at room temperature for about 30 h resulted in products containing as few as 10 residues (results not shown).

Precursors of ameloprotease-I

The partial amino acid sequence of ameloprotease-I reported in this study was found to contain part of the protein sequence of a 32 kDa non-amelogenin isolated from porcine secretory enamel

[20]. It is important to mention that no specific function for this non-amelogenin protein has been defined. In addition to the identity of 15 amino acid residues, both proteins (ameloprotease-I and the 32 kDa enamelin) were purified by 65% $(\text{NH}_4)_2\text{SO}_4$ fractionation, and their isoelectric points were the lowest among those of enamel proteins, which are usually in the range 5–7. Tanabe et al. [20] reported the 32 kDa protein as being an enamelin present mainly in the inner secretory enamel. This finding supports the notion that ameloprotease-I is active in the maturation stage of enamel formation, when massive degradation of amelogenin is taking place [13]. It has also been reported that the 32 kDa enamelins include phosphorylated glycoproteins which are rich in Pro, Glu, Gly and Asp residues [20,34].

Recently, Fukae et al. [23] reported the complete primary structure of a 89 kDa enamelin found in porcine secretory enamel at an early stage of development. Their results indicated that the 89 kDa enamelin, consisting of 627 amino acid residues, was derived from a 142 kDa enamelin. It was also shown that the 32 kDa enamelin (equivalent to ameloprotease-I reported in the present study) was derived from the 89 kDa enamelin. The 32 kDa enamelin is composed of 104 amino acid residues, starting at Leu¹³⁵ and terminating at Pro²³⁸ of the 89 kDa enamelin. As also reported by Uchida et al. [22], we show that the 89 kDa enamelin is a precursor of ameloprotease-I. This notion is strongly supported by the presence of bands near 90 kDa and at the top of the gel in the Western blot in Figure 6. The negative band (at about 90 kDa) corresponding to an amelogeninase activity in the zymogram in Figure 1(B) may also be related to the 89 kDa enamelin. It is therefore reasonable to propose that the 89 kDa enamelin is an active precursor of ameloprotease-I, while the 142 kDa enamelin is an inactive precursor.

The term 'enamelines' which was used by Fukae and Tanabe [6] for non-amelogenin proteins of molecular mass 13–142 kDa is an operational term based on the terminology initially used by Termine et al. [4]. According to Termine et al., enamelines were characterized first by their extraction procedure, and then by their property of 'closely associating with synthetic hydroxyapatite crystals under dissociative conditions' and their amino acid compositions, which were rather acidic. Here we suggest the term 'enamel proteinases' for those proteins, extracted from developing tooth enamel, showing enzymic activity against amelogenin or gelatin. Ameloprotease-I is the first enamel proteinase to be characterized in terms of primary structure and function. We present evidence that it has specific amelogeninase activity; however, its activity against other enamel proteins such as tuftelins and ameloblastins is not yet known.

Putative model for activation of ameloprotease-I

An understanding of the ameloprotease-I activation process depends on detailed knowledge of the structures and catalytic actions of its precursors. However, on the basis of present knowledge, we can present a hypothetical mechanism for the activation of this novel proteinase and also its probable pathway from inside the ameloblasts through the extracellular matrix, where enamel mineralization is initiated. We suggest that cleavage of a single specific peptide bond transforms the protein from a catalytically inactive form (the 142 kDa enamelin) to one that is active (the 89 kDa enamelin). A similar activation mechanism for π - and α -chymotrypsin from the precursor chymotrypsinogen has been reported [35]. Polypeptides are then removed from both the N- and C-termini of the 89 kDa enamelin to produce ameloprotease-I (the 32 kDa enamelin). Tanabe et al. [36] reported the formation of the 32 kDa enamelin from the 89 kDa and 142 kDa enamelines to be a result of the action of the same

proteinases that are responsible for the degradation of amelogenin, suggesting an autocatalytic mechanism for activation of ameloprotease-I.

Immunohistochemical studies of immature porcine enamel showed that the 89 kDa enamelin-like immunoreactivity was greatest in enamel prisms located in the outer layer, and gradually decreased towards the dentino-enamel junction [37]. We propose that ameloprotease-I is secreted from the ameloblasts as its inactive precursor (the 142 kDa enamelin). This proposal may well be supported by the faintly stained Golgi apparatus seen in secretory ameloblasts when an anti-(89 kDa enamelin) antibody was used for immunoreactivity studies [37]. Finally, we suggest that the 89 and 142 kDa enamelins reported by Tanabe et al. [20] and Fukae et al. [23] are indeed enamel proteinase precursors that are degraded in immature enamel soon after their secretion to form amelogeninases which are then fully functional for their role in degrading amelogenins in maturing enamel.

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