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How do enamelysin and kallikrein 4 process the 32-kDa enamelin?

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The activities of two proteases – enamelysin (MMP-20) and kallikrein 4 (KLK4) – are necessary for dental enamel to achieve its high degree of mineralization. We hypothesize that the selected enamel protein cleavage products which accumulate in the secretory-stage enamel matrix do so because they are resistant to further cleavage by MMP-20. Later, they are degraded by KLK4. The 32-kDa enamelin is the only domain of the parent protein that accumulates in the deeper enamel. Our objective was to identify the cleavage sites of 32-kDa enamelin that are generated by proteolysis with MMP-20 and KLK4. Enamelysin, KLK4, the major amelogenin isoform (P173), and the 32-kDa enamelin were isolated from developing porcine enamel. P173 and the 32-kDa enamelin were incubated with MMP-20 or KLK4 for up to 48 h. Then, the 32-kDa enamelin digestion products were fractionated by reverse-phase high-performance liquid chromatography (RP-HPLC) and characterized by Edman sequencing, amino acid analysis, and mass spectrometry. Enamelysin cleaved the 32-kDa enamelin only after it was deglycosylated. Kallikrein 4 digestion of the 32-kDa enamelin generated nine major cleavage products, six of which were successfully characterized. After 12 h of digestion with KLK4, all of the 32-kDa enamelin had been cleaved, but some cleavage products persisted after 48 h of digestion.

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The hardening of dental enamel is a consequence of mineral deposition on the sides of enamel crystallites, which form intially as long ribbons of hydroxyapatite (1–3). This mineral deposition is somehow dependent upon the degradation of the extracellular organic matrix, which is composed primarily of amelogenin, ameloblastin, and enamelin (4). There are two proteases that cleave and degrade enamel matrix proteins. The early enzyme, which is expressed from the onset of enamel matrix secretion through the early maturation stage, is known as enamelysin (MMP-20) (5–7). The late enzyme, which is expressed from the beginning of the transition stage and throughout maturation, is designated kallikrein 4 (KLK4) (8–10).

During the secretory stage, enamel proteins are secreted at the mineralization front, where they are associated with the lengthening of enamel ribbons (11). Analyses of the mix of porcine enamel proteins that are extracted from secretory-stage enamel at successive depths demonstrate that the older (deeper) enamel matrix is different from that of the newly formed (superficial) layer (12). The amelogenin C-terminal region is lost, while the rest of the protein is split into a set of relatively stable fragments. The major amelogenin derivatives that accumulate in the deeper enamel are the 20-kDa (amino acids 1–148) 13-kDa (amino acids 46–148), 11-kDa (amino acids 64–148), 7-kDa (amino acids 1–63), and 5-kDa (amino acids 1–45) cleavage products. Besides being composed of smaller pieces, the total amelogenin component is increasingly diminished in amount, which appears to be the principal explanation for enamel crystallites to grow thicker with depth (13,14).

In pig, the major ameloblastin isoform has 395 amino acids (15). The intact protein has never been isolated, but is presumed to be the slowest migrating band (70 kDa) on western blots (16). Only parts of the ameloblastin parent protein are not found in the deeper enamel (16,17). The inner enamel contains mostly fragments from the N-terminal region of ameloblastin – 17-kDa (amino acids 1–170), 15-kDa (amino acids 1–130), and 13-kDa (amino acids 33–130) fragments – and the *O*-glycosylated 13- and 11-kDa fragments from the extreme C terminus (18,19).

Enamelin is the most extreme example of this proteolysis phenomenon. The 186-kDa (amino acids 1–1104) parent protein can only be found within 1 μm of the enamel surface (20). Most enamelin cleavage products are confined to the superficial enamel (21,22). The 32-kDa enamelin cleavage product (amino acids 136–241) is the only stable domain, and accumulates to 1% of total enamel protein (23–25). It is apparent then, that the mix of enamel proteins at the secretory/mineralization front is dictated by the level of expression of enamel matrix proteins by the ameloblast. Processing and selective degradation of this inventory of secreted proteins by enamelysin determines the mix of enamel matrix protein derivatives that accumulate in the deeper enamel.

Insight into the functional reasons for the processing of enamel proteins focuses on the activities of enamelysin, which is secreted along with the matrix proteins

during the secretory stage of amelogenesis (26,27). The enamel phenotype of enamelysin knockout mice (–/–) includes disrupted enamel rod organization, reduced enamel thickness, and a tendency for the enamel to delaminate from the underlying dentin (28). In addition to these secretory-stage defects, the enamel hardness is decreased by 37%, while the weight per cent of mineral decreases by 7–16% (29). The importance of enamelysin activity for proper enamel maturation is manifest in the pigmented hypomaturation *amelogenesis imperfecta* displayed by an individual with two defective MMP-20 alleles (30).

While the role of enamelysin in enamel maturation is becoming understood in greater detail, enamel maturation is more directly associated with the activity of KLK4 (31,32). During the transition and early maturation stages, when the expression of enamelysin is waning and the expression of KLK4 is highest, the protein content of the enamel matrix decreases from 30 to 2% (wet weight) and the average size of enamel proteins is reduced (33). This decrease is associated with a significant change in the amino acid composition of total matrix protein, which is consistent with the interpretation that amelogenins might be preferentially degraded while enamelins persist in the mature enamel (34). Kallikrein 4 is not expressed by ameloblasts during the secretory stage, but is expressed at that time by the underlying odontoblasts. Kallikrein 4 is secreted by odontoblasts via their cell processes into the deepest enamel layer, where it is believed to facilitate the hardening of enamel nearest to the dentino-enamel junction (DEJ) (35). The most definitive evidence that KLK4 is necessary for enamel maturation is the finding that human *KLK4* mutations cause hypomaturation *amelogenesis imperfecta* (36).

The cleavage of recombinant amelogenin (rP172) by the recombinant MMP-20 catalytic domain (37) and by native KLK4 (38) *in vitro* has been reported in detail and is entirely consistent with the interpretation that enamelysin represents the predominant processing/degradation activity that determines the mix of amelogenin derivatives which accumulate in the deeper enamel during the secretory stage, and that KLK4 is the predominant activity during the transition and maturation stages that aggressively degrades the residual enamel matrix. Both MMP-20 and KLK4 are thought to be active against other enamel proteins, such as enamelin and ameloblastin; however, experimental evidence for such activities is lacking. In this study, we use native MMP-20 and KLK4, isolated from developing pig teeth, to digest native 32-kDa enamelin and amelogenin (P173), also isolated from developing teeth. The results provide new insights into the interplay of these molecules during amelogenesis.

Material and methods

All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Program at the University of Michigan.

Isolation of porcine KLK4

Tooth germs of permanent molars were surgically extracted from the maxillae and mandibles of 6-month-old pigs at the Michigan State University Meat Laboratory (East Lansing, MI, USA). The enamel organ epithelia (EOE) and dental pulp tissue were removed with tissue forceps. The soft, cheese-like enamel was separated from the crowns using a spatula. Early maturation-stage enamel samples, containing KLK4, were obtained by scraping the remaining hard, chalky enamel. The hard-enamel shavings were homogenized in Sörensen buffer (pH 7.4), made by mixing Na_2HPO_4 and KH_2PO_4 to achieve a final phosphate concentration of 50 mM and a pH of 7.4. Insoluble material was removed by centrifugation. The supernatant was saturated 40% by the addition of ammonium sulfate, and the precipitate was removed by centrifugation. Then, the supernatant was saturated to 65%, and the precipitate (containing KLK4) was pelleted by centrifugation. The 40–65% saturation pellet was resuspended in 2 ml of resin buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4). The same volume of benzamidine sepharose 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden), which was equilibrated with the resin buffer, was added, and the suspension was rotated overnight at 4°C. The bound resin was packed into a disposable column and washed with 10 volumes of resin buffer. Bound protein was eluted with 5 ml of 0.05% trifluoroacetic acid (TFA) and the eluate was injected onto a C18 reverse-phase (RP) column (TSK-gel ODS-120T, 7.5 mm × 30 cm; TOSOH, Tokyo, Japan). The column was eluted with a linear gradient (20–80% buffer B in 60 min) at a flow rate of 1.0 ml min^{-1}. Buffer A was 0.05% TFA, and buffer B was 0.1% TFA in 80% aqueous acetonitrile. Protein was detected by absorbance at 230 nm.

Isolation of porcine enamelysin

Enamelysin (MMP-20) was isolated from secretory-stage porcine (soft) enamel shavings, as described previously (38). In brief, the alkaline enamel extract was successively fractionated on a Q-Sepharose Fast Flow column (1.6 cm × 20 cm, Amersham Biosciences), a Superdex 200 column and, finally, on an RP column (TSK-gel Phenyl-5PW, 4.6 mm × 7.5 cm; TOSOH).

Isolation of porcine amelogenin (P173)

Uncleaved, native porcine amelogenin was isolated from secretory-stage porcine (soft) enamel shavings, as described previously (39). In brief, the alkaline enamel extract was divided into three fractions (Q1 to Q3) by anion-exchange chromatography on a Q-Sepharose Fast Flow column (1.6 cm × 20 cm; Amersham Biosciences). P173 was isolated from fraction Q2 by using a C18 RP column (TSK-gel ODS-120T, 7.5 mm × 30 cm; TOSOH).

Isolation of the 32-kDa enamelin

The 32-kDa cleavage product of porcine enamelin was isolated from secretory-stage porcine enamel shavings, as described previously (40). In brief, the 32-kDa enamelin was isolated from the neutral soluble fraction and purified by reverse-phase high-performance liquid chromatography (RP-HPLC), first using a C4 column (0.8 × 30 cm; Millipore, Bedford, MA, USA) and then by using a C18 column (41–43).

Digestion of P173 or the 32-kDa enamelin with MMP-20 or KLK4

The proteinase and substrate were incubated at a molar ratio of $\approx 1:80$ for MMP-20 and 1 : 150 for KLK4 in 50 mM Tris-HCl buffer (pH 7.4) at 37°C. Reaction aliquots were quenched by the addition of sample buffer at 0, 6, 12, and 48 h, and analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The KLK4 digestion of the 32-kDa enamelin, after the 48-h incubation, was fractionated by C18 RP-HPLC.

SDS–PAGE, western blots, and zymography

Enamel fractions are separated by SDS–PAGE on 15% gels stained with Coomassie Brilliant Blue (CBB) or silver. Duplicate gels were transblotted onto Hybond-P membrane (Amersham Biosciences) and immunostained with a polyclonal antibody raised in rabbits against recombinant pig KLK4 (30). Zymograms were performed on 10% SDS polyacrylamide gels containing 0.2% gelatin, according to published methods (37). Reactions were performed in 50 mM Tris buffer (pH 7.4) at 37°C for 24 h.

Automated Edman degradation, amino acid analysis, and mass analysis

Automated Edman degradation was performed on an Applied Biosystems Procise 494 cLC protein sequencer, amino acid analyses were performed using a Beckman Model 7300 ion-exchange instrument, and electrospray ionization mass spectrometry (ESMS) was carried out on a Micromass Q-TOF API mass spectrometer (Waters-Micromass, Milford, MA, USA) at the W.M. Keck Facility at Yale University.

Deglycosylation of KLK4

To remove N-linked oligosaccharides, KLK4 in 0.1 M citrate-phosphate buffer (pH 5.0) was incubated with 1 mU glycopeptidase A (Seikagaku America, East Falmouth, MA, USA) at 37°C for 24 h.

Results

Kallikrein 4 was purified from the hard enamel of developing pig teeth (Fig. 1). The portion of the neutral soluble enamel extract that is soluble in ammonium sulfate at 40% saturation, but which precipitates in ammonium sulfate at 65% saturation, was affinity-purified using benzamidine sepharose, which specifically binds to serine proteases. Gelatin zymography detected the KLK4 doublet at 32 and 34 kDa, but also the 76–78 kDa serine protease (Fig. 1A) (44). The eluate from the affinity purification was then fractionated by RP-HPLC (Fig. 1B), which separated KLK4 from the larger protease (Fig. 1C). Enamelysin (MMP-20), the 32-kDa enamelin cleavage product, and the intact major amelogenin isoform (P173) were also isolated from developing teeth, as demonstrated previously (39–41).

Native MMP-20 and KLK4 were both active and able to digest amelogenin (Fig. 2A). The MMP-20 digestion

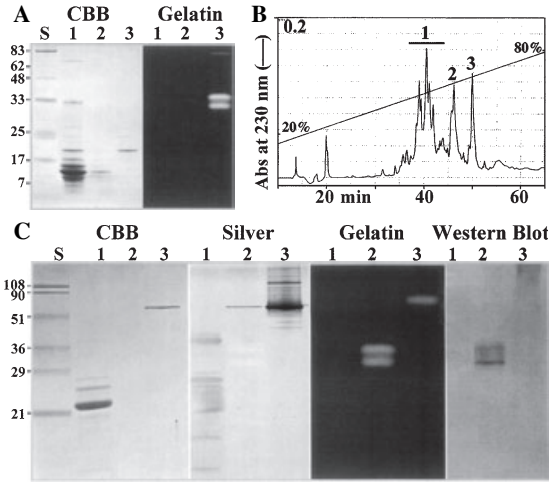

Fig. 1. Purification of porcine kallikrein 4 (KLK4). The 65% saturation ammonium sulfate precipitate was applied to a benzamidine affinity column (A). The flow through (lanes 1), wash (lanes 2), and eluate (lanes 3) were visualized, after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), by staining with Coomassie Brilliant Blue (CBB) and by gelatin zymography. Kallikrein 4 was further purified by reverse-phase high-performance liquid chromatography (RP-HPLC) (B). The three main fractions (lanes 1–3) were analyzed by SDS–PAGE and staining with CBB and silver, gelatin zymography and western blot analyses (C). Porcine KLK4, corresponding to a doublet at 32 and 34 kDa, was identified in fraction 2 and was the only gelatinolytic activity in this fraction.

produced relatively discrete cleavage products (at 18, 14, and 6 kDa), whereas KLK4 generated more of a smear and almost completely degraded amelogenin after 48 h. Although MMP-20 actively digested amelogenin, the 32 kDa enamelin was resistant to cleavage by this enzyme (Fig. 2B, left). Enamelysin (MMP-20) was able to cleave the deglycosylated 32 kDa enamelin (Fig. 2B, right), albeit slowly.

Unlike MMP-20, KLK4 actively cleaved the native 32-kDa enamelin (Fig. 3). All of the original 32-kDa protein was digested after 12 h. Many of the cleavage products showed an increase in apparent molecular weight on 15% SDS–PAGE. This anomalous behavior, however, was not observed on the RP-HPLC column: the native 32-kDa enamelin substrate had a retention time, on a C-18 column, of 63 min (Fig. 3, left), while all of its digestion products had shorter retention times (Fig. 3, right). We speculate that cleavage of the 32-kDa enamelin disrupted its fold, causing it to interact more with the polyacrylamide sieve and run more slowly during electrophoresis. The KLK4 digestion of the 32-kDa enamelin generated a complex mixture of products that resolved into approximately nine chromatographic peaks. Six of the cleavage products were characterized by amino acid composition analyses (Table 1) and also by N-terminal sequencing and mass spectrometry (Table 2). From this data, the N and C termini of the six cleavage products were deduced (Fig. 4). The five KLK4 cleavage sites identified in the 32-kDa enamelin digest were on the C-terminal sides of R180, Y205, H206,

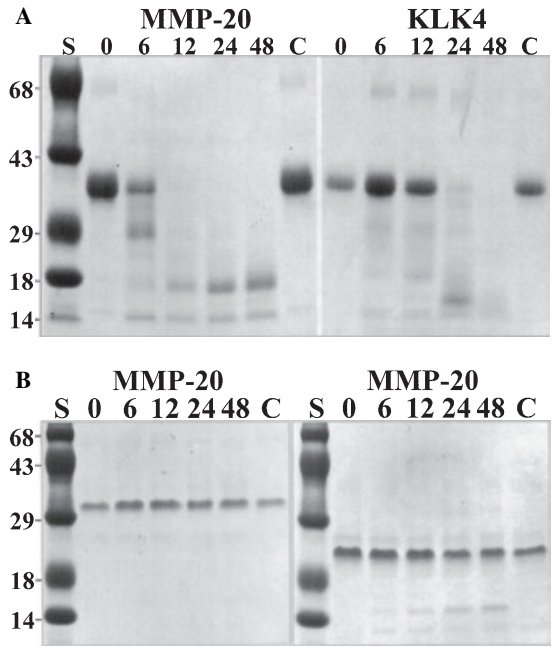

Fig. 2. Enamelysin (MMP-20) and kallikrein 4 (KLK4) digestion of enamel proteins. Porcine amelogenin (P173) was digested with MMP-20 (A, left) and KLK4 (A, right). The digestion patterns are shown after 0, 6, 12, 24, and 48 h. Control samples of P173 (lanes C) incubated for 48 h were not digested, indicating that no proteolytic activity had copurified with the amelogenin protein. Both enzymes digested amelogenin, but in different patterns. The porcine 32-kDa enamelin was incubated with MMP-20 (B, left), but no digestion occurred. Native MMP-20 was able to cleave deglycosylated 32-kDa enamel (B, right).

M219, and R262. In addition, the mass spectrometric analysis confirmed that the three specific triantennary carbohydrate complexes, previously shown to attach to N264 using biochemical techniques, are correct (45).

Discussion

During the secretory stage of amelogenesis, enamelysin and enamel matrix proteins are secreted together, at a mineralization front where enamel ribbons are growing in length. Although the mechanism of enamel biomineralization remains a mystery, a relatively detailed scenario for amelogenin has emerged. Amelogenins self-assemble into nanospheres, 15–20 nm in diameter, that separate and support the delicate enamel ribbons (46) and influence their growth (47). The nascent enamel structure may result from co-operative interactions between the forming crystals and aggregating amelogenins (48), which have their charged C termini oriented at the surface of the nanospheres (49). Some of the details of the protein–protein interactions important for nanosphere assembly have been learned. Two domains in mouse amelogenin (M180: A, amino acids 1–42; B, amino acids 157–173) are essential for amelogenin–amelogenin interactions (50). Enamelysin cleaves amelogenins in the nanospheres near their exposed C termini,

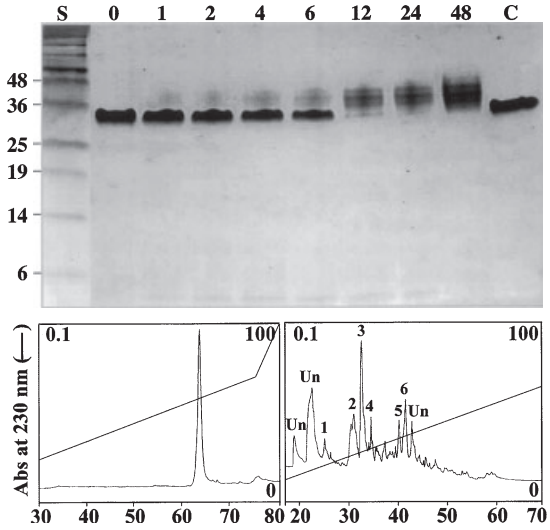

Fig. 3. Kallikrein 4 (KLK4) digestion of the 32-kDa enamelin. Porcine 32-kDa enamelin was digested with KLK4, and the cleavage products after 0, 2, 4, 6, 12, 24, and 48 h were characterized by Coomassie Brilliant Blue (CBB) staining after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Control samples of the 32-kDa enamelin (C) incubated for 48 h were not digested, indicating that no proteolytic activity had copurified with it. Reverse-phase high-performance liquid chromatography (RP-HPLC) chromatograms of the digests at $t = 0$ (left) and $t = 48$ h (right) show that the starting material, represented by a single peak with a retention time of 63 min, was fragmented into at least nine chromatographic peaks, six of which were successfully characterized. The numbers below the chromatograms indicate minutes since injection. The numbers on the right of the chromatograms represent the percentage of buffer B in the HPLC gradient, which is indicated by a thin diagonal line. The peaks are numbered, and the characteristics of their contents are shown in Tables 1 and 2. Un, undetermined.

Table 1

Amino acid compositions of the six 32-kDa enamelin cleavage products generated by kallikrein 4 (KLK4)

Amino acid	1	2	3	4	5	6
Asx	13	154 (3)	116 (6)	84 (2)	12	10
Thr	10	181 (3)	164 (7)	41 (1)	12	13
Ser	–	108 (2)	45 (2)	45 (1)	51 (1)	56 (1)
Glx	10	61 (1)	176 (8)	88 (2)	149 (2)	150 (2)
Pro	186 (1)	160 (3)	197 (9)	192 (5)	138 (2)	144 (2)
Gly	14	263 (4)	15	246 (6)	221 (3)	235 (3)
Ala	–	–	54 (2)	11	–	–
Val	148 (1)	–	60 (2)	–	–	–
Met	–	–	–	–	69 (1)	73 (1)
Ile	–	–	–	38 (1)	–	–
Leu	163 (1)	–	–	–	–	–
Tyr	–	–	–	111 (3)	145 (2)	151 (2)
Phe	–	–	49 (2)	115 (3)	67 (1)	78 (1)
His	299 (2)	–	–	33 (1)	64 (1)	16
Lys	–	–	97 (4)	–	–	–
Arg	157 (1)	73 (1)	27 (1)	27 (1)	72 (1)	74 (1)

The numbers above each column correspond to the peak number on the chromatogram of the enamelin cleaved by KLK4 for 48 h (Fig. 3). Amino acid composition values are in residues/1000. In parentheses are residues/peptide.

Table 2

Edman sequences and mass values obtained for the six 32-kDa enamelin cleavage products generated by kallikrein 4 (KLK4)

Peak	Amino acid sequence	Position	Mass (calculated)	Mass measured
1	LWHVPHR	174–180	944.11	945.55
2	GxDTSPTG	263–279	1642.66	NO
	+ $Deoxyhex_1HexNAc_5Hex_4$		3452.41	3452.51
	+ $Deoxyhex_1HexNAc_5NeuAc_1Hex_4$		3743.50	3743.57
	+ $Deoxyhex_1HexNAc_5NeuAc_2Hex_4$		4034.60	4034.62
3	FEQDFEKP…	220–262	4795.16	NO
4	IPPGYGRPP…	181–205	2858.08	2938.03*
5	HGFGGRPPYY…	206–219	1626.76	1706.70*
6	GFGGRPPYY…	207–219	1489.62	1569.64*

Two calculated peptide masses were not observed (NO) because of glycosylations. For the mono-glycosylated peptide in peak 2, mass values were observed that corresponded to the three triantenniary structureS previously shown to be attached to N264 (45).
*For peptides 3–6, the measured masses were 80 Da more than the calculated masses because of phosphorylations at S191 and S216.

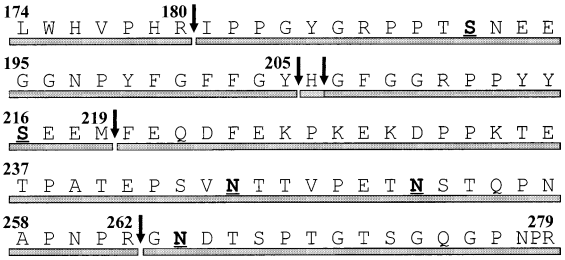

Fig. 4. Enamelin cleavages made by kallikrein 4 (KLK4). The porcine 32-kDa enamelin is a stable enamelin cleavage product that extends from L174 to R279. Arrows mark the five KLK4 cleavage sites. Bars beneath the sequences correspond to cleavage products characterized after 48 h of digestion. The five modified residues on the 32-kDa enamelin are shown in bold and underlined. The two phosphorylated serines are S191 and S219. The three N-linked glycosylations are N245, N252, and N264.

weakening their affinity for the crystals (51). Subsequent cleavages separate the A and B domains, disassembling the nanospheres. Enamelysin is able to cleave amelogenin at numerous sites. The kinetics of these cleavages is influenced by enamelysin cleavage-site specificity and by the accessibility of target sites in the protein substrates (52). Disassembly of the nanospheres exposes additional cleavage sites, and the amelogenin derivatives are slowly degraded. Digestion of amelogenin accommodates the thickening of the secretory-stage enamel crystals in the inner enamel.

The biological activity surrounding enamelin is not known in detail. It is clear, however, that enamelin is necessary for normal enamel biomineralization (25). In humans, *ENAM* mutations cause autosomal forms of *amelogenesis imperfecta*, ranging in phenotype from relatively minor localized enamel pitting to severely hypoplastic enamel (53–58). The secreted enamelin protein is the largest protein in the enamel matrix, having more than 1100 amino acids (59). The secreted protein is rapidly degraded by enamelysin, so that the only fragment which is known to accumulate in the deeper enamel is the 32-kDa cleavage product. The 32-kDa enamelin has a backbone of 106 amino acids, less than one-tenth of the original protein (21). The 32-kDa enamelin has three glycosylations and two phosphorylations, which have been extensively characterized (42,43,45). The 32-kDa enamelin does not form associations with amelogenin or itself (41).

Previously, we proposed that selective proteolysis of enamel proteins by enamelysin is a major determinant of the make-up of the enamel matrix (9). This conclusion is supported by our finding that enamelysin cannot cleave the 32-kDa enamelin *in vitro*, as enamelin glycosylations protect it from degradation. We also demonstrate that KLK4, which is a smaller protease than enamelysin (60,61), degrades the 32-kDa enamelin. We have identified five of the major KLK4 cleavage sites in the 32-kDa protein. Enamelysin and KLK4 cleave enamel proteins in different ways, suggesting that they play complementary, rather than overlapping, roles in amelogenesis.

The discovery that *MMP20* mutations cause a hypomaturation form of *amelogenesis imperfecta* indicates that MMP-20 is necessary for enamel maturation. Kallikrein 4 is apparently not able to compensate totally for the absence of MMP-20. The reason for this might relate to the finding that MMP-20 can, but KLK4 cannot, activate the KLK4 zymogen. If KLK4 activation requires MMP-20 *in vivo*, then defects in MMP-20 would reduce or eliminate KLK4 activity secondarily. Currently, there are no data concerning the mechanism of KLK4 activation *in vivo*. It is also possible that the early processing and degrading activities of MMP-20 are required because some parts of the enamel matrix are only susceptible to cleavage by MMP-20, or that KLK4 cannot penetrate the unnaturally protein-rich maturation-stage matrix that forms in the absence of MMP-20 activity.

Enamelysin is involved in enamel protein processing and degradation. It cleaves and then degrades the amelogenin C terminus. Enamelysin processes the rest of the amelogenin protein into a set of cleavage products that accumulate in the deeper enamel. Then it slowly degrades the amelogenin derivatives, reclaiming space for the partial maturation of enamel crystallites that occurs during the secretory stage. Although there are currently no *in vitro* studies to confirm this, enamelysin is assumed to be the protease that rapidly cleaves the ameloblastin and enamelin parent proteins following their secretion by ameloblasts and then selectively

degrades some of their cleavage products, while allowing others to accumulate. This study provides evidence that the 32-kDa enamelin accumulates because it is resistant to further cleavage by enamelysin.

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