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# Porcine Amelogenins

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**Abstract.** Amelogenins were extracted from the thin outer layer of porcine secretory enamel and purified by gel filtration and reverse-phase HPLC. The results of amino acid sequencing of the purified porcine amelogenins indicated the presence of at least four prototype amelogenins translated from alternatively spliced transcripts. The results of mass spectroscopy of the CNBr-cleaved pepides derived from the 25kDa amelogenin indicated that porcine 25kDa amelogenin is neither phosphorylated nor glycosylated.

**Key words:** Porcine secretory enamel — Porcine amelogenin — Plasma desorption mass spectometry — Amino acid sequence — CNBr cleavage

The organic matrix of developing enamel consists of a family of proteins and large peptides, and their proportions change during enamel maturation [1–6]. Amelogenins are the major constituents, and an amelogenin with an apparent molecular weight of 20kDa, derived from 25kDa amelogenin by the action of proteinases existing in the enamel, is the major amelogenin in porcine secretory enamel [7–9]. It has been shown that the complex mixture of amelogenins is generated by both the degradation of a single amelogenin translation product and also by a differential mRNA processing mechanism [10–16]. In the present study, we describe the isolation of porcine amelogenins and their primary structures.

## Materials and Methods

### *Extraction and Fractionation of the Enamel Proteins*

Tooth germs of permanent incisors were dissected from fresh mandibles of pigs approximately 6 months old obtained from a meat processing facility. After the removal of the surrounding soft tissues and pulpal tissue, the tooth germs were washed in cold saline and wiped carefully with laboratory tissues. A thin outer layer of enamel was scraped from the surface of the secretory stage enamel with a razor blade [9]. A pooled enamel sample was suspended in 10 volumes of 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.8) containing proteinase and phosphatase inhibitors (50 mM ε-aminocaproic acid, 5 mM benzamidine, 1 mM p-hydroxymercuribenzoic acid, 1 mM phenylmethylsulfonyl fluoride and 1 mM levamizole), and was homogenized using a Polytron homogenizer for 30 seconds at half speed and centrifuged for 15 minutes at 10,000 × *g*. This extraction procedure was repeated three times. The supernatant was concentrated by ultrafiltration (YM-5 membrane, Amicon) and applied to a column of Sephadex G-100 (4 × 100 cm) equilibrated with 50 mM sodium carbonate-sodium bicarbonate buffer (pH 10.8) containing the same inhibitors at one-tenth of the above concentrations. The effluents were continuously monitored at 280 nm. Two fractions, one eluted in the first peak from the column of Sephadex G-100 and the other in the second peak, were concentrated and desalted by ultrafiltration (YM-5 membrane, Amicon) and lyophilized. The fraction eluted in the fourth peak was lyophilized without desalination. The lyophilized samples were stored at −80°C.

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### *Purification of Amelogenins*

Each of these enamel protein samples was further fractionated by reverse-phase high performance liquid chromatography (RP-HPLC) using a Pharmacia DfB HPLC system equipped with a TSK-gel ODS-120T column (7.8 mm × 30 cm, TOSO). The column was equilibrated with 0.05% trifluoroacetic acid (TFA) and was eluted with a linear acetonitrile gradient (40–60%) containing 0.05% TFA at a flow rate of 1.0 ml/minute. Each of the peak fractions was collected and lyophilized. The purification of the peak fractions, corresponding to the amelogenins having apparet molecular weights of 27, 25, 23, 20, 18, and 6.5kDa, was performed by rechromatography using the same system. The samples, which were purified and lyophilized, were stored at −80°C.

### *SDS-Polyacrylamide Gel Electrophoresis*

The SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 15% polyacrylamide slab gel containing 1% SDS according to the method of Laemmli [17]. The lyophilized protein samples were dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, 1% SDS, and 50% glycerol at a concentration of 0.1% w/v. The electrophoresis was carried out using a current of 20 mA for 4 hours. The gels were stained with 0.125% Coomassie Brilliant Blue (CBB) and destained with 7.5% acetic acid-5% methanol solution. The apparent molecular weights of the protein bands were estimated by using Bio-Rad LMW protein standards.

### *CNBr Cleavage of 27, 23, and 18kDa Amelogenins and Purification of CNBr-Cleaved Peptides*

Each of the purified 27, 23, and 18kDa amelogenins (3 mg) was dissolved in 40 ml of CNBr solution (0.5 g of CNBr dissolved in 40 ml of 12 M formic acid) and kept at room temperature for 20 hours. After lyophilization, the cleavage products were dissolved in a small volume of 0.1 M acetic acid, applied to a column of PD-10 (Pharmacia), and eluted with 0.1 M acetic acid. The effluents containing peptides were collected and lyophilized.

The fractionation of peptides was carried out by ion-exchange HPLC (IE-HPLC) using a Pharmacia DfB HPLC system equipped with a TSK-gel CM-2SW column (7.8 mm × 30 cm, TOSO). The

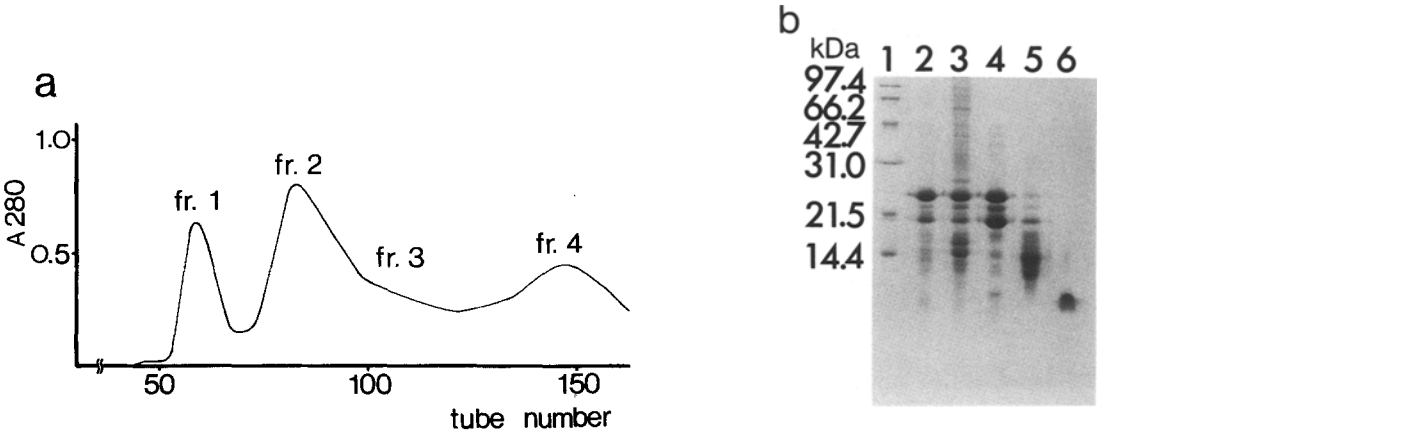


**Fig. 1.** Elution profile of porcine enamel proteins from Sephadex G-100 column (**a**) and SDS–PAGE patterns of the fractions (**b**). Lane 1: BIO-Rad low-molecular weight protein standards, lane 2: original enamel protein sample, lane 3: fr. 1, lane 4: fr. 2, lane 5: fr. 3, and lane 6: fr. 4.

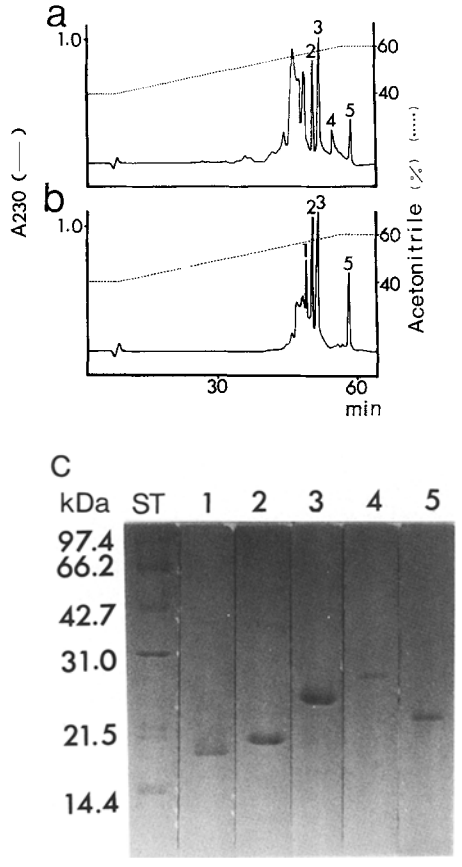


**Fig. 2.** Elution profiles of porcine amelogenins from a TSK-gel ODS-120T column and SDS–PAGE patterns of the purified amelogenins. (**a** and **b**): Chromatograms of fr. 1 and fr. 2 fractionated by Sephadex G-100 gel filtration. Peak numbers indicate 1: 18kDa, 2: 20kDa, 3: 25kDa 4: 27kDa, and 5: 23 kDa amelogenin. (**c**) lane 1: 18kDa, lane 2: 20kDa, lane 3: 25kDa, lane 4: 27kDa, and lane 5: 23kDa amelogenin.

column was equilibrated with 20 mM sodium-acetate (pH 5.0) containing 0.5% isopropyl alcohol and eluted with a linear NaCl gradient (0–1.0 M) at a flow rate of 0.8 ml/minute. The peptides were further purified by RP-HPLC with a TSK-gel ODS-120T column (7.8 mm × 30 cm, TOSO). This column was equilibrated with 0.05% TFA and eluted with a linear acetonitrile gradient (20–70%) containing 0.05% TFA at a flow rate of 1.0 ml/minute.

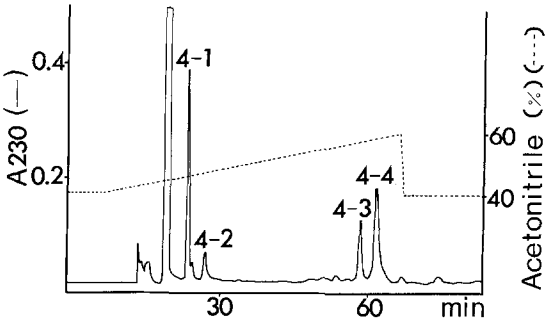


**Fig. 3.** Elution profile of RP-HPLC on a TSK-gel ODS-120T column of the fr. 4 fractionated by Sephadex-G-100 gel filtration. Peak 4-3: 6.5kDa amelogenin and peak 4-4: 5.3 kDa amelogenin.

### *Digestion of the 6.5kDa Amelogenin by Clostridiopeptidase B and Purification of Digestion Products*

Digestion of the purified 6.5kDa amelogenin (0.2 mg) was carried out in 0.1 M sodium phosphate buffer (pH 7.8) containing 1 mM $CaCl_2$ and 5 mM dithiothreitol with an enzyme/substrate ratio of 1/100 (w/w) at 30°C for 24 hours [18]. After lyophilization, the digests were applied to a TSK-gel ODS-120T column (7.8 mm × 30 cm, TOSO) and chromatographed. The column was equilibrated with 0.05% TFA and eluted with a linear acetonitrile gradient (10–80%) containing 0.05% TFA at a flow rate of 1.0 ml/minute.

### *Amino Acid Analysis*

The purified samples (0.2 mg) were hydrolyzed with 6 N HCl in a vacuum-sealed tube at 110°C for 24 hours. The amino acid analyses were performed using a JEOL JLC-300 automatic amino acid analyzer.

### *Automated Edman Degradation*

Automated Edman degradation was performed with an ABI 477A protein sequencer. The purified peptide samples were dissolved in 0.1 ml of 0.1 M acetic acid containing 30% acetonitrile, and 50 µl of the sample solution was applied to the membrane containing 3 mg of polybrene.

**Table 1.** Amino acid compositions of the amelogenins

| | 5.3kDa | 6.5kDa | 18kDa | 20kDa | 23kDa | 25kDa | 27kDa |
|---|---|---|---|---|---|---|---|
| Asp | 49 | 93 | 27 | 24 | 29 | 35 | 49 |
| Thr | 49 | 54 | 42 | 36 | 31 | 39 | 42 |
| Ser | 45 | 39 | 65 | 49 | 49 | 44 | 60 |
| Glu | 77 | 104 | 198 | 184 | 178 | 185 | 197 |
| Pro | 195 | 189 | 230 | 258 | 251 | 245 | 164 |
| Gly | 128 | 48 | 64 | 56 | 42 | 43 | 74 |
| Ala | — | 38 | 30 | 23 | 27 | 30 | 33 |
| Cys | — | — | — | — | — | — | — |
| Val | 17 | 31 | 37 | 32 | 31 | 35 | 54 |
| Met | 68 | 37 | 25 | 27 | 53 | 38 | 24 |
| Ile | 30 | 26 | 36 | 41 | 39 | 34 | 30 |
| Leu | 66 | 119 | 83 | 84 | 99 | 92 | 97 |
| Tyr | 136 | 57 | 28 | 39 | 36 | 33 | 33 |
| Phe | 26 | 22 | 18 | 24 | 19 | 18 | 25 |
| His | 77 | 64 | 89 | 94 | 88 | 84 | 72 |
| Lys | 22 | 49 | 12 | 8 | 7 | 17 | 22 |
| Arg | 15 | 29 | 16 | 10 | 6 | 11 | 22 |

Values are given in numbers of amino acid residues per 1000 total residues

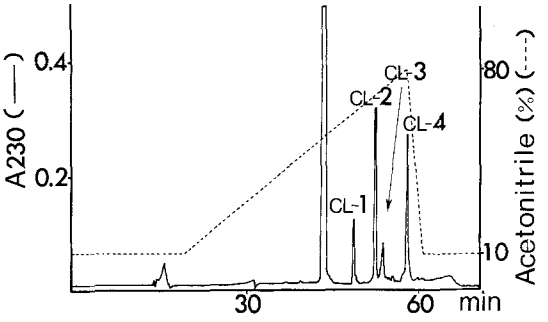


**Fig. 4.** Elution profile of reverse phase HPLC on a TSK-gel ODS-120T column of the 6.5kDa amelogenin digests by Clostridiopeptidase B.

### *Plasma Desorption Mass Spectrometry (PDMS)*

The mass of CNBr-cleaved peptides was determined by using a $^{252}Cf$ plasma desorption time-of-flight mass spectrometer (BIOION 20, ABI). The purified CNBr-cleaved peptides were dissolved in 30 ml of 40% methanol to a concentration of 1 nM/10 μl, and 10 μl of each of the solutions was applied to a nitrocellulose matrix and spin dried [19]. The spectra were collected for $1 \times 10^6$ primary ions, at +15 kV accelerating potential.

## Results

### *Purification of Amelogenins*

Figure 1 shows the elution profile of enamel proteins from the Sephadex G-100 column (Fig. 1a) and the SDS–PAGE patterns of these peak fractions (Fig. 1b). Enamel proteins extracted from the thin outer layer of the porcine secretory enamel were separated into four fractions. The 27kDa amelogenin was eluted in the first peak, which included many other protein components having higher and lower molecular weights than the 27kDa amelogenin (Fig. 1b, lane 3). The 23 and 18kDa amelogenins were eluted in the second peak, in which the 25 and 20kDa amelogenins, the major amelogenins in the enamel of the very early secretory stage, were also eluted (Fig. 1b, lane 4). Amelogenins having low molecular weights were eluted in the last peak.

Figure 2 shows the elution profiles of proteins by RP-HPLC on a TSK-gel ODS-120T column and the electrophoretic patterns of the purified 27, 25, 23, 20, and 18kDa amelogenins. The elution profile of the proteins in the first peak eluting from the Sephadex G-100 column is shown in Figure 2a, and that of the proteins in the second peak is shown in Figure 2b. The peaks, in which the amelogenins were eluted, are numbered in order of eluting. The numbers also correspond with the lane number in the electrophoretic pattern. Peaks 1, 2, 3, 4, and 5 correspond to the 18, 20, 25, 27, and 23kDa amelogenin, respectively.

Figure 3 shows the result of RP-HPLC of the last peak fraction eluting from the Sephadex G-100 column. The amelogenins were eluted in the peaks designated 4-3 and 4-4, and were identified as 6.5kDa and 5.3kDa amelogenin, respectively. Table 1 shows the results of amino acid analyses of the 5.3, 6.5, 18, 20, 23, 25, and 27kDa amelogenins. Interestingly, a low molecular weight amelogenin (6.5kDa) was rich in Asx, Leu, and Lys when compared with the other amelogenins.

### *Clostridiopeptidase B Digestion*

The 6.5kDa amelogenin was digested by Clostridiopeptidase B. The digest was resolved into four peaks, which are designated as CL-1, CL-2, CL-3, and CL-4 in Figure 4, by RP-HPLC on a TSK-gel ODS-120T. The results of Edman degradation of the 6.5kDa amelogenin and the purified peptide eluted in each of the peaks are presented in Table 2. The amino acid sequences of N-terminal 24 residues of the CL-3 and the CL-4 peptides were identical to the 25kDa amelogenin, and the sequence of C-terminal 23 residues of the CL-2 peptide was also identical to that of the 25kDa amelogenin.

### *CNBr Cleavage of Amelogenins*

The CNBr-cleaved peptides derived from the 27, 25, 23, and 18kDa amelogenins were fractionated and purified by IE-HPLC on a TSK-gel CM-2SW column. A representative

**Table 2.** Amino acid sequences of the peptides derived from the 6.5kDa amelogenin by Clostridiopeptidase B digestion

| | |
|---|---|
| 6.5kDa amelogenin | MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPSLL---- |
| CL-1 | WYQNMIR |
| CL-2 | HPSLLPDLPLEAWPATDKTKREEVD |
| CL-3 | MPLPPHPGHPGYINFSYEVLTPLK |
| CL-4 | MPLPPHPGHPGYINFSYEVLTPLKWYQNMIR |

---- Not terminated

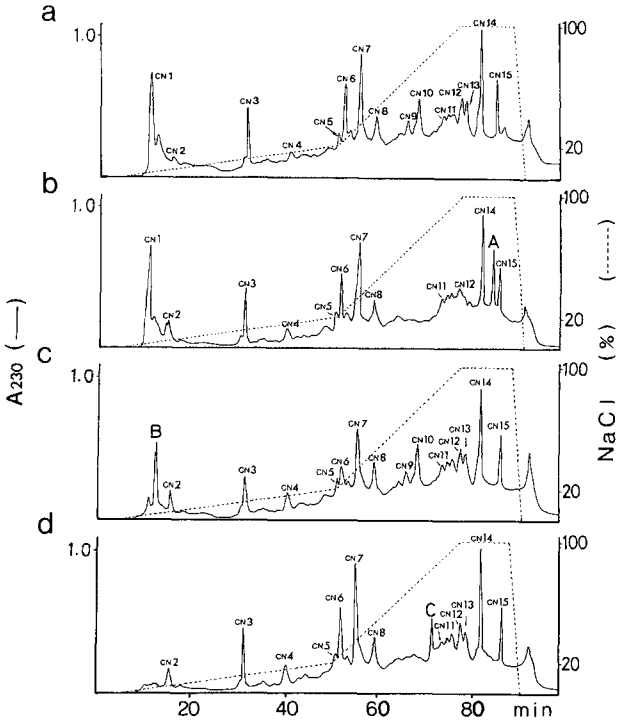


**Fig. 5.** Elution profile of ion exchange HPLC on a TSK-gel CM-2SW column of the CNBr-cleaved peptides. (**a**) CNBr-cleaved peptides of the 25kDa amelogenin; (**b**) CNBr-cleaved peptides of the 27kDa amelogenin; (**c**) CNBr-cleaved peptides of the 23kDa amelogenin; (**d**) CNBr-cleaved peptides of the 18kDa amelogenin.

chromatogram obtained by chromatography of the CNBr-cleaved peptides from the 25kDa amelogenin is shown in Figure 5a. The peptides eluted in each of the peaks in Figure 5b (the 27kDa), 5c (the 23kDa), and 5d (18kDa amelogenin) were identified by homology in the peak position and amino acid composition, to the peptides derived from the 25kDa amelogenin. Three different peptides, not identical to any of the CNBr-cleaved peptides derived from the 25kDa, were also isolated: from the 27kDa—peak A, the 23kDa—peak B, and from the 18kDa amelogenin—peak C. The amino acid sequences of the CNBr-cleaved peptides are shown in Table 3. As the amino acid sequence of the 25kDa amelogenin is already known [20], the amino acid sequences of the 27, 23, and 18kDa amelogenin were deduced from those of the CNBr-cleaved peptides (Table 4).

### *Plasma Desorption Mass Spectometry of the CNBr-Cleaved Peptides*

Because several CNBr-cleaved peptides having an identical amino acid sequence were eluted as two different peaks by HPLC (e.g., CN-2 and CN-3, CN-6 and CN-7), the masses of the CNBr-cleaved peptides derived from the 25kDa amelogenin were determined by PDMS. Table 5 summarizes calculated molecular weights and data obtained by PDMS of these peptides. The difference in mass between the calculated masses and the masses obtained (1, 23, or 46 mass units) was attributed to the fact that the molecular ion regions of the peptides consisted of $MH^+$ or sodium adduct ions. The difference in mass between two peptides having

**Table 3.** Amino acid sequences of CNBr-cleaved peptides

| | |
|---|---|
| N-terminal region of 25kDa amelogenin | MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHP----- |
| CN-1 | QSLLPDLPLEAWPATDKTKREEVD |
| CN-2 | VPAQQPGIPQQP |
| CN-3 | VPAQQPGIPQQP |
| CN-4 | HPIQPLLPQPPLPP |
| CN-5 | HPIQPLLPQPPLPP |
| CN-6 | TPTQHHQPNLPLPAQQPFQP----- |
| CN-7 | TPTQHHQPNLPLPAQQPFQPQPV----- |
| CN-8 | PLPGQHSMTPTQHHQPNLPLPAQQPF----- |
| CN-9 | PLPPHPGHPGYINFSYEVLTPLK----- |
| CN-10 | PLPPHPGHPGYINFSYEVL----- |
| CN-11 | FS |
| CN-12 | IRHPYTSYGYEP |
| CN-13 | IRHPYTSYGYEP |
| CN-14 | GGWLHHQIIPVVSQQTPQSHALQP------ |
| CN-15 | GGWLHHQIIPVVSQQTPQSHALQP------ |
| 27kDa amelogenin peak A | PLPPHPGHPGYINFSYEKSGSWGAxLTAFVSyVQVLTPLK------ |
| 23kDa amelogenin peak B | QSLLPDLPLEAW |
| 18kDa amelogenin peak C | PVLTPLKWYQN |

----- Not terminated

The letters x and y in the sequence of peak A are thought to be arginine and proline, respectively.

**Table 4.** Amino acid sequences of amelogenins

```
27kDa  : MPLPPHPGHPGYINFSYEKSGSWGAxLTAFVSyVQVLTPLKWYQNMIRHP(50)
25kDa  : MPLPPHPGHPGYINFSYE----------VLTPLKWYQNMIRHP(33)
23kDa  : MPLPPHPGHPGYINFSYE----------VLTPLKWYQNMIRHP(33)
20kDa  : MPLPPHPGHPGYINFSYE----------VLTPLKWYQNMIRHP(33)
18kDa  : MP----------VLTPLKWYQNMIRHP(17)
6.5kDa : MPLPPHPGHPGYINFSYE----------VLTPLKWYQNMIRHP(33)
5.3kDa : MPLPPHPGHPGYINFSYE----------VLTPLKWYQNMIRHP(33)

27kDa  : YTSYGYEPMGGWLHHQIIPVVSQQTPQSHALQPHHHIPMVPAQQPGIPQQ(100)
25kDa  : YTSYGYEPMGGWLHHQIIPVVSQQTPQSHALQPHHHIPMVPAQQPGIPQQ(83)
23kDa  : YTSYGYEPMGGWLHHQIIPVVSQQTPQSHALQPHHHIPMVPAQQPGIPQQ(83)
20kDa  : YTSYGYEPMGGWLHHQIIPVVSQQTPQSHALQPHHHIPMVPAQQPGIPQQ(83)
18kDa  : YTSYGYEPMGGWLHHQIIPVVSQQTPQSHALQPHHHIPMVPAQQPGIPQQ(67)
6.5kDa : ----------
5.3kDa : YTSYGYEPMGGW(45)

27kDa  : PMMPLPGQHSMTPTQHHQPNLPLPAQQPFQPQPVQPQPHQPLQPQSPMHP(150)
25kDa  : PMMPLPGQHSMTPTQHHQPNLPLPAQQPFQPQPVQPQPHQPLQPQSPMHP(133)
23kDa  : PMMPLPGQHSMTPTQHHQPNLPLPAQQPFQPQPVQPQPHQPLQPQSPMHP(133)
20kDa  : PMMPLPGQHSMTPTQHHQPNLPLPAQQPFQPQPVQPQPHQPLQPQSPMHP(133)
18kDa  : PMMPLPGQHSMTPTQHHQPNLPLPAQQPFQPQPVQPQPHQPLQPQSPMHP(117)
6.5kDa : ----------

27kDa  : IQPLLPQPPLPPMFSMQSLLPDLPLEAWPATDKTKREEVD(190)
25kDa  : IQPLLPQPPLPPMFSMQSLLPDLPLEAWPATDKTKREEVD(173)
23kDa  : IQPLLPQPPLPPMFSMQSLLPDLPLEAW(161)
20kDa  : IQPLLPQPPLPPMFS(148)
18kDa  : IQPLLPQPPLPPMFS(132)
6.5kDa : ----------SLLPDLPLEAWPATDKTKREEVD(56)
```

---- Lacked sequence
Numbers in parenthesis indicate the number of amino acid residues

identical sequences (18 mass units) was attributed to the fact that when methionine reacts with CNBr it is converted to homoserine lactone, which, even under acidic conditions, is in equilibrium with homoserine [21]; that is, one peptide corresponded to peptidyl homoserine lactone and the other to peptidyl homoserine, and these peptides were resolved into two peaks by the chromatography system used in this study.

**Table 5.** Masses of CNBr-cleaved peptides derived from the 25kDa amelogenin

| Site in 25kDa amelogenin | | Calculated | Observed |
|---|---|---|---|
| CN-1: | $^{150}$Gln – $^{173}$Asp | 2734.0 | 2757.1 (M + $Na^+$) |
| CN-2: | $^{73}$Val – $^{84}$Pro – Hse | 1342.5 | 1365.8 (M + $Na^+$) |
| CN-3: | $^{73}$Val – $^{84}$Pro – Hsl | 1324.5 | 1347.5 (M + $Na^+$) |
| CN-4: | $^{132}$His – $^{144}$Pro – Hse | 1626.9 | 1649.3 (M + $Na^+$) |
| CN-5: | $^{132}$His – $^{144}$Pro – Hsl | 1608.9 | 1654.9 (M + $2Na^+$) |
| CN-6: | $^{95}$Thr – $^{130}$Pro – Hse | 4147.6 | 4170.6 (M + $Na^+$) |
| CN-7: | $^{95}$Thr – $^{130}$Pro – Hsl | 4129.6 | 4152.3 (M + $Na^+$) |
| CN-8: | $^{87}$Pro – $^{130}$Pro – Hsl | 4977.6 | 5001.1 (M + $Na^+$) |
| CN-9: | $^{2}$Pro – $^{28}$Asn – Hse | 3248.7 | 3272.0 (M + $Na^+$) |
| CN-10: | $^{2}$Pro – $^{28}$Asn – Hsl | 3230.7 | 3253.6 (M + $Na^+$) |
| CN-11: | $^{147}$Phe – $^{148}$Ser – Hsl | 335.4 | 358.3 (M + $Na^+$) |
| CN-12: | $^{30}$Ile – $^{41}$Pro – Hse | 1565.7 | 1588.7 (M + $Na^+$) |
| CN-13: | $^{30}$Ile – $^{41}$Pro – Hsl | 1547.7 | 1571.1 (M + $Na^+$) |
| CN-14: | $^{43}$Gly – $^{71}$Pro – Hse | 3363.8 | 3364.3 (M + $H^+$) |
| CN-15: | $^{43}$Gly – $^{71}$Pro – Hsl | 3345.8 | 3369.1 (M + $Na^+$) |

Hse: homoserine; Hsl: homoserine lactone

## Discussion

The amelogenin gene of 13 different mammalian species appears to reside either exclusively on the X-chromosome or jointly on the X- and Y-chromosome [16, 22], and regulated alternative splicing can lead to the production of different amelogenins from a single primary RNA transcript [10–14]. Although no porcine amelogenin genes or cDNA have been isolated, the present results indicate that there are at least four different spliced mRNA products that are translated into four different prototype porcine amelogenins; that is, 27, 25, 18, and 6.5 amelogenin (Table 4). The difficulties involved in the reproduction of homogeneous enamel samples have not permitted us to measure the amounts of these prototype amelogenins in the enamel accurately, but, judging from the electrophoretic patterns (Fig. 1b) and the yields of purified materials (data not shown), the amounts in the very early secretory enamel can be assumed to be 25kDa $\gg$ 27kDa > 18kDa > 6.5kDa amelogenin.

Figure 6 summarizes the intron-exon structure of the primary transcript of porcine amelogenin deduced from the amino acid sequence data (Table 4) and the relationships between the resultant amelogenins and their derivatives which have been found in the secretory porcine enamel. As porcine amelogenin genes and cDNA have not been isolated, the exons, which are known to be present in the amelogenin mRNAs of other organisms and which carry the ribosome binding site and signal peptide, are omitted from the scheme of the intron-exon structure of the primary transcript. So far as we know, the 27kDa amelogenin consists of 190 amino acid residues and is the largest amelogenin to have been isolated from porcine secretory enamel. The 25kDa amelogenin is the major amelogenin, which ameloblasts synthesize and secrete into the enamel, consists of 173 amino acid residues and lacks the $Lys^{19}$-$Gln^{35}$ region of the 27kDa amelogenin. It is converted either to the 20kDa amelogenin, which is the most abundant amelogenin in the secretory porcine enamel, or to the 23kDa amelogenin by the action of pro-

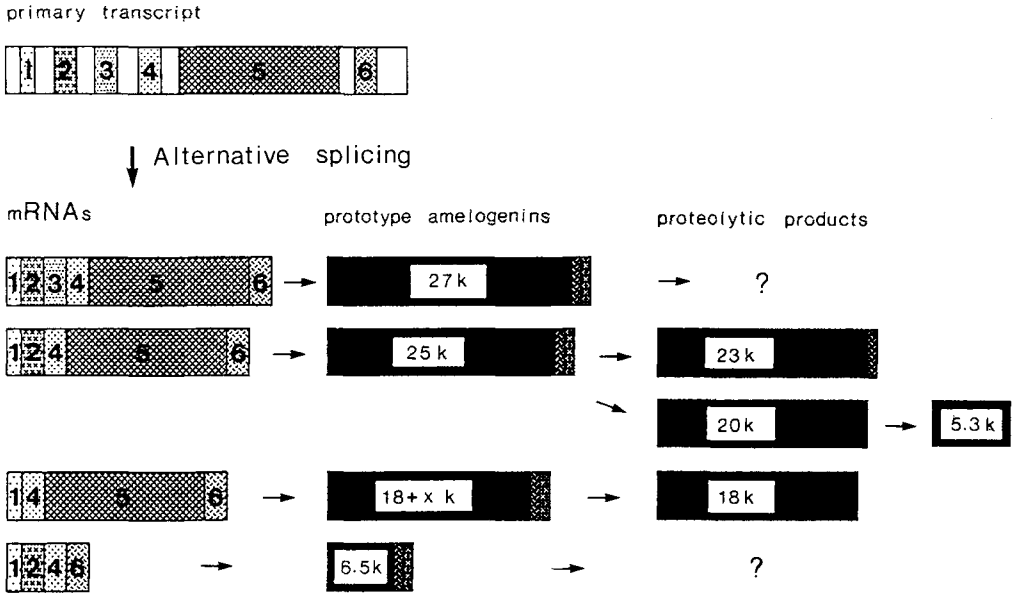


**Fig. 6.** Relationship of amelogenin family.

teinases (76 and 78kDa proteinase) found in the thin outer layer of the enamel [9]. The proteinases could cleave the 25kDa amelogenin at the site of $Ser^{148}$-$Met^{149}$ to yield the 20 kDa amelogenin or $Trp^{161}$-$Pro^{162}$ to yield the 23kDa amelogenin. It has been shown that the 20kDa amelogenin is further degraded by serine proteinases (30 and 34kDa proteinase) found in the inner layer of porcine secretory enamel [23]. Although the serine proteinases appear to be active in the conversion from 25kDa into 20kDa amelogenin, these enzymes cleave mainly the 20kDa amelogenin into two large fragments either at $Trp^{45}$-$Leu^{46}$ or at $His^{62}$-$Ala^{63}$ [24, 25]. The 5.3kDa amelogenin corresponds to the $Met^{1}$-$Trp^{45}$ fragment of the 20 kDa amelogenin. The 18kDa amelogenin lacks the $Leu^{3}$-$Glu^{18}$, $Lys^{19}$-$Gln^{35}$ and $Met^{166}$-$ASP^{190}$ regions of the 27kDa amelogenin. The possible precursor of the 18kDa amelogenin having the region corresponding to $Met^{166}$-$Asp^{190}$ of the 27kDa amelogenin, which is common to all the other prototype amelogenins, has not been isolated. However, the fact that the sequenced 20kDa amelogenin derived from the 25kDa amelogenin does not contain this region could indicate the proteolytic removal of this region from the 18kDa amelogenin precursor. The 6.5kDa amelogenin, the smallest prototype amelogenin, lacks the $Lys^{19}$-$Gln^{35}$ and $Tyr^{51}$-$Gln^{167}$ regions of the 27kDa amelogenin, and this amelogenin corresponds to a bovine amelogenin termed *leucine-rich amelogenin polypeptide* (LRAP) by Fincham et al. [26].

It has been reported that $Ser^{16}$ of a bovine amelogenin of 170 amino acid residues is phosphorylated [27] and the phosphoserine content for rat 25kDa amelogenin corresponds to a single phosphoserine residue per molecule [28]. Also, several immunohistochemical and lectin-binding data have indicated the presence of glycocompounds in developing enamel [29–31]. However, as shown in Table 5, there are no significant differences between the calculated mass values and the obtained mass values of CNBr-peptides. This indicates that phosphorylation and/or glycosylation of porcine 25kDa amelogenin does not occur. The most drastic procedure used during the preparation of CNBr-cleaved peptides in this study is that of CNBr cleavage itself. However, CNBr cleavage had been used successfully for the preparation of phosphorylated or glycosylated peptides from phosphoprotein and glycoprotein [32–36]. Our preliminary results indicate that not only is phosphoserine not dephosphorylated, but also that carbohydrate moieties joined to an enamelin polypeptide are not removed during the processes of CNBr cleavage (data not shown). This further suggests that dephosphorylation and deglycosylation does not occur prior to the measurement of the mass. However, the present findings do not eliminate the possibilities that a phospho- and/or glyco-25kDa amelogenin is synthesized within the ameloblast and deglycosylated and dephosphorylated prior to or immediately upon secretion from the cell, or that some other amelogenin(s), other than the porcine 25kDa amelogenin, are phosphorylated and/or glycosylated.

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