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Enamelins in the Newly Formed Bovine Enamel

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Summary. The possibility of using the antisera raised in rabbits against the porcine 25 kDa amelogenin, 32 and 89 kDa enamelins, and the 13–17 kDa nonamelogenin for the differentiation and identification of the protein components in bovine immature enamel was examined. Although the immunoreactivities of these antisera against bovine enamel proteins were weaker than those against the porcine proteins, it was found that these antisera could differentiate and demonstrate immunohistochemically a characteristic distribution of three different kinds of enamel protein components in the bovine secretory stage enamel similar to those observed in the porcine immature enamel. Of the several high molecular weight proteins being reactive to the anti-porcine 32 and 89 kDa enamelin sera, the 130 kDa protein, having the highest molecular weight, was extracted and purified from the bovine enamel sample which was obtained by peeling approximately 30-μm thickness of the outermost layer of the secretory stage enamel. The amino acid composition of the 130 kDa protein was similar to the known bovine enamelins, and was rich in aspartic acid, glutamic acid, proline, and glycine. The results could suggest that the enamelins of lower molecular weight than this protein, which are found in the bovine secretory stage enamel, are derived from this precursor protein.

Key words: Bovine enamelin – Amelogenin – Newly formed enamel – Western blot.

The major structural organic constituents of developing dental enamel are the amelogenins, and the complete amino acid sequence of several species has been elucidated by deducing from the cDNA sequences or amino acid sequence analysis [1–4]. The other structural constituents are the enamelins which are acidic proteins associated with the enamel crystals [5]. Recently, a protein has been reported by Deutsch et al. [6] which they called "tuftelin" and which they considered to be a kind of bovine enamelin, and its amino acid sequence was deduced from the cloned cDNA sequence.

In porcine immature enamel, enamelins have been isolated as those having molecular weights of 32, 89, and 142 kDa [7, 8], and it was suggested that the 32 and 89 kDa enamelins were derived from the 142 kDa enamelin. However, in the bovine enamelin fraction, the presence or absence of enamelin of higher molecular weight than the 70 kDa enamelin is not certain [9].

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The present paper describes the immunochemical identification of three kinds of enamel proteins, especially those of the enamelin family, in newly formed bovine enamel.

Materials and Methods

Preparation of Immature Enamel and Extraction of Total Soluble Proteins

Two immature enamel samples were prepared according to the method of Fukae and Shimizu [10] from the developing permanent incisors dissected from the mandibles of approximately 1-year-old cattle. One outer enamel sample, corresponding to approximately 30-μm thickness of the newly formed outermost layer of the enamel in the secretory stage [11, 12], was peeled off from the cleaned labial surface of the teeth using a razor blade. After the removal of a further, approximately 30-μm thickness layer, which was discarded, the inner enamel sample was obtained by scraping from the remainder of the secretory enamel. All samples were stored at −80°C.

Each pooled sample was dissolved in 0.5 M acetic acid for the extraction of the whole of the soluble enamel proteins. Approximately 28 mg (wet weight) of the outer enamel sample and 620 mg of the inner enamel sample were used. After centrifugation to remove trace amounts of insoluble material, the extract was concentrated and desalted by ultrafiltration with a YM-5 filter (Amicon), lyophilized, and stored in a freezer (−30°C). Protein samples were also prepared from the outer and inner enamel samples obtained from the developing permanent incisors of 6-month-old pigs by the same procedures described above.

Production of Antisera Against Porcine Enamel Proteins

The details of the production of the antisera against porcine 25 kDa amelogenin, 89 kDa enamelin, and 13–17 kDa nonamelogenin have been described in a previous paper [11]. Polyclonal antibodies were raised in rabbits against the 25 kDa amelogenin, the 89 kDa enamelin, and the 13–17 kDa nonamelogenin which were isolated from the outermost layer of porcine secretory enamel. The term "nonamelogenin" was first used by Fukae and Tanabe [7] to denote the 13–17 kDa proteins obtained from secretory porcine enamel which had a different amino acid composition from enamelin and amelogenin. For the preparation of C-terminal specific anti-25 kDa amelogenin serum, the anti-25 kDa amelogenin serum was adsorbed with an excess amount of the purified 20 kDa amelogenin.

The antiserum against a synthetic peptide having the amino acid sequence HVPGRIPPGYGRPPT, corresponding to the 3rd–17th residues from the N-terminal region of the porcine 32 kDa enamelin, was prepared as described previously [8].

Immunochemical Detection

Bovine enamel protein samples were electrophoresed on a 12%

polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) [13] under nonreducing conditions. The proteins on the electrophoresed gel were electrotransfered onto Durapore GVHP membrane (Millipore, Yonezawa, Japan) in a Marysol Transblot Apparatus (Tokyo, Japan) with cooling to 4°C [14]. The membrane was immunostained by the avidin-biotin complex method [15] with antisera raised against porcine enamel proteins, as described in a previous paper [11].

Immunohistochemistry

Tooth germs of permanent incisors were dissected from the mandible of approximately 1-year-old cattle and immersed in 4% paraformaldehyde containing 0.4% picric acid and 0.2% glutaraldehyde in sodium phosphate buffer, pH 7.4. After decalcification with 10% EDTA (pH 7.4) at 4°C for about 2 weeks, the specimens were washed well in phosphate buffer, dehydrated in graded N,N-dimethylformamide, and embedded in glycol methacrylate at −20°C. Ultrathin sections were cut, mounted on gelatin-coated glass slides and were stained by the immunogold silver staining method using the antisera raised against three different kinds of porcine enamel proteins [11].

High Performance Liquid Chromatography (HPLC)

The samples of enamel proteins from the bovine and porcine immature enamel were dissolved in 50 mM carbonate buffer (pH 10.8) containing several kinds of proteinase and phosphatase inhibitors (50 mM ε-aminocaproic acid, 5 mM benzamidine, 1 mM p-hydroxymercuribenzoic acid, 1 mM phenylmethylsulfonyl fluoride, 1 mM levamisole), and chromatographed at room temperature on a column (7.5 mmID × 60 cm) of TSKgel G3000PW(TOSOH, Tokyo) attached to a Shimadzu LC-3A HPLC system (Kyoto, Japan). The column was eluted with 50 mM carbonate buffer (pH 10.8) containing 0.1 mM levamisole at the flow rate of 0.4 ml/minute. The effluent was monitored at 280 nm. The proteins eluted in each peak were desalted using Sephadex PD-10 column equilibrated with 0.1 M ammonium bicarbonate and lyophilized. For the purification of high molecular weight enamel protein, the molecular sieve chromatography was carried out on a HPLC system under denaturing conditions. A tandem column (7.5 mmID × 120 cm as total length) of TSKgel G4000 PW was equilibrated and eluted with 4 M guanidine HCl-30 mM Tris buffer, pH 7.0. The effluent was monitored at 230 nm.

Analytical Procedures

Analytical electrophoreses were carried out according to the method of Laemmli [13] under nonreducing conditions. The gel was stained with Coomassie Brilliant Blue (CBB). The apparent molecular weights of protein bands were estimated by using Bio-Rad LMW and HMW protein standards. The amino acid analyses were performed using a JLC 300 automatic amino acid analyzer (JEOL, Tokyo) after the hydrolysis of protein samples in 6 N HCl in an evacuated sealed tube at 110°C for 24 hours.

Results

Figure 1 shows the electrophoretic patterns of the whole soluble enamel protein sample obtained from the bovine outer immature enamel under reducing and nonreducing conditions. Some protein bands, for example, the bands designated a, b, and c which were electrophoresed to the positions corresponding to 55, 45, and 40 kDa, respectively, under the nonreducing conditions, migrated to different positions a′(66.2 kDa), b′(53 kDa), and c′(43 kDa) under the reducing conditions. However, as there was no difference

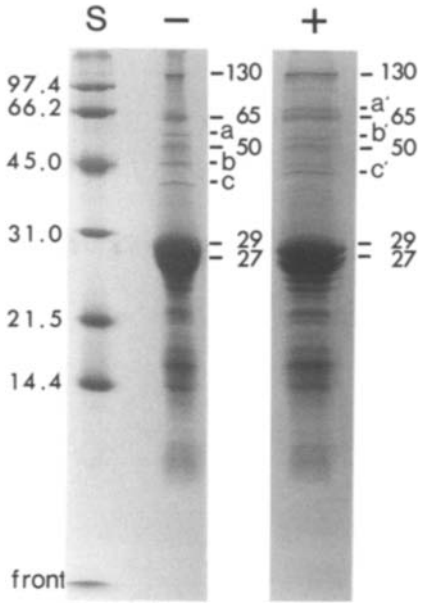

Fig. 1. Electrophoretic patterns of enamel proteins obtained from the outer layer of bovine secretory stage enamel. The gels were stained with CBB. S: Bio-Rad LMW protein standards, − = under nonreducing condition and + = under reducing condition (reduced in 5% 2-mercaptoethanol).

under nonreducing and reducing conditions in the electrophoretic migration of protein bands corresponding with the 130 kDa, 65 kDa, and others that are discussed in this study, all the procedures of electrophoresis and chromatography were carried out under nonreducing conditions.

The electrophoretic patterns of the whole soluble enamel protein samples obtained from the bovine outer and inner immature enamel are shown in the left two lanes of Figure 2a. For reference, the patterns of the porcine enamel proteins are shown in the right 2 lanes of Figure 2a. The thick protein bands (26 kDa, 27 kDa, and 29 kDa in molecular mass) stained with CBB in the bovine samples are amelogenins as demonstrated by their immunoreactivity against the anti-porcine 25 kDa amelogenin serum (Fig. 2b). The largest molecular weight 29 kDa protein corresponds to a protein regarded by Shimokawa et al. [2] as the prototype of bovine amelogenin.

Besides these components, several high molecular weight narrow protein bands were detected with CBB staining in the bovine sample (Fig. 2a). When their immunoreactivity against the anti-porcine 89 kDa enamelin serum was examined, the main bands reacting to the serum were the bands corresponding to 130 kDa, 65 kDa, 50 kDa, and 45 kDa in the bovine outer enamel sample, and those of 65 kDa, 50 kDa, 45 kDa, and 32 kDa in the inner enamel sample (Fig. 2c). These bands also reacted with the anti-porcine 32 kDa enamelin (Fig. 3) but did not react with either anti-porcine 25 kDa amelogenin or anti-13-17 kDa nonamelogenin sera (Fig. 2b and d).

The antisera against the porcine 25 kDa amelogenin, the 13–17 kDa nonamelogenin and the 89 kDa enamelin, and the C-terminal specific anti-25 kDa enamelin serum demonstrated a staining characteristic of bovine immature enamel (Fig. 4). The immunoreactivity against the anti-25 kDa amelogenin serum was strongest at the outer layer of the immature enamel and gradually decreased toward the dentino-enamel junction (Fig. 4a), and that of the C-terminal-specific

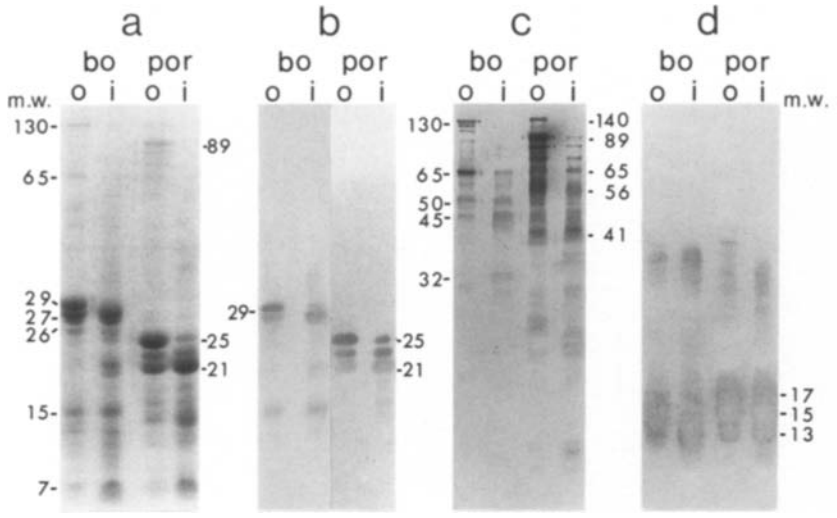

Fig. 2 a–d. Detection of enamel proteins obtained from the outer or inner layer of both bovine and porcine secretory stage enamel by SDS-PAGE. **(a)** CBB staining after electrophoresis on 12% acrylamide gel. **(b–d)** Western blots followed by immunodetections using anti-porcine 25 kDa amelogenin serum **(b)**, anti-porcine 89 kDa enamelin serum **(c)**, and anti-porcine 13–17 kDa nonamelogenin serum **(d)**. For the detection of bovine amelogenin 5000-fold diluted anti-amelogenin serum was used, and for that of porcine amelogenin, the 20,000-fold diluted serum was used. The anti-89 kDa enamelin serum and anti-13–17 kDa nonamelogenin serum were diluted 30,000-fold and 40,000-fold, respectively. bo: bovine, por: porcine, o: whole soluble proteins of the outer enamel sample, i: whole soluble proteins of the inner enamel sample.

25 kDa amelogenin was restricted to the outer layer of the enamel, and the deeper layer of the enamel reacted very faintly (Fig. 4b). The anti-13-17 kDa nonamelogenin serum reacted with prism sheaths, producing a honeycomb pattern over the entire thickness of the enamel (Fig. 4c). On the contrary, the anti-89 kDa enamelin serum stained the prisms in the outer layer of the enamel, producing a reverse honeycomb pattern which was not observed in the deeper portion of the enamel (Fig. 4d).

In order to purify the bovine 130 kDa protein which had the highest molecular weight among the proteins reactive to the porcine anti-89 kDa and 32 kDa enamelin, molecular sieve chromatography of the protein samples containing all the soluble proteins obtained from the outer enamel sample were carried out by the HPLC system using a TSKgel G3000PW column at pH 10.8. The results of analytical electrophoresis of each peak fraction showed that the 130 kDa protein was eluted into the first two peak fractions (Fig. 5). These first two fractions were then combined, lyophilized without desalting, dissolved with 4 M guanidine HCl solution, and chromatographed using a TSKgel G4000PW column in the dissociative condition with 4 M guanidine HCl solution. The 130 kDa protein eluted in the second peak (Fig. 6) was further purified by re-chromatography on the same column system. Although the bovine 130 kDa protein was highly purified, the preparation still contained traces of contaminant. The amino acid composition of this purified bovine 130 kDa protein was rich in aspartic acid, glutamic acid, proline, and glycine, and was similar to that of the 65 kDa enamelin (Table 1).

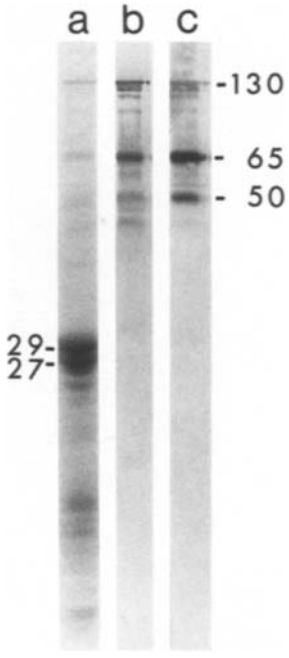

Fig. 3. Detection of enamelins in the protein sample obtained from bovine outer enamel. **(a)** CBB staining, **(b,c)** Western blots followed by immunodetection using anti-porcine 89 kDa enamelin serum **(b)**, and anti-porcine 32 kDa enamelin serum **(c)**. The anti-89 kDa enamelin serum and anti-32 kDa enamelin serum were diluted 30,000-fold and 2000-fold, respectively.

Discussion

Because the difficulties to obtain sufficient amounts of bovine immature enamel samples, especially in the outer enamel sample, did not permit us to prepare the purified prototype amelogenin and other enamel protein components used for the production of antisera, we therefore used the antisera raised in rabbits against the porcine 25 kDa amelogenin, 32 and 89 kDa enamelins, and 13–17 kDa nonamelogenin for the differentiation and identification of the protein components in the bovine immature enamel used in this study. Although the immunoreactivities of these antisera against bovine enamel proteins are 5–10 times weaker than those against the porcine proteins (data are not shown), it does show that these antisera are available for the differentiation of three kinds of enamel protein components in bovine immature enamel, as shown in Figures 2 and 3. Also, these antisera could demonstrate immunohistochemically a specific distribution of the three different immunoreactivities in the bovine secretory stage enamel similar to that observed in the porcine immature enamel [11]; that is, (1) the 25 kDa amelogenin-like immunoreactivity over the bovine secretory stage enamel decreased gradually from the enamel surface to the middle layer, and in the inner layer the immunoreactivity was concentrated in the regions of the prism sheaths; (2) the

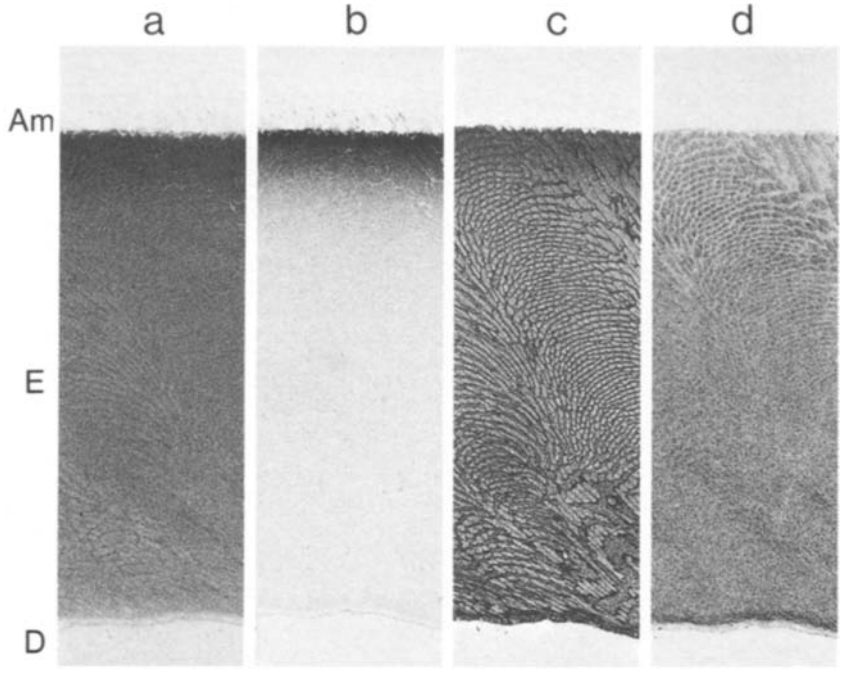

Fig. 4. Light micrographs (×200) showing immunoreactivity of bovine immature enamel fixed with 4% paraformaldehyde containing 0.4% picric acid and 0.2% glutaraldehyde in phosphate buffer, pH 7.4. Antisera used were the 8000-fold, diluted anti-25 kDa amelogenin serum **(a)**, the 4000-fold, diluted C-terminal, specific anti-25 kDa amelogenin **(b)**, the 16,000-fold diluted anti-13–17 kDa nonamelogenin serum **(c)**, and the 3000-fold diluted anti-89 kDa enamelin serum **(d)**. Am: Layer of secretory ameloblasts, E: immature enamel, D: dentin.

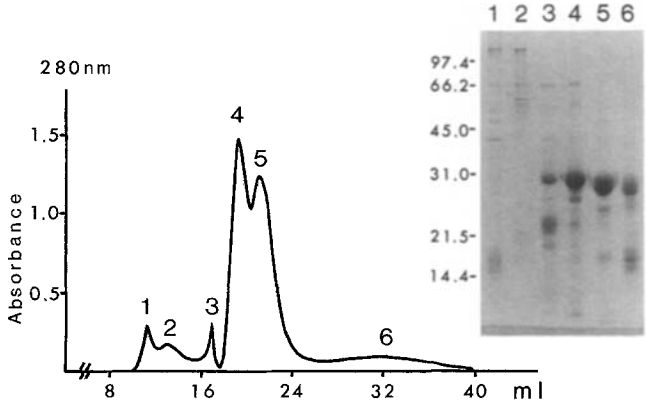

Fig. 5. HPLC profile of the whole soluble proteins obtained from bovine outer enamel sample on the column of TSKgel G3000 PW. Fractions indicated were analyzed by SDS polyacrylamide gel electrophoresis as shown in the inset. The protein sample (8.3 mg) was dissolved with 0.4 ml of 50 mM carbonate buffer (pH 10.8), and was eluted with the same buffer.

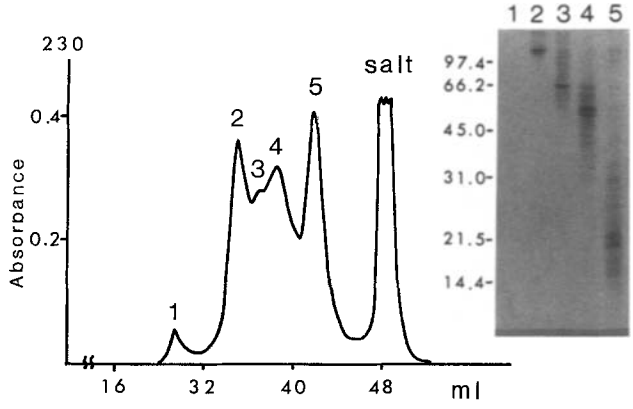

Fig. 6. HPLC profile of the mixture of Fr 1 and 2 shown in Figure 5 on a tandem column of TSKgel G4000PW equilibrated and eluted with 4 M guanidine HCl-30 mM Tris buffer, pH 7.0. Fractions indicated were analyzed by SDS electrophoresis, as shown in the inset.

prism sheaths were intensely stained by the antiserum to the 13–17 kDa nonamelogenin; and (3) the 89 kDa enamelin-like immunoreactivity was intense over enamel prisms at the outer layer of the enamel and was rather homogeneously distributed in the inner layer. These results indicate the presence of at least three different molecular species of protein being common to both pig and cattle as comprising the structural organic constituents in the secretory stage enamel; for example, (1) the amelogenin whose amino acid sequence is known and is the major protein constituent in the secretory stage enamel, (2) the enamelin first described by Termine et al. [5] has been characterized to have an affinity to hydroxyapatite crystals, and (3) a protein being reactive to the porcine 13–17 nonamelogenin antiserum, which is distributed mainly in the prism sheath regions, and for which may be proposed the name "sheath protein" for convenience of description. In addition to these three proteins, the "tuftelin" described by Deutsch et al. [6] would be added as a protein constituent in the enamel.

Furthermore, the results of SDS-PAGE (Fig. 2a,b) and the distribution of the immunoreactivity against the C-terminal specific 25 kDa amelogenin antiserum (Fig. 4) confirm the view that the prototype amelogenin is transformed by excision of its C-terminal small peptide. This process results in the 27 kDa amelogenin in the bovine enamel [17–19] and the 20 kDa amelogenin in the porcine enamel [10] which are the major amelogenin components in the secretory stage enamel.

The 65 kDa enamelin is the major enamelin component found in the bovine immature enamel in the secretory stage (Fig. 2a,c), and may correspond to the known 70 kDa enamelin described by Termine et al. [5] and Ogata et al. [16]. Ogata et al. suggested the 70 kDa enamelin as the initial form

Table 1. Amino acid composition of bovine 130 kDa and 65 kDa enamelin (residues/total 1000 residues)

Amino acid	130 kDa	65 kDa
Asp	104	108
Thr	57	54
Ser	98	74
Glu	159	150
Pro	113	104
Gly	112	110
Ala	48	53
Cys	0	0
Val	41	37
Met	11	12
Ileu	24	25
Leu	38	51
Tyr	42	31
Phe	35	35
His	37	56
Lys	37	40
Arg	44	58

of bovine enamelin. However, as shown in Figures 2 and 3, in the protein sample extracted from the outer layer of the bovine enamel in a very early secretory stage, the 130 kDa protein which was reactive to both anti-porcine 89 kDa and 32 kDa enamelin serum was detected, and the result of amino acid analysis of the highly purified 130 kDa showed a close similarity to the amino acid composition of the 65 kDa enamelin, and closely resembled those of bovine enamelins reported by other investigators [5, 16]. It is important that not only was there no difference in the electrophoretic migration of the 130 kDa protein under denaturing reducing and nonreducing denaturing conditions (Fig. 1), but also that no cysteine and/or cysteic acid was detected by amino acid analysis of both the 130 kDa protein and the 65 kDa enamelin (Table 1). These facts could rule out the possibility that the 130 kDa protein is an aggregate or a complex of the 65 kDa enamelin desulfide linked to other unrelated proteins. These present results might indicate that the 130 kDa protein is a prototype bovine enamelin, the 130 kDa enamelin being transformed into the 65 kDa enamelin which is the major enamelin in an early secretory stage of bovine enamel formation.

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