Sequencing of Bovine Enamelin ("Tuftelin") a Novel Acidic Enamel Protein*

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Enamelin is a major group of 28–70 kDa acidic proteins rich in aspartic acid, glutamic acid, serine, and glycine, and are rich in proline, histidine, and glutamine, and acidic proteins. They have been associated with the mineralization and structural organization of the tooth. In an attempt to elucidate the primary structure of enamelin, a 2674-base pair cDNA isolated from a bovine ameloblast-enriched, lambda Zap 2 expression library, was sequenced. The identity and localization of the deduced protein was confirmed by amino acid composition, enzyme-linked immunosorbent assay, Western blotting, indirect immunohistochemistry, and high resolution protein-A gold immunocytochemistry. The immunological techniques were used employing antibodies directed against synthetic peptides corresponding to the protein sequence deduced from the cloned cDNA sequence. The results reveal the deduced protein to be a novel acidic enamel protein. It contains 389 amino acids and has a calculated molecular weight of 43,814. Its amino acid composition is similar to that of "tuft" proteins (enamel matrix protein fragments remaining in the tissue) but contains few cysteine residues. Southern hybridization of the cloned cDNA with genomic bovine DNA indicated the existence of a single gene with one or more introns.

Enamel, a unique and highly mineralized ectodermal tissue covering vertebrate teeth, is synthesized and secreted by specialized cells of the enamel organ called the ameloblasts. During the process of development and mineralization the extracellular enamel matrix, which is originally rich in protein but relatively poor in mineral, loses most of its protein and acquires mineral ions calcium and phosphorus, the tissue finally becoming highly mineralized (96% by tissue weight), hard, and mature (1-4). Two major groups of protein comprise the developing extracellular matrix: the hydrophobic amelogenins, rich in proline, histidine, and glutamine, and the acidic enamelines, rich in glutamic acid, aspartic acid, serine, and glycine (6). The amelogenins constitute about 90% of the matrix proteins, and their molecular mass is 28 kDa and less. They are localized primarily in the intercrystalline space (3, 6-10) and are selectively lost during the process of mineralization (11-14). The enamelines, on the other hand, comprise 10% of the forming matrix, have masses up to 70 kDa, and are partially retained in the mature tissue (6, 15, 16).

The functions of these two major matrix protein groups are still not clear, but they are thought to be involved in the mineralization and structural organization of enamel. The acidic enamelines are secreted at a very early stage of enamel formation (3, 13, 17-19), and are tightly bound to the surface of the growing crystallites. They have been reported to possess β-pleated sheet structures (potential nucleating structures for hydroxylapatite (20)), and under certain in vitro conditions can inhibit crystal growth (21). For these reasons, enamelines have been suggested to be involved in the nucleation and regulation of enamel crystal growth. Unlike the amelogenins, however, which have been cloned and were sequenced first in the mouse (22) and later in the bovine (23), no amino acid sequence of enamelines has been published. This is probably one reason for the uncertainty of its identity (24-26). Enamelines have been shown to possess common immunologically reactive epitopes with enameloid of aquatic species spanning 450 million years of vertebrate evolution (i.e., shark and hagfish), suggesting that they are highly conserved, and again indicating their importance in the development and mineralization of enamel (27, 28). The present work describes the sequencing of bovine enamelin protein (tuftelin), a novel acidic protein.

MATERIAL AND METHODS

Construction and Identification of the Bovine Enamelin cDNA Clone—The construction and preliminary identification of the enamelin cDNA clones have been previously reported (5). In brief, tooth organs (mandibular permanent molars) were removed from 3-month old Holstein calves. The teeth generally contained only the forming stage of enamel (first stage). Enamel organ (ameloblast-enriched tissue) was separated from the adjacent enamel, pooled, and then extracted for total RNA. Poly(A) mRNA was selected by affinity chromatography on oligo(dT)-cellulose and 20 μg of poly(A) mRNA was used to make cDNA (29). The cDNA was size-selected to be enriched in molecules ≥ 1.0 kb and inserted into the EcoRI site of a lambda Zap 2 expression library (Stratagene). The amplified cDNA expression library was screened using affinity-purified polyclonal antibodies against the enamelin 66-kDa protein (30). Two types of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) M64924.

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§ The abbreviations used are: kb, kilobase(s); ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; FITC, fluorescein isothiocyanate.

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enamelin clones were identified, one containing a 2.8-kb insert with an internal EcoRI site and the other an insert of 1.8 kb with no internal EcoRI site (5). The present paper describes the sequencing of the 2.8-kb cDNA enamelin clone, tuftelin.

cDNA Sequencing—The cloned 2.8-kb cDNA was sequenced using both subcloned fragments in M13 (31) and double-stranded sequencing of the denatured plasmid DNA, with T4, T7, and synthetic oligonucleotides using the di-deoxy chain termination method (33). For primer walking (32) synthetic oligonucleotides, 19–21 bases long, were designed every 250 bases in both directions of the cDNA. DNA sequences were analyzed by the program of the University of Wisconsin, Genetic Computer Group (WGGC).

Genomic DNA Extraction and Southern Analysis—High molecular weight bovine genomic DNA was extracted from a fetal bovine liver and aliquots of 10 μg digested with excess amounts of various restriction endonucleases for 3 h at 37 °C using buffers recommended by the manufacturer. After enzyme treatment, fragmented DNA was resolved through 0.8% agarose gels and transferred to nitrocellulose using the method of Southern (34). DNA bound nitrocellulose was hybridized with full length tuftelin cDNA which was previously

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**Fig. 1.** Nucleotide sequence of bovine enamelin (tuftelin) cDNA and deduced amino acid sequence. Nucleotide residues are numbered on the right. A Kozak consensus sequence for eukaryotic initiation sites is underlined. A possible polyadenylation signal in the 3'-untranslated sequence is underlined. A termination signal (TAG), potential N-glycosylation site (Asn-Lys-Ser), and tripeptide (Glu-Ser-Leu), the phosphorylated form of which appears in all mineralizing tissues, are also marked. Cysteine residues are marked with arrows. The deduced protein sequences to which synthetic peptides (73, 74, 75) were produced, and to which anti-sera LF-73, LF-74, LF-75 were made, are underlined.
Fig. 2. A comparison between the amino acid composition of tuftelin (bold line) to a tuft protein (thin line) reported by Robinson et al. (41). The amino acid composition of the proteins are represented as rose diagrams according to the method of Robinson et al. (41).

Peptide Synthesis—The sequences used for the synthetic peptides (73, 74, 75; see Fig. 1) were derived from the deduced protein sequence (Fig. 2) and from sequence homology. Hydrophilicity was predicted by the method of Kyte and Doolittle (35). Isoelectric point analysis was carried out by the method of Towbin et al. (36). The tissue specimens were incubated with the first antibody for 1 h and the second antibody for 7 min. Longitudinal tooth sections treated with corresponding preimmune sera served as a control.

High Resolution Protein A-Gold Immunohistochemistry—High resolution protein A-gold immunohistochemistry was carried out according to the methods of Bendayan (48) and Nanci et al. (49). Briefly, nondecalcified developing bovine incisor teeth were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, and postfixed with potassium ferrocyanide-reduced osmium. The tissues were dehydrated and then embedded in Epon. Sections were then prepared and stained with Amido Black and others were processed for immunodetection using synthetic peptide antisera (LF-73) as first antibody and protein A-1125.

Indirect Immunohistochemistry and Immunofluorescence—Indirect immunohistochemistry using synthetic peptide antisera as first antibody (LF-73, LF-74, LF-75) and goat antirabbit IgG coupled to horseradish peroxidase (second antibody) was carried out on 5-µm sections of undecalcified paraffin-embedded developing bovine teeth. Indirect and immunofluorescence using synthetic peptide antisera as first antibody, and fluorescein-conjugated antirabbit (FITC) as second antibody, was performed on Cryostat sections (5-µm) of nonetched and HC1-etched calcified developing bovine teeth and on nonetched and HC1-etched enamel protein extracts and proceeding with ELISA, using peroxidase conjugated second antibody.

Western Blotting and Immunodetection—Bovine enamelin-enriched extract (6) was electrophoresed separately on 10-18% SDS-polyacrylamide gel electrophoresis (17). Electroblotting was according to the method of Towbin et al. (38). Nitrocellulose electrotransfers were stained with Amido Black and others were processed for immunodetection using synthetic peptide antisera (LF-73) as first antibody and protein A-1125.

ELISA—Relative titers of synthetic peptide antisera (LF-73, LF-74, LF-75) and affinity-purified polyclonal antibodies against enamelin (30) were determined by coating Dynatech microtiter plates with enamel protein extracts and proceeding with ELISA, using peroxidase conjugated second antibody.

Dyneatech microtiter plates were coated overnight at 4°C with either 1.0 µg of purified enamelin and 73, 74, and 75 peptides (not conjugated to chick serum albumin) or with dentin matrix proteins. The plates were then blocked with 125 µl of 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature. 100 µl of antisera (LF-73, LF-74, LF-75, and affinity-purified polyclonal antibody against enamelin) at increasing dilutions (1:50 to 1:6400) (in 2% BSA solution) were then added. Incubation with the respective preimmune sera served as controls. The plates were then incubated for 1 h at room temperature, washed for 3 × 10 min (PBS with 0.05% Tween 20) and incubated in a 1:2,000 dilution of peroxidase-conjugated goat anti-rabbit IgG coupled in 2% BSA for 1 h. After washing 3 × 10 min in PBS-Tween, the wells were incubated in 100 µl of 0.2% 3,3′-azino-di-3-ethylbenzthiazine sulfate) for 30 min, then monitored at 405 nm in a Dynatech microplate spectrophotometer.

Computer Analysis—The National Biochemical Research Foundation protein data base and GenBank nucleic acid data base were searched for sequence homology. Hydrophilicity was predicted by the method of Kyte and Doolittle (35). Isoelectric point analysis was carried out by the method of Towbin et al. (36). The tissue specimens were incubated with the first antibody for 1 h and the second antibody for 7 min. Longitudinal tooth sections treated with corresponding preimmune sera served as a control.

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cDNA-derived Enamelin Protein Sequence and Localization

RESULTS

cDNA Sequence Analysis—In a preliminary report we described the isolation of cDNA encoding bovine enamelin (5). The cDNA clone, 2,674 base pairs long, was isolated by antibody screening (affinity-purified antibody against enamelin, (30) from a bovine ameloblast-enriched cDNA library in expression vector or lambda Zap 2. Northern blot analysis showed that it hybridized to an mRNA species of 2.7 kb and a shorter mRNA of 1.0 kb (5).

The deoxynucleotide sequence for the 2.7-kb enamelin cDNA clone and the predicted amino acid sequence of the protein it encodes is illustrated in Fig. 1. A methionine codon at base 182 surrounded by a Kozak (39) consensus sequence (GCCATGG) for eukaryotic initiation sites is followed by an open reading frame extending to a stop codon (TAG) at base 1349. A polyadenylation signal (AAUAAA) begins at position 2637. The sequence contains 5 cysteine residues and a potential N-glycosylation site (Asn-Ser). It also contains a Glu-Ser-Leu peptide, the phosphorylated form of which has been shown to exist in all mineralizing tissues (40). 181 bases (1-181) from the 5’ and 1322 (1532-2674) bases at the 3’ ends are untranslated sequences.

Deduced Protein Characteristics—A search in the National Biochemical Research Foundation protein data base and GenBank nucleic acid data base revealed that the cDNA encodes a novel protein. Its composition resembles tuft proteins (protein remaining in the mature enamel, the origin of which is mainly the enamelines secreted in the forming stage (41, 42) (Fig. 2). This figure shows the comparison of amino acid composition of the deduced bovine enamelin protein (“tuftelin”) with one of the tuft protein sequences reported by Robinson et al. (41). The amino acid compositions are represented as rose diagrams according to the method of Robinson et al. (41). The calculated molecular weight of the deduced protein is 43,814.

Computer analysis of the deduced amino acid sequence revealed the protein to be highly hydrophilic (Fig. 3) and acidic with an isoelectric point of 5.2. This isoelectric point is similar to that already reported for this protein (18, 44).

Analysis of Tuftelin Genomic DNA—In order to determine the nature and approximate copy number of the tuftelin gene, bovine genomic DNA was fragmented with enzymes that either cut the cDNA, or not at all. EcoRI, which cuts the cDNA into two asymmetric fragments of 1.8 and 1.0 kb, also generated two hybridizable genomic fragments that were distinct in size from the cDNA (Fig. 4). The restriction enzymes BamHI and HindIII, which do not cleave the cDNA, resulted in multiple hybridizable genomic fragments ranging in size from ~2.5 to 1.6 kb (Fig. 4). These data imply the probable existence of only one gene copy per haploid genome containing one or more introns.

Immunological Analysis of the Deduced Tuftelin Protein—Since no amino acid or cDNA sequences typical of enamelin were available in the literature, to confirm the identity of the deduced protein, antisera against three chick serum albumin-conjugated synthetic peptides (corresponding to 73, 74, 75, respectively) were reacted against forming and mature enamel (indirect immunohistochemistry and immunofluorescence) and dentin enamelin (ELISA, Western blotting). Fig. 5 are ELISA analyses of these synthetic peptide anticorss. Fig. 5a shows the ELISA reaction of synthetic peptide antisera (LF-73, LF-74, LF-75) made to the deduced tuftelin protein sequences (73, 74, 75) (see Fig. 1) with (i) nonconjugated peptides (73, 74, 75), (ii) dentin noncollagenous extracellular matrix proteins. Reaction with pre-immune sera served as control. The results revealed (i) that the antisera had indeed been produced against the peptides of concern, and (ii) that they did not react with dentin noncollagenous extracellular matrix protein. Fig. 5b shows the ELISA reaction of synthetic peptide antisera (LF-73, LF-74, LF-75) made to the deduced tuftelin protein sequences with sequentially extracted (6) and purified (15) enamelin protein. Reaction with preimmune sera served as controls. The results reveal strong reaction with the enamelin protein extracted from developing extracellular bovine enamel. Fig. 5c shows the ELISA reaction of affinity-purified polyclonal antibody against enamelin (30) (used originally to screen the enamelin cDNA clones from the expression library) with nonconjugated synthetic peptides (73, 74, 75) produced according to the tuftelin-deduced sequences. Reaction with preimmune sera served as control. The results indicate that all three synthetic peptides made to the deduced protein reacted with the polyclonal antibody against enamelin and that the highest activity occurred with peptide 75. Fig. 6 shows the immunological relationship of the deduced tuftelin protein to enamel protein extracts using Western analysis. In this experiment an enamelin-enriched fraction (obtained by the method of Termine et al. (6)) (see also Refs. 5 and 49) was reacted against synthetic peptide antisera LF-73 (antibody produced to the peptide sequence near the COOH-terminal end of the deduced protein). The results reveal that the antisera reacted with 66-, 58-, 48-, and 28-kDa protein bands. Indeed, enamelines have been reported to possess molecular masses of 66, 58, 48, and 28 kDa (37, 19, 44, 45).

Expression and Ultrastructural Localization of Tuftelin in Enamel—In order to determine the expression and ultrastructural localization of tuftelin, indirect immunohistochemistry, indirect immunofluorescence, and high resolution protein Ag-gold immunocytochemistry were performed employing the synthetic peptide antisera (see Figs. 7 and 8). Fig. 7A shows the indirect immunohistochemistry (using horseradish peroxidase as second antibody) and immunofluorescence (using FITC antibody as second antibody) on paraffin-embedded and cryostat sections (5 μm) of forming bovine enamel, respectively, employing synthetic peptide antisera (LF-73, LF-74, LF-75). Reaction with preimmune sera served as controls. The antibodies reacted with the ameloblast cells, in particular

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**Fig. 4. Southern analysis of bovine genomic DNA fragmented with the endonucleases EcoRI, BamHI, HindIII, PstI, and KpnI.** Nitrocellulose bound DNA was hybridized with a full length cDNA fragment of tuftelin (see Fig. 1) previously labeled with P32. The deoxynucleotide sequence for the 2.7-kb enamelin cDNA clone and the predicted amino acid sequence of the protein it encodes is illustrated in Fig. 1. A methionine codon at base 182 surrounded by a Kozak (39) consensus sequence (GCCATGG) for eukaryotic initiation sites is followed by an open reading frame extending to a stop codon (TAG) at base 1349. A polyadenylation signal (AAUAAA) begins at position 2637. The sequence contains 5 cysteine residues and a potential N-glycosylation site (Asn-Ser). It also contains a Glu-Ser-Leu peptide, the phosphorylated form of which has been shown to exist in all mineralizing tissues (40). 181 bases (1-181) from the 5’ and 1322 (1532-2674) bases at the 3’ ends are untranslated sequences.

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with the secreting region (Tomes's process) but not with the odontoblasts (dentin-secreting cells) Fig. 7A(1). In addition, the antisera reacted with developing enamel extracellular matrix but not with dentin (Fig. 7A(2)). Fig. 7B shows the indirect immunofluorescence (using FITC antibody as second antibody) on 30–40-μm longitudinal ground sections of mature, erupted, unetched, and HCl-etched bovine and human enamel, employing the synthetic peptide antisera as first antibodies. Reaction with preimmune sera served as control. The results show reaction of antibodies with bovine (Fig. 7B(1)) and human (Fig. 7B(2)) tuft proteins radiating from the dentin-enamel junction toward the enamel surface. The reaction on a light-microscopic level was localized mainly interprismatically, between the enamel prisms. In the human mature enamel, the typical horseshoe pattern was seen (Fig. 7B(2)). No definite reaction occurred with the underlying dentin. In order to determine the ultrastructural localization of tuftelin protein in developing bovine enamel, the protein-A gold method (48, 49) was used with synthetic peptide antisera as first antibodies and conjugated gold particles of 10 nm in size. The grids were inspected under a Phillips 300 electron microscope. Fig. 8A shows reaction of tuftelin antisera with developing bovine enamel and Fig. 8B the control, using preimmune sera. These results reveal that tuftelin, present in the extracellular enamel, is mainly associated with the crystal component.

**DISCUSSION**

We have determined for the first time the DNA sequence for an enamel-specific gene coding for one acidic enamelin protein, tuftelin. The results reveal that the protein deduced from the sequenced cDNA is a novel protein with many hydrophilic residues and an isoelectric point of an acidic
The deduced protein using synthetic peptide antibody of the enamelin-enriched fraction proteins. Sections of developing bovine tooth reacted with LF-75 synthetic preimmune sera were used as control. Indirect immunohistochemistry of paraffin-embedded thin sections (5 μm thick) of a developing bovine tooth depicting ameloblast organelles of ameloblasts, indicating that some components of tuft are early secretory products (nonamelogenin enamel-like) of ameloblasts.

Their antisera also reacted with some minor components (25 kDa), which the authors suggest were not amelogenin. Furthermore, Amizuk and Ozawa (47) showed that specific polyclonal antibodies against enamelin proteins cross-reacted with components of tuft proteins. Our present indirect immunohistochemical studies (Fig. 7) show that the synthetic peptide antiserum, which reacted with purified enamelin protein (Figs. 5 and 6), also reacted well with the tuft proteins radiating from the enamel dentin junction towards the enamel surface both in bovine and human mature enamel. For this reason, we propose the name tuftelin for this specific enamel protein.

On a light-microscope level, the reaction of the synthetic peptide antiserum with the mature enamel occurred mainly interprismatically, surrounding the enamel prisms. In the human tooth (Fig. 7), this reaction occurs in a typical horseshoe pattern. This agrees well with recent high resolution protein-A gold studies of Amizuk and Ozawa (47), who reacted polyclonal antibodies against enamelin with mature enamel proteins.

Recently, our studies using high resolution protein-A gold immunohistochemistry employing these synthetic peptide antisera confirmed earlier findings (3, 6–10) that enamelins in the developing extracellular enamel matrix are localized mainly at the crystal surfaces (Fig. 8).

Our results reveal cross-reactivity between enamelins of
the secretion of relatively high concentrations of enamelin at the dentin enamel junction area, already in very early stages of development, as reported by Robinson et al. (13), Deutsch et al. (17), and more recently by Slavkin et al. (18), has suggested to us (5, 17) that these enamelin proteins are associated with the mineralization of the hypermineralized enamel region adjacent to the dentin enamel junction area present in the very early forming enamel of all species, thus creating a mineralization front in enamel. The exact role, however, is still unclear. Some have suggested enamelin is a template or nucleator for mineralization (3, 18, 20). More recently, it has been suggested (51) that a component of enamel matrix protein (enamel sheath, which represents in part the presence of enamelines) could bind to collagen fibers of the underlying dentin. Such chemical interactions between the two matrices may serve to promote enamel crystal nucleation. Preliminary results show that some reaction of our enamelin synthetic peptide antisera on the ultrastructural level (protein-A gold method) could be seen at the dentin enamel junction area in the vicinity of the collagen fiber tip surrounding the enamel crystals.

Finally, an NH2-terminal decapeptide sequence of a 22-kDa unidentified protein band isolated from enamelin extract has just been published (52). It contains a -Pro-Ser-Ser-X-Ala-Gln- sequence. A homologous sequence can be found in our tufelin sequence: -Pro-Ser-Pro-Pro-Ala-Gln- (previous sequence has an extra serine). This may indicate the published peptide sequence to be a tufelin derivative or fragment.

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