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Characterization of two distinctly different mineral-related proteins from the teeth of the Camarodont sea urchin *Lytechinus variegatus*: Specificity of function with relation to mineralization

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Abstract

The majority of the mineral phase of the *Lytechinus variegatus* tooth is comprised of magnesium containing calcite crystal elements, collectively arranged so that they appear as a single crystal under polarized light, as well as under X-ray or electron irradiation. However, the crystal elements are small, and in spite of the common alignment of their crystal axes, are not the same size or shape in different parts of the tooth. The toughness of the tooth structure arises from the fact that it is a composite in which the crystals are coated with surface layers of organic matter that probably act to inhibit crack formation and elongation. In the growth region the organic components represent a greater part of the tooth structure. In the most heavily mineralized adoral region the primary plates fuse with inter-plate pillars. Using Scanning Electron Microscopy; TOF-SIMS mapping of the characteristic amino acids of the mineral related proteins; and isolation and characterization of the mineral-protected protein we report that the late-forming inter-plate pillars had more than a three-fold greater Mg content than the primary plates. Furthermore, the aspartic acid content of the mineral-related protein was highest in the high Mg pillars whereas the mineral-protected protein of the primary plates was richer in glutamic acid content. These results suggest that the Asp-rich protein(s) is important for formation of the late developing inter-plate pillars that fuse the primary plates and increase the stiffness of the most mature tooth segment. Supported by NIDCR Grant DE R01-01374 to AV.

Keywords

sea urchin tooth; mineral-related proteins; high magnesium calcite; TOF-SIMS; SEM

1 Introduction

The flanged, camarodont sea urchin tooth has a very complex structure that has many features designed for maximizing both strength and toughness. The complexity is emphasized in two rather different spheres: the arrangement of the cellular components of the tooth which allows continuous growth and regeneration, in response to tooth wear and abrasion during feeding; and the arrangement of the mineral phase structure comprised from distinctly different high

magnesium containing calcite (magnesian) crystal elements. It is known from previous studies that the Mg is not distributed evenly throughout the calcite. The calcite plates have a 4% to 14% (mol) MgCO_3 content while the columns joining the calcite plates may have contents as high as 40% (mol) MgCO_3 [1–4]. The individual crystal elements are small and have different sizes and shapes depending upon location. Before the columns link the plates, each is surrounded by an organic sheath, and these plates share a common crystallographic alignment, appearing as a single crystal under polarized light and X-ray or electron diffraction. The crystal axes of the later forming, very high Mg columns grow aligned with the adjacent plates [4].

We have hypothesized that the formation of the crystal elements is guided and regulated by the components of the organic sheaths constituted from the cell syncytial membranes and extracellular or surface-associated proteins [5–7]. In the present work we have focused on the nature of the proteins directly related to the mineralized phase and the relationship between the magnesium content and the protein composition.

2 Material and methods

2.1 Structure of the intact *Lytechinus variegatus* tooth in the mineralized region

2.1.1 Identification of mineral ion and protein amino acid distributions—The intact adoral mineralized portion was cut into transverse cross-sections, which were mechanically polished. These surfaces were scanned by time of flight secondary ion mass spectroscopy (TOF-SIMS) using a PHI THRIFT III system with a Ga ion source operating at 25 kV. Spectra were examined for Ca, Mg, Na and K ion masses, and for the characteristic fragment ions corresponding to amino acids Asp and Glu following the procedures described by Robach et al. [3].

2.1.2 Scanning electron microscopy of fractured tooth segments—The mature, highly mineralized intact adoral portion of *L. variegatus* teeth was examined in a Hitachi S-4500 cold cathode field emission SEM at 10 kV.

2.2 Isolation of mineral-related proteins

Lytechinus variegatus teeth were collected, frozen in liquid nitrogen, and then crushed to a powder using a mortar and pestle. After thawing, the powder was washed with saline containing protease inhibitors, then extracted with 4.0 mol/L guanidine HCl to remove any exposed, non mineral-protected cellular constituents. The mineral-protected organic components were then extracted by demineralization in 0.6 mol/L HCl at 4°C [8]. These mineral-phase proteins were fractionated by hydroxyapatite affinity (HA). Both the HA binding protein and the non-bound proteins were isolated, trypsin digested, and the peptides sequenced. The sequences obtained were used to clone the proteins from *L. variegatus* total mRNA based cDNA library, based on homology to proteins in the *Strongylocentrotus purpuratus* data bank. Complete sequences were obtained for two differentially isolated proteins. The details of cloning, the primers used, and the determination of the complete protein sequences are described in detail elsewhere [9] and generally followed [8].

2.3 Properties of the urchin proteins

The sequences of the proteins, as deduced from the N-terminal sequencing and subsequent cloning from their cDNAs were analyzed for various properties. Most importantly they were analyzed for potential intracellular, transmembrane and nuclear localization, and possible post-translational modifications using the various predictive algorithms from the CBS prediction servers (www.cbs.dtu.dk/services/), particularly phosphorylation and glycosylation. The proteins isolated by the extraction and fractionation schemes were subjected to analysis for phosphorylation by dephosphorylation with potato acid phosphatase (Sigma, St. Louis, MO),

and for Ca ion binding by using the isolated protein bound to nitrocellulose and measuring ^{45}Ca binding by the procedure of Maruyama et al. [10].

3 Results

Fractured sections from the highly mineralized adoral segment of the *L. variegatus* teeth (the primary plate region of the flange) show the calcite plates and connecting columns, and reveal the many channels where the syncytial cytosol and enclosed cell nuclei had not yet been eliminated from the densifying structure (Fig. 1). Note the great variability of the channel sizes and the variability of the column pattern. Where the column-plate interface is exposed, both the calcite plates and column surfaces appear smooth. The fractured calcite plates have a distinct midline that appears to have polymeric components that penetrate the central region of each plate. In contrast, the lateral surfaces of the columns are not at all smooth but fracture in a different manner. The prisms and needles (Fig. 2) are also covered with similar flat-surfaced, cementing columns with, at this stage of mineralization, space for cellular organic components.

Polished *L. variegatus* tooth surfaces revealed an interesting spatial distribution of protein fragments when examined by TOF-SIMS. The SEM image of Fig. 3 (top row, right) shows the region investigated with SIMS, a region of the mature tooth containing the tooth stone part (St), flange primary plates (PP) and needles (N). The SIMS images (top left and top center of Fig. 3) are from the same area as the SEM image and show maps of Mg and of one ion-fragment spalled from the surface, respectively. The fragment mass and presence of subsidiary mass peaks corresponds to $\text{C}_3\text{H}_9\text{O}_2\text{N}$, a product of the breakdown of peptide bound aspartic acid. In the SIMS maps, the brightest yellow color represents the highest concentration of species in question; red presents a lower concentration; and the lowest concentration appears black. In each case, the concentrations are relative, and qualitative, not quantitative. Nevertheless, it is evident that the St has a higher average Mg content than the bulk of the PP or N regions does.

The two lower panels of Fig. 3 show higher magnification SIMS maps of the two boxed areas in the SEM image. The middle panel shows area A from the SEM, comprised of primary plates and cementing columns, and the bottom panel shows area B, an area of needles and columns. The Mg maps are shown on the left of these two panels, Asp data appear in the middle, and colocalization maps of Mg and Asp (the brighter green pixels show positions of corresponding Mg and Asp signal) are on the right. The lower Mg calcite plates are labeled PP, and, as expected, the inter-plate columns contain more Mg. The positions of highest Asp signal mirror those with highest Mg content (colocalization map) but not all of the Asp occurs in the columns: there is a less amount of Asp in the lower Mg primary plates and needles.

The proteins extracted from the entire heavily mineralized adoral part of the teeth were fractionated by their apatite binding properties and separated into Unbound and Bound fractions, and, as indicated in the Experimental section, were each separated by gel electrophoresis. Proteins differentially present in each fraction were isolated, digested with trypsin and the tryptic peptides were isolated by HPLC and selected peptides were sequenced, and then their cDNAs cloned from a *L. variegatus* cDNA library. The protein from the Unbound fraction, was the *L. variegatus* analog of predicted mineral-related protein *S. purpuratus* P19. The Bound fraction was the *L. variegatus* analog of the *S. purpuratus* P16, another mineral-related protein [11,12]. To distinguish the *L. variegatus* tooth proteins from the *S. purpuratus* predicted proteins, we have designated them as UTMP16 and UTUM19.

The UTMP 19 polypeptide sequence is shown in Fig. 4 as an acidic protein that has no signal sequence or membrane spanning sequence, so it is likely to be a strictly intracellular protein. Interestingly it has distinct, consensus nuclear localization and nuclear exit sequences, so it

may be important in the cell nucleus. It is rich in Thr and Glu residues, particularly in the amino-terminal part of the molecule. Analysis of post-translational modifications indicates that residues 61T, 62S, 63T and 78S have a greater than 90% probability of phosphorylation.

On the other hand, UTMP16, which is shown in Fig. 5, does have a signal sequence and a C-terminal region membrane spanning domain, suggesting that it is an extracellular protein that may be anchored to the syncytial membrane. Most importantly, the anchored central region, from residues 75 to 100 has an Asp-Ser-rich domain which is a substrate for the same antibody that recognizes vertebrate DPP, the phosphorylated vertebrate tooth matrix protein linked to mineralization. The Ser residues in this domain have a high probability of being substrates for the casein kinases, CKII and CKI.

Direct studies of phosphatase activity have shown that both UTMP19 and UTMP16 are phosphorylated and that the phosphates are substrates for potato acid phosphatase. Direct binding studies of UTMP19 and UTMP16 immobilized on nitrocellulose showed that UTMP16 avidly bound to ^{45}Ca , whereas UTMP19 did not, as shown in Fig. 6.

Preliminary localization studies, using anti-DMP2, the antibody which recognizes the Ser-Asp rich phosphoprotein of vertebrate dentins, show the localization of UTMP16 (or another similar protein), to the syncytial membrane at the mineralization front in an *L. variegatus* tooth section where primary plate mineralization is just beginning, as shown in Fig. 7.

4 Discussion

These data suggest that as Cheers and Etensohn [12] and Livingston et al. [11] had proposed the P19 and P16 proteins of the mineralizing embryonic spicules of the sea urchin are mineral-related proteins. In this work we show that they are also in adult tissue, at least in the continuously growing tooth, and that they are associated with the mineralized phase. However, the proteins are quite distinct. UTMP19 is an intracellular, possibly a nuclear, protein with poor Ca binding properties. Thus, it is likely that it may serve a role such as that of a transcription factor, regulating the appearance of other mineral-related proteins. UTMP16, which is a secreted extracellular protein, appears to have a more direct function in the mineral deposition since it has strong Ca binding properties. It has a carboxyltail region that appears to remain in the syncytial cytosol, and a neighboring transmembrane spanning region that fixes the protein to the membrane. Thus, one can speculate that the phosphorylated Ser-Asp rich region may be a part of the localization mechanism for nucleation of the high Mg columns that fuse the low Mg calcite plates together while toughening the mechanical properties of the mature, adoral, incisal part of the tooth [13].

Obviously, much remains to be done to verify these hypotheses. Work is underway to specifically localize these proteins and to understand the timing of their expression. One fact is evident, i.e., one cannot simply on the basis of protein composition alone assign proteins to the group of directly active participants in the mineralization process, as distinct from those proteins with a more biosynthetic regulatory function.

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Fig. 1. Fracture surface in primary plate region (Arrows show both narrow channels and wide spaces for syncytial contents and cell nuclei)

Fig. 2.
Isolated prisms from the keel (showing columns on the surface)

Fig. 3.

SEM and TOF-SIMS images of the complex of primary plates PP, stone part St and needles N in the center of a mature, mineralized *L. variegatus* tooth. The top row of images shows the same area: Mg map (left), Asp map (center), SEM image (right). The horizontal white line crosses the same plane in both panels. The other white lines depict the stone part boundaries. SIMS maps of areas A and B (SEM) are shown in middle and bottom panels, respectively, with Mg at the left and Asp in the middle. The panels in green (right) represent colocalization of Mg and Asp using the NIH Image J procedures.

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* Protein: UniProt: P08001
* Accession: P08001
* Name: UTMP16L
* Length: 168
* Description: UTMP16L
* Species: Homo sapiens
* Gene: UTMP16L
* Organism: Homo sapiens
* Taxonomy: Eukaryota; Metazoa; Chordata; Mammalia; Primates; Hominidae; Homo
* Molecular Weight: 16.8 kDa
* Isoelectric Point: 4.5
* Molecular Weight: 16.8 kDa
* Isoelectric Point: 4.5

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Fig. 5.
The protein sequence of UTMP16L

Fig. 6.
⁴⁵Calcium binding property of isolated UTMP19, Lane 1, and UTMP16, Lane 2. Protein immobilized on nitrocellulose.

Fig. 7. Mineralization “front” for plate formation (tip of arrow), early in tooth development. Stained with anti-DMP2 (red fluorescence). Black areas show new mineral crystals.