摘要

齒舌是軟體動物的攝食器。笠螺及青螺的齒舌在不同的發育階段會相繼累積鐵和矽元素，是研究生物礦化極佳的材料。齒舌的發育及礦化是一連續且同時性的事件，亦即可以在一條齒舌上可關察到一個攝食器官的整個發育過程。我們利用蛋白質體的方法分析參與細線青螺與花笠螺齒舌不同發育階段生物礦化的蛋白質。在不同發育階段，S^{35}標記的蛋白質在 SDS-PAGE 上顯示了不同的模式，指出齒舌的發育會經過某些變化，而這些變化可能與齒舌的生物礦化過程有關。新合成的蛋白質發現被運送至細胞外的齒舌牙腔內。花笠螺和細線青螺在二維蛋白質電泳分析中分別出現了一千及三百個點，比較齒舌的不同發育階段後發現，分別有 17 及 19 個蛋白質在齒舌形成的不同發育階段有差異性的表現。例如我們發現在花笠螺中，一系列蛋白質（點 17、20、21、22、23、24、25）和點 53 只有在齒舌囊和第一階段出現。點 155 的密度在第一階段大幅度的增加。點 79 在第三階段表現出高量的 S^{35} 標記。在細線青螺，點 40 及 41 的蛋白質被放射線顯影。點 262 在第二階段出現並持續至第三階段。點 53 在第三階段表現出高量的 S^{35} 標記。上述的這些蛋白質可能在齒舌生物礦化的過程中扮演著重要的角色。觀察齒舌發育階段的蛋白質表現的多樣性，有助於進一步探討生物礦化過程中的生物學意義。

Abstract

The candidate proteins that are involved in biomineralization in the radula of two limpet species *C. toreuma* and *P. striata* were studied during different developmental stages by proteome analysis and *de novo* protein synthesis. Protein patterns revealed by S^{35}-labeling on SDS-PAGE gel differed at different developmental stages in both species, indicating the radulae undergo through some changes and a part of these changes may be related to the biomineralization process. Proteins were also found to be synthesized *de novo* and transported to the extracellular compartment. Among 1000 and 300 spots resolved in the two-dimensional gels in *C. toreuma* and *P. striata* respectively, comparison of different developmental stages of radulae revealed that 19 and 17 protein spots showed differential occurrence at different developmental stages of radulae formation. We found that for example in *C. toreuma*, a series of spots (spot No.19, 20, 21, 22, 23, 24, 25) and spot 53 appeared only around the radular sac and during stage I. The intensity of spot 155 greatly increased at stage I. Spot 79 presented high intensity ^{35}S-label at stage III. In *P. striata*, the autoradiography of two protein spots 40 and 41 were found in radular sac. Spot 262 appeared at stage II through early stage III. Spot 53 presented with high intensity ^{35}S-label at stage III. The preceding proteins might play a major role in the mineralization of the radulae. The broad spectrum of protein variability in expression observed during developmental stages of radulae might justify some speculation upon further biological implications of the biomineralization process.

Introduction

Biomineralization, simply stated, is the process by which organisms convert soluble ions into insoluble minerals. To complete this definition it should added that the conversion is the result of cellular activities that make possible the necessary physical chemical changes for mineral formation and crystalline growth(Sinkiss and Wilbur 1989).

Biomineralized structures are composite materials formed in diverse biological systems. Their composition, distribution and functions are described together with several technological applications(Webb, Brooker et al. 1997). Such examples include the use of coral in bone reconstructive surgery and the development of flexible macro-defect-free-concrete. Recent studies of biomineralization in the prominent feature of radular teeth structure in the marine mollusks, chitons and limpets have further extended understanding of the initial stages of iron biomineral formation(Mann, Perry et al. 1986). In the marine gastropod *Crystoplax striata*, a novel motif of tooth structure is reported, where granules (300nm-1.5 μm) are embedded in the organic matrix which similar to the quality of sandpaper(Mann, Perry et al. 1986). Further studies of biomineralization at the nanoscale level, utilizing the hollow spherical protein ferritin and several polysaccharides, reveal the nanotechnological possibilities of such research, which can produce varied multi-layered nanocomposites and extended arrays(Webb, Brooker et al. 1997).

Limpets (Mollusca: Gastropoda) together with chitons (Mollusca:

Polypla-cophora)(Fig. 1), are common members of the intertidal and shallow water molluscan community which occur on rocky shores. Limpets are well know marine invertebrates that feed by using developed tongue or radula (Figs. 2, 3 and 4) which are extruded from the mouth to scrape encrusting algae and sponges from the intertidal rocks on which they live (Steneck and Walting 1982). The radula is one of the most characteristic organs of the phylum Mollusca. The basic pattern of most radulae consists of numerous transverse rows of radular teeth with bilateral symmetry relative to the main axis, supported by a thin radular membrane. This structure has been shown to vary both with the type and more particularly with the hardness of the food material consumed (Steneck and Walting 1982). In several species of limpets, the radula teeth are mineralized and are thus able to excavate the rocks over which they browse with the anterior part of the radula. The recurved hook-like teeth are forced against the substrate by dragging over it(Jones, McCance et al. 1935; Lovenstam 1962; Lovenstam 1967; Runham, Thornton et al. 1969; Lovenstam 1971; Lovenstam 1981; Steneck and Walting 1982)(Figs. 2D,3, and 4E). The teeth are abraded during this process and need to be continuously replaced(Runham, Thornton et al. 1969). Consequently, the radula on which the individual teeth are borne consists of a series of teeth in various stages of development(Webb, Macey et al. 1989). Initially, the teeth are composed of only a soft organic framwork (Figs. 2B, 4A and B), which is secreted by odontoblast cells of the posterior end of the radula sac(Runham 1963)(Figs. 2A,4A). As the teeth move down the radula, cells of the superior epithelium formed adjacent to the odontoblast cells move with them(Runham 1963) and are intimately involved in the deposition of the various minerals involved in the biomineralization(Kim,

Macey et al. 1989). Mineral deposition within each tooth cusp is very site specific, and by the time the teeth are approaching maturity each of the various minerals are found in architecturally discrete compartments(Lowenstam 1967). In limpets, where both Fe and Si are mineralized. The Fe appears as acicular crystals of goethite(a-FeOOH), while silicon is found as extensive deposits of amorphous opaline silica($SiO_2 \cdot nH_2O$)(Simkiss and Wilbur 1989).

The formation of the radula has been described by Mackenstedt and Märkel(Mackenstedt and Markel 1987). The most posterior cells or odontoblasts initially secrete the matrix of the cusp and distal tooth surface. The entire ribbon of teeth then progresses anteriorly so that the next odontoblasts on coming in contact with the forming tooth secrete more matrix and thus contribute to the ongoing enlargement of the tooth. As the developing radula moves anteriorly, the base is added by other cells until a final shaping of the matrix takes place. Thus, odontoblasts in linear arrangement demonstrate a marvelously integrated sequence. Not only do different cells secrete specific amounts of matrix for particular portions of the tooth but they shape it as well. To complete the process, another group of cells, the superior epithelia, adds ions and organic compounds, which results in a hardening process. The radula is degraded at its anterior end (Figs. 3E, 4F) and is constantly replaced by the newly formed regions as they advance anteriorly(Simkiss and Wilbur 1989).

Mineralization of each tooth cusp in these animals begins with goethite impregnation at the posterior region and consists of these strands of minerals (15-20 nm wide) arranged with their 001 crystallographic axis

parallel to the tooth wall(Simkiss and Wilbur 1989). The process continues with an increase in the number and thickness of the crystals. The mature crystals are well ordered but with extensive growth distortions attributed to the filamentous organic matrix. Silica impregnates the matrix at a later stage in mineralization(Simkiss and Wilbur 1989). This confirms the earlier work of Runham et al.(Runham, Thornton et al. 1969)that suggested that the iron oxide and silica phases were spatially separated. It seems to be generally agreed, therefore, that silica infiltrates the radula and fills spaces left between the goethite crystals. Such composites are rare in biomineralization and deserve further study.

Mineralization of the limpet's radula is a very complex phenomena, involving a sequence of differential stages of mineral deposition(Runham 1961; Runham, Thornton et al. 1969; Mann, Perry et al. 1986). Following the scheme described by Kirschevink and Lowenstam(Kirschevink and Lowenstam 1979), teeth on the radulae teeth were assigned to four developmental stages: clear, unmineralized teeth (stage **I**)(Figs. 2B, 4A and B), teeth of the transitional zone where iron mineralization begins (stage **II**)(Figs. 2C, 4B and C1). By stage**III**, because of the capping of iron oxide, the cusp of the teeth become reddish brown due to the presence of goethite(Figs. 3 and 4D), and stage**IV** where as the teeth are largely matured(Figs. 3C, D,E and 4E, F).

Two limpet species, *Cellana toreuma* (Fig. 1B) and *Patelloida striata* (Fig. 1C) occur in close sympatric association in the northeast coast of Taiwan, R.O.C, these two species being found browsing mainly on the

algae which cover the limestone rocks found in this area. Following the scheme described above, and combine with the research carried out by H, K Lu et al(Lu, Huang et al. 1995) the radula teeth were divided into four developmental stages.

Stage Ⅰ:

Situated after the odontoblasts, newly formed teeth which are unmineralized, consisting purely of the organic matrix framework, clear, soft (Figs. 2B, 4Aand B). The organic framework is composed mostly by α-chitin together with smaller amounts of proteins (a polysaccharide). This material is structured as a series of rope-like fibers which vary in their arrangement depending on the position in the tooth cusp(Evans, Macey et al. 1990; Evans, Macey et al. 1994), which later acts as a framework and directs the deposition of inorganic salts. It is also this structure which gives the tooth structure tensile strength and flexibility(Evans, Macey et al. 1990).

Stage Ⅱ:

Classified by the onset of mineralization and is characterized by a deep red color due to the acute deposition of iron(Towe and Lowenstam 1967)(Figs 2C, D,3A and 4B). At this early stage of mineralization in limpets, deposition of goethite is first found. For chitons, ferrihydrie ($5FeO_3 \cdot 9H_2O$), lepidocrocite (?-FeOOH) and magnetite (Fe_3O_4) are deposited.(Li, Chin et al. 1989; Evans, Macey et al. 1990; Evans, Macey et al. 1991; Evans, Macey et al. 1992; Evans, Macey et al. 1994)

Stage**III**:

The appearance of a dramatic event leading to a partial change in the tooth cusp structure occurs approximately at this stage and was interpreted as being indicative of final changes occurring in the mineralization process (Figs. 3B, C, 4D and E). The anterior of each tooth cusp turned to a reddish brown color due to the capping with iron minerals. The more it moves torwards the radular cone (Figs. 3E, 4F), the darker it becomes. At this stage, deposition of apatite occurs in chitons, whereas deposition of opal occurs specifically in limpets. A new mineral berthierine, $Fe_3Si_2O_5(OH)_4$ was found in the *C.toreuma* species(Lu, Huang et al. 1995).

Stage**IV**:

Beyond this point the teeth are considered fully mineralized (Figs. 3Dand E, 4E and F).

It is concluded that radula biomineralization is subject to highly complex but precisely controlled cytological-biochemical processes and that different parts of each young tooth are subjected simultaneously to different biomineralization pathways(Rinkevich 1986). Furthermore, such biomineralized structures are composite materials highly appropriate to their biological function and as such are prime sources for the design and synthesis of novel composite materials(Lee, Webb et al. 1998).

There is a growing body of information on the structure, composition and synthesis of the macromolecules, but the key and the exciting question is to identifying the molecular process that produce minerals of precise form with uniform particle size, novel crystal morphology and specific

crystallographic orientation. How do the proteins and polysaccharides of the substrate interact to regulate solid-state chemical reactions? Key to these processes is the need to develop improved enzymatic pathways in these organisms. In the initial step, the proteome of the organism must be completely characterized. To gain a broad overview of cellular activities upon biomineralization, we performed a comprehensive proteome analysis to study biomineralization between different developmental stages in the radulae of the limpets. This cell system was chosen because much is known about the ultrastructure and mineral composition of each developmental stage. And the teeth along the radulae occur in sequential stages, the onset of mineralization occurs in a quite synchronous manner.

The totality of proteins expressed from the genome of a cell is referred to as proteome. The proteome is highly dynamic and depends on many different parameters affecting cells. Proteomic analysis may be preformed by high resolution, two-dimensional gel electrophoresis, separating proteins according to their molecular weight and electric charge, which yields highly reproducible and characteristic 2D protein patterns. In this study we invested proteome alterations and proteins that are newly synthesized in each developmental stage of the radula.

17 of more than 1000 protein spots resolved in *P. straita* and 19 of more than 300 protein spots resolved in *C. toreuma* have shown differential occurrence at different developmental stages of radula formation. Which of these might play a major role related to the mineralization of the radular. This study demonstrates that many data on a large number of proteins may be obtained by proteome analysis, providing new insights into the role of

cells during biomineralization.

Materials and Methods

Materials- Specimens of *Cellana toreuma* (2.8 to 3.5 cm shell length) and *Patelloida striata* (2.5 to 3.1 cm shell length) were collected from the rocks of tidal pools at Shrmen Shiang, Taipei, Taiwan, R.O.C (latitude 25.3º N, longitude 121.6º E), brought back to the institution, maintained in the laboratory aquaria with running sea water, and sacrificed within 24h after collection.

Metabolic Labeling with Radionuclieotides- *De novo* protein synthesis was revealed by L-[^{35}S] methionine (SJ-123, Amersham) labeling complemented with serum- and methionine-free RPMI medium (RPM1-1640 medium, Sigma) at a concentration of 1600 μ Ci/mL. Labeling was performed by injection of 50 μ L of the medium into each specimen. After the 3-h labeling time elapsed, specimens were sacrificed and the radulae were dissected out of the animals and washed twice with 20mM phosphate buffered saline (PBS, 137mM NaCl, 2.7mM KCl, 8.6mM Na_2HPO4, 1.4mM KH_2PO_4, pH7.3). The scheme was modified for the present study where by devised by Kirschvink and Lowenstam[2], each radulae was then cut into segments representative to four development stages, some of which were further subdivied into seven sections: 1. radular sac, 2. clear unmineralized teeth (stage **I**), 3. a section marked all or most of the radula characterized by a deep red color (stage **II**). 4-7. At the microscope level it is very difficult to distinguish between stages **III** and **IV**. Stage **III** was subsequently reassigned as the end of stage **II** and stops

at the radular cone, which was reassigned as stage **IV**. Stage **III** was then equally divided into three smaller sections, redesignated as stage **III** α, β and γ. Seven specimens of each species (1 as whole, 4 dissected as the scheme described above, two by the scheme in four stages) were used in each SDS-polyacrylamide gel electrophoresis. Ten specimens of each species (2 as whole, 8 were dissected in a similar way, only stage **III** was equally divided into half) were treated with 3%collagenase (C-0150, Type **I**, Lot 10k1142, SIGMA) at room temperature for 5h until the cells were released from the teeth. The teeth were subsequently boiled in SDS for 10 minutes, washed twice in PBS and also used in SDS-polyacrylamide gel electrophoresis. Ten specimens of each species (2 as complete, 8 in the scheme described above) were analyzed with two-dimensional electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography-All cell experiments, labeling, and SDS-polyacrylamide gel electrophoresis results presented were performed more than three replicates. The protein samples were dissolved in 40 μL of 2x sample buffer (31.25M Tris-HCl, pH 6.8, 4% SDS, 28.8mM 2-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) homogenized, boiled for 10 minutes and centrifuged (5000g, 10min) to remove precipitated proteins before loading. 25 μL of each sample were loaded into each well of 0.75mm thick 4.75% stacking gels and 15% separating gels. The gels were run at room temperature in electrode buffer (0.1%

SDS, 25mM Tris-HCl, pH8.3, 192mM glycine) at 25mA for 4h. Gels were stained by Coomassie Brilliant Blue (CBB) R250. The gels were laid on Whatman 3MM chromatography paper, covered with Saran Wrap, and dried at 80°C using the Slab Dryer Model 583 (Bio-Rad), or by sealing the gel in glass paper and stored in the cabinet for 3 days until dry. Exposure of BIOMAX^{TM} MR X-ray films (Kodak, Rochester, NY) were carried out at -70°C in autoradiography cassettes for 9 days.

Two dimensional Electrophoresis and Autoradiography-Two-dimensional gel electrophoresis was carried out by modification of the method of Hochstrasser et al(Hochstrasser, Harrington et al. 1988), using GT series gel electrophoresis unit (Amersham). The protein samples were dissolved in 40 μL of 9.5M urea, 4% Triton x-100, 5% (v/v) 2-mecaptoethanol, 5% ampholyte (Bio-Rad) (2 volume pH 3-10, 3 volume pH5-7) solution mixture. Each protein sample was centrifuged(5000g, 10 min) before loading. Isoelectric focusing (IEF) was prepared by a prerun at 600 V-h in a stepwise fashion (15min at 200V; 30min at 300V; 1hr at 400V) and applied protein samples were preformed at 12,400 V-h in a stepwise fashion (4h at 400V; 12h at 800V; 1hr at 1200V) in 4% acrylamide (Merck), 0.1% bis-acrylamide (Bio-Rad) 2-mm x 16-cm tube gels. The gel buffer contained 8.6M urea, 1.875% Nonidet P-40, and 4.69% ampholytes (Bio-Rad) (1 volume pH 3-10, 4 volume pH 5-7), 10mM NaOH served as catholyte, and 10mM H_3PO_4 served as anolyte. For SDS-polyacrylamide gel electrophoresis the extruded tube gels were placed on top of 0.75-mm thick 12.5% polyacrylamide separating gels.

After a 1h equilibration with 2% SDS, 125mM Tris-HCl, pH6.8, 8% glyceral, 5% 2-mercaptoethanol, 1% bromophenol blue, the gels were run at room temperature in electrode buffer (0.1% SDS, 25mM Tris-HCl, pH8.3, 192mM glycine) for 4h. Gels were silver-stained by the method of Heukeshoven, J. et al(Heukeshoven and Dernick 1988). The gels were subsequently sealed in glass paper, and stored in the cabinet for 3 days till dry. Exposition of BIOMAX^{TM}MR x-ray films (Kodak, Rochester, NY) was carried out at -70℃ in autoradiography cassettes for 9 days.

Evaluation of 2D Data-Scanning of gels and films and comparative spot pattern analysis were accomplished with a scanner (perfection 1200s, EPSON), using the Image Master^{TM}2D V3.1 software package (Amersham). The gels were analyzed as two versions, the first is the 2D dried gels, and the 2D autoradiographs as the second version. Each version was spot calculated in wizard mode, background subtracted by manual mode, normalized, matched, and calculations of molecular weight were also performed by means of this software.

Results

The rationale of these experiments was based on the theory that the development of the radulae progresses in a sequential way, and the mineral deposition occurs at different stages, with each mineral deposition depended on a different pathway. The onset of the mineral deposition is due to the onset of protein function, either by the pH adjustment in a cell or the specific proteins that are precisely controlled by genes or both. The latter assumption was chosen and preformed in these tests. Each stage was carefully separated and analyzed in a comprehensive way by SDS-polyacrylamide gel eletrophoresis and two-dimensional electrophoresis.

C. toreuma and *P. striata* were metabolically labeled for 3h with [^{35}S] Met. This time was selected to allow the [^{35}S] Met to be adequately absorbed and incorporated by the radular tissue cells. The resulting previously Coomassie Blue stained SDS-polyacrylamide gel and silver stained two-dimensional gels were autoradiographed, which allowed us to record ^{35}S incorporation for *de novo* protein synthesis.

Limpet radulae were dissected following the four stages outline described previously. The length of each stage are varied, but they all occur in an average about 1:1:4:1 ratio (Stage **I** : Stage **II** : Stage **III**: Stage **IV**, data not shown), almost identical in both species. Then radular sacs, where all radular cells arise, was separated from Stage **I** . Stage **III** was further divided into three equal parts:

Protein pattern and de novo synthesis differed between stages in C. toreuma-Most significantly, some of the newly appearing protein bands displayed high ^{35}S labels. A 22.2-kDa band (Figs. 5 and 7) stained prominently at stage **II** in Coomassie Blue stained gels, and which corresponded to a band labeled autoradiographically (Figs. 6 and 7) extending from stage **II** to stage **III**a. The complete absence of this protein in the radular sacs and its faintly staining appearance in stage **I** indicates the *de novo* synthesis of this protein. Thus this suggests that this is a stage **II** specific peptide. Compared with the work of Lu et al(Lu, Huang et al. 1995), this might be the 26-kDa subunit of ferritin in the limpet *C.toreuma*.

An 18.8-kDa band (Fig. 5) appeared between stages **I** and stage **III***r* in the Coomassie Blue stained gel with the correspondingly parallel band revealed at stage **I** and ending at stage **III***a* on the autoradiographic films (Fig. 6). The synthesis of this band occur primarily during stages **I** to **II**.

More than a dozen ^{35}S labeled proteins were recorded per lane, some of which were not detectable by Coomassie Blue staining. During different stages, 9 bands (Fig. 6) were found specifically at certain stages, including a predominate 25.8-kDa protein specific to the radular sac, two proteins (19.4-kDa, 19.2-kDa) in stage **I**, five proteins (26.8-kDa, 20.9 -kDa, 19.7-kDa, 19.0-kDa, 18.7-kDa) in stage **III***a*, while two (20.9-kDa, 19.0 -kDa) extended all the way to **III**?, and one (19.7-kDa) to stage **III**ß.

Three proteins(62.1-kDa, 55.1-kDa, 21.7-kDa) predominated stage **IV** lanes.

Protein pattern and de novo synthesis differed between stages in P. striata-A 21.5-kDa band (Figs. 7 and 8), similar to the *C. toreuma* species was also spotted at stage **II** as a prominent band stained, which was also recorded on the autoradiography at that stage (21.7-kDa)(Figs. 7 and 9), but in a faint way compared to *C. toreuma* species. This may due to the appearance of stage **II** in P. striata which was not as prominent as in *C. toreuma*. Stage **II** occurred transiently, and appeared less reddish in *P. striata* thus harder to define between stages.

Two bands (19.0-kDa,18.9-kDa) appeared at stage **II** through stage**III**? in Coomassie Blue stained gel (Fig. 8) with the corresponding band (21.8-kDa, 21.3-kDa)(Fig. 9) revealed at stages **II** to **III** a on the autoradiographic films. Another band (130.5-kDa)(Fig. 8) appeared at stage**III**? to **IV** and was also synthesized (210.6-kDa)(Fig. 9) at the same stage.

More then a dozen ^{35}S labeled proteins were recorded per lane (Fig. 9), some of which were not sufficiently detectable by Coomassie Blue staining. During different stages, representative 10 bands were found specifically at certain stages, including a predominantly stained band of 24.9-kDa protein for radular sac and stage **I** , two bands (20.9-kDa, 20.9-kDa) in stage**III**(a,

ß, ?), four bands (27.0-kDa, 22.9-kDa, 22.4-kDa, 22.0-kDa) at stage **III**ß to **III**?, and three bands (27.8-kDa, 22.3-kDa, 22.1-kDa) in stage **IV**.

Proteins synthesized de novo and transported to the extracellular compartment-After a 3h labeling, the radular tissue was treated with collagenase to disaggregate the cells and the teeth. The teeth (base and cusp) were then analyzed with SDS-polyacrylamide gel electrophoresis. In *C. toreuma*, seven bands (135.0-kDa, 59.6-kDa, 47.0-kDa, 28.9-kDa in stage **I-II**; 20.4-kDa, 20-kDa, 18.6-kDa in stage **I-III**) were revealed by Coomassie Blue staining (Fig. 10). five bands were recorded on the x-ray film(Fig. 11), three of which (75.0-kDa, 50.8-kDa, 36.5-kDa) appeared in late stage **III** and were not detected by Coomassie Blue staining. Two major bands (20.4-kDa, 20.1-kDa) stood out prominently from stage **II** through early stage **III** (Figs. 10, 11 and 13), which indicates that these two proteins were synthesize *de novo* at stage **II-III** and transported out to the extracellular space where teeth are formed.

In *P. striata*, five bands were revealed by Coomassie Blue staining (Figs. 12 and 13). Two bands (155.2-kDa, 133.1-kDa) appeared at stage **I**, three bands ranged from stage **II** to **III** (18.5-kDa, 18.0-kDa, 17.4-kDa) (Figs. 12 and 13). No image was recorded on the autoradiographic film.

It has been estimated that there are about thousand genes which are

expressed as proteins in any one distinct cell type. This potential level of complexity is, therefore, not amenable to analysis by a single one-dimensional electrophoretic procedure. Another limitation of this SDS-polyacrylamide gel electrophoretic method is that, proteins are separated on the basis of only one of their physico-chemical property (i.e. size). Consequently, the discrete bands which are detected after electrophoresis do not necessarily represent homogeneous proteins. These factors have led the research to use a better electrophoretic method with the potential to separate very complex samples containing several hundreds of proteins and to resolve proteins which sharing similar physico-chemical properties, such as two-dimensional electrophoresis.

From the Coomassie Blue stained gels and *de novo* synthesis protein patterns we obtained in SDS- polyacrylamide gel electrophoresis, we were encouraged to use a more advanced technique to seek a further detail.

Two-dimensional Electrophoresis and protein synthesis-The radulae of two species, *C. toreuma* and *P. striata* were dissected according to four stages, seven different parts and analyzed by two-dimensional gel electrophoresis. Computer-assisted comparative analysis of the respective silver-stained spot patterns revealed a total of 1143 spots of the radular organ and 157 spots being newly synthesized for the *P. striata* species. Due to the high salt concentration in marine gastropods or the factor of the PBS wash after labeling, the proteome of two-dimensional electrophoresis was partially retarded and was only able to analyze a portion of the spot pattern for the *C. toreuma* species. After omitting the distorted protein

pattern by filtering out proteins above 23-kDa, a total of 303 spots were revealed. Fortunately, the autoradiography of the gel was little affected by the disrupted area and revealed 300 newly synthesized protein spots.

Then the two versions of protein patterns in each species were matched manually, and each spot checked and carefully compared to the spots that were specifically found in certain developmental stages. Spots that showed to increased intensity at certain stage were also included. Due to the possibility that the radulae were dissected in unequal parts, thus causing unequal loading protein amounts, spots that appear in a faint intensity were omitted.

Protein spots obtained from the limpet C. toreuma (Figs.14-29) - Two gels of *C. toreuma*, radular sac and Stage II were damaged severely caused by a power supply malfunction, was not able to be detected by the 2D analysis software. Thus the detection of spots of these two gels were carried out manually.

14 silver-stained spots and 14 ^{35}S labeled spots showed exclusivity in the *C. toreuma* species. From the basic part to acidic part of the gel, a 22.9-kDa spot (spot No.142) appeared at stage II to IV, 24.8-kDa (spot No.76) and 24.8-kDa (spot No.78) spots appeared at all stages, while autoradiography of the spots appeared only at radular sac to stage II zone. A 22.9-kDa (spot No.169) appeared only at stage II. A 22.9-kDa (spot No.155) spot appeared at all stages, the intensity of which greatly increased at stage I (Fig. 31). A series of spots (39.4-kDa, 38.856-kDa, 37.7-kDa,

37.9-kDa, 37.4-kDa, 37.6-kDa, 37.2-kDa, spot No.19, 20, 21, 22, 23, 24, 25) appeared only around sac and stage **I** and the autoradiography also showed they were synthesized at sac and stage **I** (Figs. 22, 23, 24 and 30). Six spots (see appendix spot report) determined autoradiographically were undetected by silver-staining. 24.7-kDa (spot No.93) and 24.7-kDa (spot No.85) spots appeared at stage**III**(a, ß, ?). A 24.7-kDa (spot No.94) spot appeared at stage **II** though stage, while a 24.8-kDa (spot No.80) spot also appeared at stage **II** but ended at stage**III**a. A 24.7-kDa (spot No.87) was found only at stage **II**. A 26.3-kDa (spot No.53) spot was stained faintly by silver-stain at radular sac but was not detected by the 2D analysis software, the autoradigraphy of this spot appeared at sac to stage **I**. Unexpectedly, the 24.8-kDa (spot No.79) was negatively stained by silver-stain (Figs. 26, 27, 28 and 32), thus not detected by the 2D analysis software. This negatively stained spot ranged from stage**III**a to *?* and presented high intensity of ^{35}S labeling.

Proteins spots obtained from the limpet P. strata (Figs. 33-48)-ten silver-stained spots and eight ^{35}S labeled spots showed exclusivity in the *P. striata* species. From the basic part to acidic part of the gel, the 21.6-kDa (spot No.273) spot and the 21.5-kDa (spot No.283) spot must have a close pI value, this means they are both close in size and charge. The 21.6-kDa (spot No.273) spot appeared at stage **I** through stage**IV**, and showed increased intensity at stage**II** and stage**III**a. The ^{35}S labeled spot also

showed that this protein is synthesized during stage **I** and **II** (Spot report 94). The 21.6-kDa (spot No.283) spot started at the radular sac and ended at stage**III**?. The 21.6-kDa (spot No.274) spot was evident from stage **II** to stage**III**ß. A 21.7-kDa (spot No.262) protein appeared at stage **II** though stage**III**a, and was synthesized at stage **II** (Spot report 86) (Figs. 36, 37, 44, and 50). 21.6-kDa (spot No.282) was found only at radular sac. A series of acidic peptides with similar molecular weight (21.9-kDa, 21.3, 21.7-kDa, 21.7-kDa, 21.7-kDa, spot No.248, 261, 260, 269, 271)(Figs. 33-40, and 52) all of which appeared at the most right hand side of the protein pattern were selected because of their significance in presenting the acidic molecules which are rare in proteins. The autoradiography of this part did not match to the silver-stained gels, which was probably due to the unequal amount of samples loading. 22-kDa (spot No.238) spot ranged from radular sac to stage**III**a. six spots of autoradiography were undetected by silver-staining. Three of them had a close molecular weight, but differed in pI value (30.5-kDa, 31-kDa, 30.6-kDa, spot No.36, 37, 38)(Figs. 41, 42), furthermore they only appeared at the anterior of radulae where the cusps undergo maturation (stage**III**?-**IV**). Two spots (28.2-kDa, 26.265-kDa, spot No.40, 41) were faintly stained by silver-stain in radular sac and were limited only to this area (Figs. 41, 42, and 49). A 23.3-kD (spot No.58) spot showed high ^{35}S labeling at stage**III**(a, ß, ?) (Figs. 45-47, and 51).

Features of 2D- analysis shared by the two limpet C. toreuma and P. striata-Interestingly, two features are shared by *C. toreuma* and *P.*

striata in two-dimensional electrophoresis. One is the 26.3-kDa (spot No.53) and 26.3-kDa (spot No.41) spots shown in the autoradiographys in *C. toreuma* and *P. striata* respectively (Figs. 22-24, 30, 41, 42, and 49). They are all expressed in the radular sac, positioned almost at the same site in each gel, and undetected or faintly stained by silver-staining. Two other spots that are the 24.8-kDa (spot No.79) (Figs. 18-20) and 23.4-kDa (spot No.58) (Figs. 45-47) spots were also undetected or stained negatively by silver-staining. Although not expressed in the same area, both however displayed high ^{35}S labeling at stage**III**(a, ß, ?).

Discussion

Within the biomineralizing organisms, the process of mineralization is restricted to specific tissues or organs(Simkiss and Wilbur 1989). There are two kinds of specificity, one relating to control over the type of mineral deposited and the other to the metabolism of the tissue or organ that brings about mineral deposition and exploits its properties(Simkiss and Wilbur 1989).

It was the aim of this study to gain a more comprehensive insight into the cellular mechanisms underlying and activated during different development stages of radulae by using the proteome analysis. While the availability of full genomic reference sequences provides a set of road maps as to what is possible, and measurements of the expressed RNAs tell us what might happen, the proteome is the key that tells us what really happens. Based upon isotope labeling, two versions of protein patterning was generated and analyzed simultaneously, to precisely establish changes in expression. 17 protein spots and 19 protein spots in the limpets *P.striata* and *C.toreuma* respectively displayed transcription activity in different developmental stages determined silver-stained two-dimensional gels. In addition, proteins below silver stain sensitivity displayed an interesting phenomenon as detected by ^{35}S autoradiography. The broad spectrum of protein variability in expression observed during developmental stages of radulae might justify some speculation upon further biological implications of the biomineralization process.

Chitin and organic matrix framework-

Biogenic minerals usually adopt characteristic forms defined by the geometry of their organic matrix frameworks. This is called an "organic matrix-mediated" process(Lowenstam 1981). In general, the organism constructs an organic framework or mold into which the appropriate ions are actively introduced and then induced to crystallize and expand. The organic framework that initially makes up the tooth is composed of the polysaccharide chitin(Evans, Macey et al. 1990), with various proteinaceons components, such as protein rich in tyrosine (Runham 1961). In chitons, aspartic and glutamic acids are also found, sometimes with phophorylated proteins(Evans, Macey et al. 1990; Evans, Macey et al. 1991).

Chitin (poly-N-acetylglucosamine) has been previously identified in the radulae of *C. toreuma* (Lu, Huang et al. 1995). Chitin is highly negatively charged due to the sulfate or carboxyl groups on most of its sugar residues. With this characteristic, compared to the result in 2D-analysis, two groups of proteins were found in *C. toreuma* (Figs. 12-27) and *P. striata* (boxed in Figs.28-34). The nature of these two groups of proteins remain to be investigated.

The role of proteins in iron biomineralization-

Biological mineralization of iron is now known to occur widely, yet studies of the complete process are few. In the case of chitons, it was pointed out that the magnetite is deposited on the teeth through lepidocrocite (?-FeOOH). In limpets, however, the synthesis terminates in

the form of a-FeOOH. The iron must be transported in the soluble ferrous form across the cell membrane(Lowenstam 1981). In the superior epithelial cell of the radula, ferritin was found to disassemble through autophagy or heterophagy before exocytosis (Lu, Huang et al. 1995). In fact, the ferritin micelles are observed to be at various stages of demineralization at specific areas on the surface of the tooth in the form of ferrihydrite(Nesson and Shiller 1968). The iron is then redeposited in specific areas on the surface in the form of ferrihydrite(Lowenstam 1981).

In the synthesis of magnetite in vitro, careful control of the reaction conditions, particularly of pH, is needed because Fe(II) systems can give rise to goethite, ferrihydrite and/or magnetite/maghemite as well(Schwertmann and Cornell 1991). So it is not surprised to find that chitons synthesize magnetite whereas limpets synthesize goethite. Many minerals occur in a range of living organisms, extending from microorganisms to higher vertebrates. Reports in the literature list oxides and hydroxides including magnetite (Fe_3O_4), maghemite (?-Fe_2O_3), goethite (a-FeOOH), lepidocrocite (?-FeOOH), ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$) and amorphous ferrihydrates. Their relationships are shown in the following scheme(Mizota, Webb et al. 1998):

Fe^{3+} chelate ? Fe^{2+} ? Hydrous ferric oxide ? Fe_3O_4 ? ?-Fe_2O_3

Ferritin Transferrin Ferrihydrite Magnetite Maghemite

(Insoluble Iron) a-FeOOH

(Goethite)

ß-FeOOH

?-FeOOH

(Lepidocrocite)

There is undoubtedly a competition among the reactions that produce three different crystalline phases from ferrihydrite, but the modulators of this competition have not yet been identified(Mizota, Webb et al. 1998).

The ferrihydrite to goethite transformation involves dissolution and reprecipitation. The *in vitro* synthesis of iron oxide phases can indicate factors that affect the transformation among those phases(Schwertmann and Taylor 1989). Many chemical factors have in fact been reported to affect *in vitro*, hydrolysis and precipitation of Fe(**II**) and Fe(**III**) giving rise to different reaction products(Mizota, Webb et al. 1998).

Goethite may be synthesized from either Fe(**III**) or Fe(**II**) systems. The synthesis from Fe(**III**) systems involves holding a freshly precipitated ferrihydrite in KOH (pH13.5) at a temperature of 70°C for three days (Atkinson, Posner et al. 1968). Under alkaline conditions the precipitate dissolves to release soluble Fe(**III**) species ($Fe(OH)_4^-$) from which the less soluble goethite nucleates and grows(Schwertmann and Cornell 1991).

Synthesis from Fe(II) systems involves oxidative hydrolysis of Fe(II) solutions. The bioprecipitation of goethite rather than other iron oxides must be related to the thermodynamic and kinetic properties of the mineralization environment. Magnetite (Fe_3O_4) formation such as in chiton teeth(Towe and Lowenstam 1967)is probably inhibited owing to an unfavourable redox potential. Lepidocrocite (?-FeOOH) (also mineralized in some chiton teeth (Lowenstam 1967), although thermodynamically less stable than goethite, is often kinetically favored in precipitation reactions. Thus goethite formation is only favored when the rate of oxidation of Fe^{II} is slow, for example, in the presence of CO_2/HCO^-_3, (Schwertmann and Fitzpatrick 1977; Taylor and Schwertmann 1978) Al^{3+} (Taylor and Schwertmann 1978), and Mn^{2+}(Detournay, Ghodsi et al. 1975). Since the goethite particles are well ordered single crystals, the rate of crystallization must be slow and probably occurs by direct precipitation from solution species rather than from a green rust intermediate (which would favor lepidocrocite)(Mann, Perry et al. 1986).

There should be various types of biological and chemical control over iron biomineralization, and enzymes must play important roles to terminate in favorite minerals among the possible iron phases(Mizota, Webb et al. 1998).

Silica biomineralization-

The fixation of silica by marine organism, apparently in the form of opal (SiO_2. nH_2O)(Lowenstam 1971), is generally identified with certain unicellular algae, heterotrophs, and Protozoa and with cellular-grade

Metazda. They include the diatoms, some dinoflagellates, the silicoflagellates, ebridians, most radiolarians, the Hyalisporgisa, many Demospongia, and the coralline sponges(Vinogradov 1953).

In many organisms the soluble form of silica, i.e. silicic acid, $Si(OH)_4$, permeates the cell and is transported outward by metabolic process associated with the cell membranes(Simkiss and Wilbur 1989). The role of silica is critical in the structural integrity of the tooth since the mineral infiltrates space not occupied by the goethite phase and also impregnates it(Mann, Perry et al. 1986). It should also be noted that the presence of soluble Si can severely retard iron oxide crystallization(Schwertmann 1970; Quin, Long et al. 1986).

Insights for speculation of protein roles in biomineralization-

Three general mechanisms for biomineralization enunciated by Lowenstam(Lowenstam 1981) may provide additional insights:

(a) biologically induced mineralization where biology provides the conditions for mineralization, for example, by trans-membrane pumping of bicarbonate to yield a high local [CO_3^{2-}] concentration appropriate for extracellular calcification to occur.

(b) Biologically controlled mineralization where the biological system includes, for example, organized polymeric arrays on which nucleation and growth of the biomineral occurs, that is, the polymer "controls" the biological mineralization process.

(c) Facilitated assembly using foams, emulsions and vesicles whose templating roles produce complex but organized arrays of micro crystals

serve as materials.

Acidic macromolecules and biomineralization-

The presence of protein spotting (boxed in Figs.12-30, 36, 37) located at the most acidic part of the two-dimensional gel deserves some comment. This characteristic pattern was present in *C. toreuma* samples, three samples in *P. striata* including the whole radulae, radular sac, and stage **I**, and corresponding autoradiographic recording respectively. With the appearance they were stained by silver-stain, and the way they expand on the acrylamide gel. This implicates that these macromolecule are glyco-proteins with polysaccharide chains. Weiner (1986) in a review of five different types of mineralized tissue has noted that acidic macromolecules are always present. It has been suggested that in molluscan shells, for example, specific sites on acidic proteins act as nucleation centers for mineral formation (Mann 1988).

Implications of proteome analysis of SDS-page gel-

Interestingly, in the observation made by SDS-polyacrylamine gel analysis, the postulated protein ferritin (Figs. 5, 6, 7, 8) was found to decrease or disappear in later stages indicive of protein degradation. With the appearance in stage **II** and *de novo* synthesis starting at stage **II**, vanishing at stage**III** implies this protein is under precise control.

Although we can not point out the bands that standout during each different stage as single peptides or that they have certain specific functions

in SDS-polyacrylamine gel analysis, but in general we can see peptide pattern differences at different stages which indicate that the radulae are going through some changes and a part of these changes should be related to the biomineralization process. And with a more advanced technique, the two-dimensional electrophoresis, detail made available and is ready for further studies.

Limitations and improvement of experiments-

There are of course several limitations which arise in the course of the present study and which need to be discussed and addressed:

(a)The concentration of [^{35}S] Met being absorbed by the radular tissue was not easily controlled due to several factors: specimens were not uniform in their sampling. The portion of [^{35}S] Met being taken up by the radular tissue was not as readily accessible as in the case of confluent cell culture, and therefore, the [^{35}S] Met medium was not able to be equally absorbed by the different sections of the radular organ. Most of all, because of the nature of marine gastropods, due to tension or the maintenance of keeping homeostasis, body fluid, including [^{35}S] Met medium was simultaneously taken up and excreted from the limpet.

(b)The protein that appeared in each developmental stage might be related to the cell cycle since the growth of radulae is in a sequential fashion, there is no way we could distinguish the routine cell cycling protein expression and those specific to the mineralization process.

(c)By computer-assisted comparative analysis, only about 80% of the 2D

spots were matched to the modified reference gel. This might arise from the factor of unequal loading amounts of samples, and/or defects in IEF gel such as tiny air bubbles trapped in the gel and unavoidable stretching during equilibrating and transfer between the first and second dimension.

Due to these factors of uncertainty, the proteins that are related to the complex biomineralization process are probably far underestimated. The data presented so far were selected for the most notably stained spots observed on the gels and films. Protein below silver stain sensitivity displayed *de novo* synthesis as detected by ^{35}S autoradiography. Some effects were found to be stage specific with respect to the different developmental stages. However, all variables, such as relative protein amount (determined by silver staining), *de novo* synthesis (^{35}S label) could be recorded for each detectable protein of the different developmental stages and analyzed accordingly to those stages, and therefore a three dimensional set of data can be obtained, which may yield new mechanistic insights.

For a better proteome analysis result, a modified experiment procedure is required. First, the radioisotopic labeling method could be modified according to the suggestions in "The Protein Protocols Handbook(Walker 1996)." Subsequent to the dissection of the radulae, a brief wash in PBS could reduce the amount of contamination. Furthermore, separation of each stage as previously described. And finally the suggested placement of the tissues into 1mL of tissue-culture medium containing 10% DFCS, lacking methlionine but supplemented

with 200 μCi/mL [^{35}S] Met with a 5% CO_2/95% air mixture could help to standardize the amount of isotope to be taken up by the tissue. The samples would then be placed in a shaking water bath at 25° C, and labeled for 2h. Secondly, the loading amount of sample must be determined and controlled. Third, for a better reproducible protein spotting, and the scale of the pI value, replacement of IEF (isoelectric focusing) with IPG (immobilized pH gradients) strips is recommended. Finally, an improved silver-stain procedure which presents a better probe product (spotting), and thus eliminates the negatively staining by silver-stain.

Proteome analysis demonstrated that the biomineralization process, as mentioned before is a very complex phenomenon. It is associated with the environment of the cell, pH value, the minerals it deposits, kinetics, enzymes and peptides. The candidate proteins I pointed out in this thesis are just a tiny part of the solution, and therefore more work is needed. The detail of every deposition stage, the characteristics of the deposited minerals, the pH value in the compartment during the mineralization process. But most of all, subsequent to two-dimensional electrophoresis, sampled materials require further analysis by two-dimensional Western blots and mass spectrometry analysis of tryptic digests to specifically determine candidate peptides.

References

Atkinson, R. J., A. M. Posner, et al. (1968). "Crystal nucleation in Fe(**III**) solutions and hydroxide gels." J. inorg. nucl. Chem 3**0:** 2371-2381.

Detournay, J., M. Ghodsi, et al. (1975). "Influence de la temperature et de la presence des ions etrangers sur la cinetique et le mecanisme de formation de la goethite en milieus aqueux." Z. Anorg. Allg. Chem. 4**12:** 184-192.

Evans, L. A., D. J. Macey, et al. (1990). "Characterization and structure organization of the organic matrix of the radula teeth of the chiton A*canthopleura hirtosa*." Philosophical Transactions of the Royal Society B**329:** 87-96.

Evans, L. A., D. J. Macey, et al. (1991). "Distribution and composition of matrix protein in the radular teeth of the chiton A*canthopleura hirtosa*." Marine biology 1**09:** 281-286.

Evans, L. A., D. J. Macey, et al. (1992). "Calcium biomineralization in the radular teeth of the chiton, Acanthopleura hirtosa." Calcif Tissue Int 5**1(**1): 78-82.

Evans, L. A., D. J. Macey, et al. (1994). "Matrix heterogeneity in the radula teeth of the chiton A*canthopleura hirtosa*." Acta Zoologica 7**5:** 75-79.

Heukeshoven, J. and R. Dernick (1988). "Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels." Electrophoresis 9**(**1): 28-32.

Hochstrasser, D. F., M. G. Harrington, et al. (1988). "Methods for increasing the

resolution of two-dimensional protein electrophoresis." Anal Biochem 1**73(**2): 424-35.

Jones, E. I., R. A. McCance, et al. (1935). "The role of iron and silica in the structure of the radular teeth of certain marine molluscs." J. exp. Biol 1**2:** 59-64.

Kim, K.-S., D. J. Macey, et al. (1989). "Iron mineralization in the radula teeth of the chiton A*canthopleura hirtosa.*" Proceedings of the Royal Society B**237:** 335-346.

Kirschevink, J. L. and H. A. Lowenstam (1979). "Mineralization and magnetization of chiton teeth: Paleomagnetic, sedimentologic, and biologic implications of organic magnetite." Earth and Planetary Science Letters 4**4:** 193-204.

Lee, A. P., J. Webb, et al. (1998). "In situ raman spectroscopic studies of the teeth of the chiton A*canthopleura hirtosa.*" JBIC 3**:** 614-619.

Li, C. W., T. S. Chin, et al. (1989). "Growth of chiton teeth evidenced from magnetic measurement and microstructure characterization." IEE transactions on magnetics 2**5:** 3818-3820.

Lovenstam, H. A. (1962). "Gothite in radular teeth of recent marine gastropods." Science 1**37:** 279-280.

Lovenstam, H. A. (1967). "Lepidocrocite, an apatite mineral, and magnetite in teeth of chitons (Polypplacophora)." Science 1**56:** 1373-1375.

Lovenstam, H. A. (1971). "Opal preciptation by marine gastropods (Mollusca)." Science 1**71:** 487-490.

Lovenstam, H. A. (1981). "Minerals formed by organism." Science 2**11:** 1126-1131.

Lu, H. K., C. M. Huang, et al. (1995). "Translocation of ferritin and biomineralization of goethite in the radula of the limpet Cellana toreuma reeve." 2**19:** 137-45.

Mackenstedt, U. and K. Markel (1987). "Experimental and comparative morphology of radula renewal in pulmonates (Mollusca, Gastropoda)." Zoomorphology 1**07:** 209-239.

Mann, S., C. C. Perry, et al. (1986). "Structure, morphology, composition and organization of biogenic minerals in limpet teeth." Proc. R. Soc. Lond. B **227:** 179-1986.

Mizota, M., J. Webb, et al. (1998). "Mossbauer spectroscopic study on iron-containing biominerals in the limpet radula." J. Phys. S. J. 6**7:** 679-681.

Nesson, J. W. and W. R. Shiller (1968). "Taste thresholds to bitter compounds during a submarine patrol. Rep. No. 538." Rep US Nav Submar Med Cent 5**38:** 1-5.

Quin, T. G., G. J. Long, et al. (1986). "Study of the influence of silicon and phosphorus dopants on the structural and magnetic properties of synthetic goethites." Inorg. Chem.: In the press.

Rinkevich, B. (1986). "Does germanium interact with radular morphogenesis and biomineralization in the limpet Lottia gigantea?" Comp Biochem Physiol C 8**3(**1): 137-41.

Runham, N. W. (1961). "The histochemistry of the radula of P*atella vulgata*." Q. Jl microsc. Sci. 1**02:** 371-380.

Runham, N. W. (1963). "A study of the replacement mechanism of the pulmonate radula." Quarterly Journal of Microscopical Science 1**04:** 271-277.

Runham, N. W., P. R. Thornton, et al. (1969). "The mineralization and hardness of the radular teeth of the limpet Patella vulgata L." Z Zellforsch Mikrosk Anat 9**9(**4): 608-26.

Schwertmann, U. (1970). "Der Einfluss einfacher organischer Anionen auf die Bildung von Goethite und Hamatit aus Amorphem Fe3 Hydroxid." Geoderma 3**:** 207-213.

Schwertmann, U. and R. M. Cornell (1991). Iron Oxides in the Laboratory. Weinheim, Vch Pub.

Schwertmann, U. and R. W. Fitzpatrick (1977). "Occurence of lepidocrocite and its association with goethite in Natal soils." Soil Sci. Soc. Am. J 4**1:** 1013-1018.

Schwertmann, U. and R. M. Taylor (1989). Minerals in Soil Environments. Madison, U.S.A, Soil Science Society of America.

Simkiss, K. and K. M. Wilbur (1989). Biomineralization: Cell biology and mineral deposition. San Diego, C.A, Academic press.

Steneck, R. S. and L. Walting (1982). "Feeding capabilities and limitation of herbivorous molluscs: A functional group approach." Mar. Biol. 6**8:** 299-319.

Taylor, R. M. and U. Schwertmann (1978). "The influence of aluminium on iron oxides. Part 1. The influence of A l on Fe oxide formation from the Fe(II) system." Clays Clay Miner 26: 373-383.

Towe, K. M. and H. A. Lowenstam (1967). "Ultrastructure and development of iron mineralization in the radular teeth of Cryptochiton stelleri (Mollusca)." J. ultrastruct. Res 17: 1-13.

Vinogradov, A. P. (1953). The Elementary Chemical Composition of Marine Organisms. New Haven, Conn., Yale Univ. Press.

Walker, J. M. (1996). The protein protocols handbook. Totowa, New Jersey, Human Press.

Webb, J., L. R. Brooker, et al. (1997). "Studies of biominerals and related novel nanoscale composite materials." S. Afr. J. Chem.: 195-202.

Webb, J., D. J. Macey, et al. (1989). Biomineralization of iron in molluscan teeth. Weinheim, VHC Verlagsgesellschaft.

Figure Legends