INTRODUCTION

Biominerals are the inorganic phases of minerals found in biological systems, which are distributed from microorganisms to the highest animals and plants (Mann *et al.* 1989, Simkiss and Wilbur 1989, Weiner and Lowenstam 1989, Mann 2001). Although minerals composed of calcium predominate, biominerals of barium, strontium, iron, and silica are also well known. Biominerals generally appear in the formula containing oxides, hydroxides, phosphates, and carbonates derivatives (Webb *et al.* 2001).

Biosilicification, the biological formation of opal-like amorphous hydrated silica, occurs in a wide variety of organisms including diatoms, sponges, mollusks, and higher plants (Voronkov *et al*. 1977, Lowenstam 1981, Simpson and Volcani 1981). Among various organisms with the capability to deposit silica, diatoms and sponges are the most thoroughly investigated taxa. In the study of diatoms, species-specific polypeptides with molecular weight ranging from 25 kDa to 3.5 kDa isolated from *Cylindrotheca fusiformis* were identified to co-precipitate with amorphous silica during the cell wall formation process (Kröger *et al.* 1997 1999). These polypeptides are so tightly associated with the amorphous silica that they can only be extracted after dissolution of the cell wall in hydrogen fluoride (HF) to remove the silica. The HF-extractable polypeptides were named as silaffins and were demonstrated to act as regulating molecules during biosilica formation in diatoms (Kröger *et al.* 1999 2000 2001). Later studies showed that other than the polyamine moieties, native silaffins are also heavily phosphorylated (Kröger *et al.* 2002). All 7 serine residues found in native silaffin-$1A_1$ are phosphorylated, and this high level of phosphorylation is essential for silaffins' ability to precipitate silica in ambient conditions as well as to regulate the activities of

silica-forming biomolecules (Kröger *et al.* 2002, Poulsen *et al.* 2003). A phase-separation model was proposed to account for the self-similar silica patterns observed in diatom cell walls based on the knowledge obtained in the study of silaffin molecules (Sumper 2002).

Siliceous sponges deposit silica in needle-like spicules that support the organism and provide defense against predation. Each spicule contains a proteinaceous axial filament, later named silicatein, which serves as a template or otherwise directs silica deposition (Shimizu *et al.* 1998, Uriz *et al.* 2000). Protein analysis of silicateins of several different sponge species indicated that silicatein sequences are conserved across sponge species, and the sequences are similar to those of the cathepsin L subfamily of papain-like cysteine proteases (Shimizu *et al.* 1998, Cha *et al.* 1999). Identities include the six cysteine residues that form intramolecular disulfide bridges in the protease, making it likely that the three-dimensional structure of silicatein α is similar to that of cathepsin L. Clusters of the hydroxy amino acids, serine, tyrosine, and to a lesser extent, threonine, constitute one of the most distinctive features of silicatein α. Serine-rich sequences including Ser-Ser-Cys-Thr-Tyr, Ser-Ser-Arg-Cys-Ser-Ser-Ser-Ser, two Ser-Xaa-Ser-Xaa-Ser sequences, and the Ser-Tyr sequence at the site of the serine-replaced active site of the protease are notable examples of the high localized concentrations of hydroxyls in this protein. Cathepsin L is a typical lysosomal proteolytic enzyme in both human and sponge tissues, whereas silicatein α is presumably localized in the silicalemma, which forms the membrane-enclosed vesicle in which silica deposition occurs in the sponge sclerocytes. The relationship between these proteins thus apparently includes the nature of their subcellular localizations as well as their molecular structures, further supporting the suggestion of a common evolutionary ancestry. Interestingly, the catalytic cysteine (sulfhydryl) residue at

the active site of the proteases is replaced in silicatein α with a serine (hydroxyl). Consistent with this replacement, the silicateins do not display esterase activity when tested with synthetic chromogenic substrates. The gene encoding silicatein has been cloned in some species, and how it coordinates and functions in biosilicification processes in sponges have been unraveled (Krasko *et al.* 2000, Műller *et al.* 2003, Bavestrello *et al.* 2003, Werner *et al.* 2003, Weaver and Morse 2003, Pozzolini *et al.* 2005).

In mollusks such as limpets and chitons, silica deposits are found in radulae, feeding organs of mollusks, where it is co-precipitated with iron in the construction of an efficient feeding apparatus (Mann *et al.* 1986, Liddiard *et al.* 2004). Limpets are common organisms that can be found in intertidal regions throughout the world. Previous studies focusing on the radulae of the limpet species, *Patella vulgata* (Runham and Thronton 1967, Mann *et al.* 1986) demonstrated that limpet radulae consist of a continuous series of teeth in various stages of mineralization, ranging from soft, unmineralized structures through fully mineralized teeth, which makes them ideal experimental organisms to investigate the mechanisms of mineral deposition. Various elemental analyses carried out on *Patella vulgate* revealed that the major teeth contain up to 12% ferric oxide in the form of goethite (α-FeOOH), 7%-16% silica as hydrated amorphous opal ($SiO_2 \cdot nH_2O$), and small amounts of calcium other than the organic components (Jones *et al.* 1935, Lowenstam 1962, Runham *et al.* 1969, Grime *et al.* 1985, Mann *et al.* 1986). Later studies concentrating on *Patelloida alticostata*, *Patella peronii* (Burford *et al.* 1986), and *Cellana toreuma* (Lu *et al.* 1995) indicated that the deposition of Fe is probably mediated by ferritin translocation. Furthermore, more than fifty thousand proteins related to iron transportation were identified and reported, but studies focusing on the mechanisms of silica deposition are relatively

scarce. Although silica is known to play essential roles in tooth growth and maintaining radular structural integrity in limpets (Mann *et al.* 1986), the process by which it infiltrates into the organic matrix and through what mechanism it is deposited remain elusive. To further understand the molecular mechanisms controlling biosilicification, it is rational to begin with characterization of the biomolecules encapsulated within biologically silicified structures. Studies of these various structures such as diatom cell walls and sponge spicules have been directed towards discovering the underlying principles of the micro-architecture within the mineralized tissues, including the relationships between the inorganic phase and the organic matrix of protein or polysaccharides. The radulae are especially attractive in efforts to unravel silica deposition mechanisms because they contain teeth at various mineralization stages from unmineralized soft tissue to fully mineralized complex teeth. Studies of such series of teeth at different mineralization stages may provide insights into the processes and mechanisms of silica deposition.

In this study, the limpet, *Notoacmea schrenckii,* was selected as the experimental material. This particular species belongs to the Phylum Mollusca, Class Gastropoda, Order Archaeogastropoda, and Family Acmaeidae. The radulae of *N. schrenckii* can be segmented into four stages according to the extent of mineralization based on our observations under stereomicroscope. Stage I is composed of the odontoblast and an organic framework of tooth that grows from the odontoblast, no mineral deposition is observed at this stage. Cusps at the onset of mineral deposition turn light orange, and these rows of teeth are defined as in stage II. In stage III, the mineral deposition of teeth is almost complete, and the cusps in this stage are solid and firm, and exhibit a brownish color; while in stage IV, mineral deposition of the teeth is complete and the teeth are ready to

replace worn-out ones.

In situ analysis utilizing electron microscopy and inductively coupled plasma mass spectrometer (ICP-MS) with the radulae of the limpet *N. schrenckii* may provide clues to decipher the distribution of silica in limpet teeth and a possible time frame for silica uptake in radula maturation. Possible re-deposition of amorphous silica spheres after the radulae being subjected to ammonium fluoride treatment and subsequent addition of silicate suggests that the biomolecules which induce silica deposition can be released from the mineral, which in turn guide silica precipitation repetitively if sufficient ambient silicate is supplied.