MATERIALS and METHODS

1. Collection of limpet specimen and isolation of radulae

   N. schrenckii specimens were collected at low tide from the intertidal zone of Nan Liao Fishing Port, Hsinchu, Taiwan. Following collection, animals were placed in fresh sea water and transported back to the laboratory immediately. Radulae were dissected out under a stereomicroscope and treated with 3% collagenase (Sigma) in 100 mM phosphate-buffered saline (PBS), pH 7.2, 25 ^{o}C for 3 hrs to remove the surrounding epithelial cells. After the digestion process, radulae were washed with ddH_2O and were then positioned individually between glass slides to keep flat for further processing. Cells were collected by centrifugation at 150 g, 15 min at 25 ^{o}C for further analysis.

2. Optical Microscopy (OM)

   2.1 The radulae together with the surrounding epithelial cells

   Radulae dissected out under a stereomicroscope were washed with 20 mM PBS containing 5% sucrose (by weight) twice and stained with Prussian blue for the presence of Fe^{3+}.

   2.2 Histological sections of the radulae

   Radulae dissected out under a stereomicroscope and segmented according to the mineralization stages were washed with 20 mM PBS containing 5% sucrose (by weight) twice and fixed with a mixture containing 2.5% (by volume) glutaraldehyde and 4% para-formaldehyde (EM grade) in 100 mM PBS at pH 7.2 for 1 hr and washed three times with the same PBS buffer. Serial dehydration with ethanol concentrations in 30%, 45%, 60%, 75%, 90%, and 100% were performed. Ethanol was replaced by xylene before the radulae were embedded into paraffin. The paraffin blocks were

trimmed and sections of approximately 5 μm were obtained with a Leica rotary microtome. The sections were stained with both conventional haematoxylin and Eosin-Y stains as well as Prussian blue for the presence of Fe^{3+} before observation under an optical microscope.

3. Scanning electron microcopy (SEM) and energy dispersive spectroscopy (EDS) elemental analysis

   Cleaned radulae were fixed in the mixture containing 2.5% (by volume) glutaraldehyde and 4% para-formaldehyde (EM grade) in 100 mM PBS at pH 7.2 for 1 hr, and washed three times with the same buffer. Radulae were then fully dehydrated through a gradient of alcohol. Radulae destined for morphological observation were then dried with critical point drying (CPD), mounted on stubs, and sputter-coated with gold. Radulae assigned for energy dispersive spectroscopy (EDS) were roughly sectioned into lengths ideal for analysis and embedded in Spurrs' resin blocks after serial alcohol dehydration. The blocks were ground and polished so that the teeth can be presented longitudinally on a flat surface and the exposed surfaces were sputter-coated with gold. SEM observation was carried out using a Hitachi S-4700 type II scanning electron microscope. The EDS analysis was performed using Horiba EMAX-ENERGY EX-300 model.

4. Transmission electron microscopy (TEM) and EDS elemental analysis

   Fresh radulae were separated into 4 fragments consistent with the four stages of mineralization. Radulae were fixed with a mixture containing 2.5% glutaraldehyde and 4% para-formaldehyde in 100 mM PBS, pH 7.2 at 25 ^{o}C for 1 hr, washed with 100 mM PBS three times, then post-fixed with 2% osmium tetraoxide for 30 min. Dehydration with continuous ethanol gradient was performed immediately after fixation and the radulae were then

flat-embedded in Spurrs' resin. Both longitudinal sections and cross sections of the radulae in 90-100 nm thick were obtained using Leica Ultracut R ultramicrotome. Sections were mounted on 3 mm nickel grids and post-stained with uranyl acetate and lead citrate. Transmission electron micrographs were obtained utilizing a Hitachi H-7500 electron microscopy operating at 100 keV. TEM EDS analysis was performed on a Joel JEM-2010 analytical transmission electron microscope operating at 150 keV.

5. Inductively coupled plasma-mass spectrometer (ICP-MS)

Radulae sectioned based on the mineralization extent were treated with 3% collagenase to remove surrounding epithelial cells. Cells were collected by centrifugation at 150 g for 15 min, both cells and radula were digested with 67% HNO_3 for 3 days. Silica and iron contents in soluble state after the HNO_3 digestion process were measured using a Perkin Elmer SCIEX ELAN 5000 inductively coupled plasma-mass spectrometer.

6. Extraction of peptides possibly involve in silica precipitation

Three different groups of peptides were collected based on different initial extraction sources.

6.1 Peptides extracted from radulae with surrounding epithelial cells attached

Radulae freshly dissected from the organisms with the surrounding epithelial cells remained were homogenized in 20 mM PBS with a hand-held homogenizer. After centrifugation at 70 g for 10 min at 25 ^{o}C, the supernatant was collected and lypholized. 1% SDS/10 mM Tris-HCl buffer at pH 6.8 was added to the freeze-dried sample and the mixture was boiled for 10 min. The resulting solution was subjected to dialysis and lypholization before further analysis.

6.2 Extraction of peptides from the surrounding epithelial cells

Dissected radulae were treated with 3% collagenase at 25 ^{o}C for 3 hrs to remove epithelial cells surrounding the radulae. The cells were collected by centrifugation at 150 g, 15 min at 25 ^{o}C. The collected cells were homogenized in 20 mM PBS and the supernatants were collected by centrifugation at 150 g, 25 ^{o}C for 15 min, lyhpholized, extracted with 1% SDS/10 mM Tris-HCl buffer, pH 6.8, and finally boiled for 10 min. Dialysis was carried out using Spectrum Dialysis tubing with MWCO of 6000 to 8000 Da in ddH_2O overnight at 4^{o}C. The resulting solution was then lypholized before further analysis.

6.3 Extraction of peptides within the cusps

Radulae free of the surrounding epithelial cells were either washed twice with 20 mM PBS to remove any residues and air-dried, or treated with 65% HNO_3/98% H_2SO_4 (v/v) to yield solely the cusps before the solution of 4 M HF buffered with 8 M NH_4F was applied. The application of HF disrupted any structures containing silica deposited in the cusp and base formed during the teeth formation process so that the peptides co-precipitated within the mineral complex can be released. The radulae and the cusps were allowed to react at 25 ^{o}C for 3 days and the samples were dialyzed in ddH_2O and lypholyzed for further analysis.

7. Electrophoresis

Peptides obtained from step 6 were analyzed with SDS-polyacylamide gel using Bio-Rad Miniprotein II vertical electrophoresis system. The concentration of stacking gels was 10% and the resolving gels were either 15% or 20%. The electrophoresis was performed under fixed voltage, that is, 80 V for running the stacking gel and 100 V for running the resolving gel.

The gels were stained with either silver stain or Coomassie Blue.

8. Amino acid composition analysis

   Peptides obtained from either cusps or surrounding epithelial cells were transferred to PVDF membrane after electrophoresis. Peptides on the PVDF membrane were solvated with 50% acetonitrile containing 0.1% trifluoroacetic acid for amino acid composition with Pico.Tag Amino Acid Analyzer System (Waters Amino Acid Analyzer).

9. Generation of antibodies

   Peptides extracted from the cusps with molecular weight of 16 kDa and 18 kDa were collected using 1.5 mm electrophoretic gels, with a total yield of approximately 2 to 10 mg. The generation of polyclonal antibodies was entrusted to MDBio, Inc. (http://www.mdbio.com.tw/) with the colleted peptides using rabbits; therefore, the antibodies produced were rabbit anti-16 kDa and rabbit anti-18 kDa antibodies.

10. Western blotting

    After electrophoresis, peptides were transferred from 0.75 mm gel to the PVDF membrane at 4 ^{o}C, 80 V for 2 hrs. Blocking was performed with 3% gelatin at 25 ^{o}C for 1 hr and then washed with TTBS buffer for 5 min three times. Primary antibodies were then applied at 25 ^{o}C and incubated for 1 hr. After washed with the TTBS buffer 3 times, secondary antibody diluted in the ratio of 1:2000 was added at 25 ^{o}C and incubated for 1 hr. Excess secondary antibodies were removed by washing the membrane with TTBS 3 times and with TBS once before rinsed with alkaline phosphatase developing buffer. Alkaline phosphatase developing reagent was then applied for color development for 30 min.

11. Silica re-deposition experiment

Freshly dissected radulae treated with 3% collagenase for 1 hr at 25 ^{o}C to remove the surrounding epithelial cells. After washing with PBS, the radulae were divided into four experimental groups. The first group was treated with 10 M NH_4F for 30 min at 25 ^{o}C to gently remove the outermost layer of silica deposits. Radulae were then washed with 1 M sodium acetate (NaOAc) so the pH value was adjusted to 5.0, and followed by the addition of 1 M tetramethyl orthosilicate (TMOS), which served as the supplement of silica source, at 25 ^{o}C for 5 min before wash with NaOAc buffer again. The radulae were then treated with 10 M NH_4F for 30 min again. The second group was treated the same way as the first one except that the final NH_4F treatment was omitted. The third experimental group was treated only with 10 M NH_4F, while the fourth remained intact after removal of the surrounding epithelial cells. All four groups were then subjected to fixation, serial dehydration and critical point drying processes before observed with SEM. The SEM images were all acquired from teeth of stage III of the radulae.