RESULTS

1. Morphology observation

1.1 OM observation

1.1a Anatomy

N. schrenckii has a one-piece shell shaped like a wide-brimmed hat and can often be found adhered to the rocks in the intertidal area. They graze the algae or diatoms grown on the rock with the radulae. Previous studies suggested that limpet radula can be segmented into four stages according to the extent of mineralization (Mann *et al.* 1986), and the same principle was applied in this study. Specifically, stage I refers to the segment constituted by the odontoblast and the unmineralized teeth, stage II refers to the segment undergoes rapid iron deposition and exhibits an orange to reddish color in both the radulae and the surrounding epithelial cells, stage III refers to the segment where teeth come to maturation to give a brownish color, and stage IV refers to the segment with fully matured teeth and those teeth in immediate use. Part of the radula could be readily observed once the abdominal foot was removed; however, the major part of the radula was exposed after the removal of either the ovary or seminal vesicle, depending on the gender of the individual (Fig. 1).

The odontoblast, from which the new, unmineralized teeth emerged, could be clearly identified when the whole radula is removed from the limpet (Fig. 1B, 2A). The teeth could be divided into two parts, the cusps refer to the pointed, sickle-shaped part, which were responsible for scraping during the feeding process, and the bases are where the cusps stand. Since the direct staining method for silica is not available, Pussian Blue was selected to

detect iron so the approximate initiation stage of mineralization can be determined. The junction zone refers to the interface between the cusps and the bases which can be readily identified under a stereomicroscope once stained with Prussian Blue. At stage II, both the unstained cusps and the surrounding epithelial cells showed a reddish-orange color (Fig. 1B), after staining with the Prussian Blue, this particular region turned blue, indicating the existence of abundant iron ions (Fe^{3+}) (Fig. 2B, 2C). From stage II on, the junctions between cusp and base were stained blue but the color of the epithelial cells gradually faded as the extent of maturation increases (Fig. 2D, 2E). When stage II cusps were pierced off following the Prussian Blue staining process and the exposed surfaces exhibited an extensive blue color, revealing a concentrated iron reservoir (Fig. 2F)

1.1b Histological sections

Histological sections ranging from 5 to 7 μm were obtained using a rotary microtome. Images of different mineralization stages of the radula along with the surrounding epithelial cells from the radular gland stained with Prussian blue are shown in Figures 3. In addition to the Prussian Blue stain, sections were also subjected to conventional haematoxylin/Eosin-Y staining. The newly formed stage I teeth were stained red but exhibited no change in color after Prussian Blue staining, suggesting that they were composed by organic materials and no iron influx occurred at this stage (Fig. 3A). Epithelial cells, denoted by arrow heads, and the bases, denoted by the arrows, of early stage II were stained blue (Fig. 3B, 3C, 3D), indicating the existence of amorphous Fe^{3+}. At later stages, as cusps a further mineralized, the influx of iron declined and was finally absent in epithelial cells in stages III and IV; nevertheless, traces of amorphous Fe^{3+} ions ciuld

still be found in bases (Fig. 3E, 3F).

1.2 SEM observation and EDS analysis

Radulae of *N. schrenckii* were composed of about 150 transverse rows of teeth, each row could be divided into two parts, and each part was constituted by cusp and base. The cusp consisted of two major lateral teeth sharing a single base and flanked by a small marginal tooth, which could be clearly observed under SEM (Fig. 4A, 4B). The scraping surface of the major teeth was denoted as the posterior end while the other surface was defined as the anterior end (Fig. 4A). Energy disperse X-ray mapping of polished tooth embedded in resin showed that silica was the major component of base and part of the cusp (Fig. 4C to 4E) while iron predominantly located in both posterior and anterior tips of cusp. Line profile of polished tooth from stage II was shown in Figure 4F. It revealed that the distribution of silica, the blue curve in the figure, extended from tooth base to the cusp, and the base was basically composed of silica, while iron, the red curve in Fig. 4F, was not detected in base but relatively abundant in cusp. In addition, the overlapping of silica and iron curves in the cusp region suggested that the major scraping surface was strengthened by silica and iron complexes.

1.3 TEM observation and EDS analysis

Sections of immature, unmineralized stage I radula, together with the odontoblast and the surrounding epithelial cells, revealed the arrangement of internal fibrous organic matrix as well as the relationship between adjacent cells and the radula (Figure 5). The rudiments of teeth, comprised of orderly arranged organic skeleton, appeared quite early as in the odontoblast (Fig. 5A). Some microvilli were found adjacent to the posterior region of

the tooth cusp, which might involve in part in the communication between the radula and the adjacent epithelial cells (Fig. 5A). Minerals were absent during this early stage. Junction zone, a band-like region separateing base and cusp, was also evident in this early stage, yet whether minerals enter this region at this stage is not determined (Fig. 5B). Vesicular transportation was found in the region adjacent to the junction zone as well as the anterior side of the cusp, indicating possible means of mineral transport (Fig. 5C to 5E).

In early stage III, granules with approximately 100 nm in diameter were observed at the base (Fig.6A). Further magnification showed that there were bundles of filaments measured about 2 nm in width penetrate the granules, which might be the organic skeleton synthesized by the odontoblast (Fig. 6B). Energy-disperse X-ray analyses were carried out on the particles as well as tooth base and junction zone. The results were showed in figure 7. In Fig. 7A, the element analysis of the junction zone showed that the major composition of this area was iron, while small amount of silica was also detected. The signals of copper and nickel came from the sample grid and the specimen holder. Diffraction analysis further indicated that iron detected in junction zone region was deposited in an amorphous form (Fig. 8). Tooth base, on the other hand, was composed primarily of silica as the prominent silica peak evident in the elemental analysis diagram (Fig. 7B). EDX analysis was also performed on the granules observed in Figure 6B and the result showed that they were silica granules (Fig. 7C)

2 ICP-MS Analysis

Results of elemental analysis utilizing ICP-MS were shown in Figure 9. The soluble iron level exhibited a dramatic increase in stage II both in the radula

and epithelial cells, in consistent with that of Prussian blue staining in optical microscope observation, in which both the epithelial cells and the radula exhibited prominently blue in stage II. In stage III, the level of both soluble silica and iron dropped in epithelial cells (Fig. 9A) but remained relatively constant in radula through stage III and IV (Fig. 9B). The amount of soluble silica in radula declined rapidly during the transition of stage I to stage II and remained relatively stable through stage III and stage IV (Fig. 9B).

3 Results on peptide analysis

3.1 Identification of low molecular weight peptides

Peptides co-precipitated along with the minerals within the cusps during the teeth forming process were extracted with 4M HF/8M NH_4F. Those harvested were analyzed by 15% SDS-PAGE, after silver staining, THE peptides with molecular weight ranging from 15 to 20 kDa could be identified (Fig. 10A). Three major bands with molecular weight of 18 kDa, 17 kDa, and 16 kDa, respectively, could be identified with silver stain after electrophoresis. However, the amount of peptides obtained solely from the cusps was too scarce for further analysis, the possibility that the same species of peptides might exist in the surrounding epithelial cells was exploited. Peptides in similar molecular weight were obtained from the epithelial cells covering the radula after the cells were treated with boiling SDS (Fig. 10B), but peptides smaller than 16 kDa were lost probably due to the boiling process. Almost identical group of peptides and the two dominant peptides could also be obtained from the intact radulae, with surrounding epithelial cells remained, were physically ground and extracted with SDS-boiling method (Fig. 10C). The result of electrophoresis exhibited a rather complex pattern, which was not ideal for subsequent

collection of the target peptides.

3.2 Amino acid sequence and composition analysis

After electrophoresis, two major bands with molecular weight of 18 kDa and 16 kDa were selected and submitted for sequence analysis, however, no conclusive results could be obtained, possibly due to *N*-terminal blocking of the target peptides.

The same peptides were also submitted for amino acid composition analysis and the data yielded (Table 1) was analyzed with AACompIdent of SWISS-PROT. The compositions of the peptides of interest were found to be related to mitochondrial deoxynucleotide carriers in the database (Table 2, Table 3).

3.3 Western Blotting

Two dominant peptides with molecular weight of 16 kDa and 18 kDa identified from electrophoresis were selected for antibody generation. The primary antibody produced by MDBio, Inc. was used in Western blotting to identify whether the same species of peptides also existed in the epithelial cells surrounding the radulae. In the case of crude extract of the epithelial cells, both bands corresponding to 18 kDa and 16 kDa peptides were identified by the antibodies. Intriguingly, several minor peptides with molecular weight ranging between 18 kDa and 16 kDa could also be recognized by the antibodies (Fig. 11A). Peptides extracted either from SDS-boiling of the intact radulae or the surrounding epithelial cells or HF-treated cusps could all be recognized by the anti-18 kDa antibody and anti-16 kDa antibody (Fig. 11B).

4 Results of silica re-precipitation experiment

SEM image of control experiment, the native radula without any treatment

was shown in Fig. 12A. The surface of the cusp seemed to compose of small granules in the size ranging from 40 nm to about 110 nm aligned together. In the experiment set where radulae were sequentially treated with NH_4F, tetramethyl orthosilicate (TMOS), the morphology of cusps' surface appeared as if the granules observed in Fig 12A were fused and grown during the experimental processes (Fig. 12C). In SEM image of experiment set where radulae were sequentially treated with NH_4F, tetramethyl orthosilicate (TMOS) and then NH_4F again, a few granules in the size of about 150 nm aggregated on the cusp surface were observed and most of the exposed cusp surface displayed a porous morphology (Fig. 12D). The EDS analysis indicated silica as the major component of the granules. SEM image of radulae treated only with NH_4F to remove the surface silica possessed similar granule alignment pattern as that observed in the control experiment (Fig. 12B). However, in this experiment set, the granules appeared to become smaller than those observed in Fig. 12A and the surface appeared to have been eroded and holes can be readily seen.