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In situ Studies of Biomineral Deposition in the Radula Teeth of Chitons of the Suborder Chitonina

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Abstract: The major lateral radula teeth of chitons (Mollusca: Polyplacophora) are composite materials, incorporating a variety of biominerals within an organic scaffold. While magnetite is ubiquitous to these teeth in all Polyplacophorans whose radulae have been described to date, this is not the case for the biominerals of the tooth core. *In situ* analsysis, using energy dispersive spectroscopy, to determine the distribution of elements in chiton teeth, and Raman spectroscopy, to identify the biominerals present, has been undertaken in the mature teeth of seven chiton species representing three families in the suborder Chitonina. The results show the tooth core to be comprised of a variety of elements, with the main biominerals identified as limonite, lepidocrocite and hydroxyapatite. Along with *Ischnochiton australis*, all five representatives of the Chitonidae deposit an apatitic mineral in their tooth core, while *Plaxiphora albida* does not deposit any calcium biomineral. With the exception of *Acanthopleura echinata*, the hydrated iron (III) oxide, limonite, is found in all species, including *I. australis*, which has relatively small amounts of iron in its tooth core. The lack of any evidence for a phosphate mineral in species that possess high levels of phosphorus in their core, challenges the long accepted notion that the presence of phosphorus implies its deposition as a biomineral. The combined techniques of energy dispersive spectroscopy and Raman spectroscopy provide a simple and effective means to evaluate, *in situ*, the biomineralisation strategies employed by chitons. While the results from this study are inconclusive in determining whether biomineralisation strategies reflect phylogenetic affinities in chitons, an extension of the study to include a wider range of chiton taxa could provide a basis for the utilisation of radula tooth biomineralisation as a systematic tool in this class of molluscs.

Keywords: biomineralisation, systematics, mollusc, iron, calcium

Introduction

The continuous nature of tooth development and replacement in the chiton radula presents a unique opportunity to study, *in situ*, the complexities of the biomineralisation processes in these structures. While numerous studies have investigated the external magnetite layer of the major lateral radula teeth (see for example: Lowenstam, 1962, 1967; Kim *et al.,* 1989; van der Wal *et al.,* 1989; Webb *et al*., 1991; Numako *et al.,* 1995; Numako & Nakai, 1997; Brooker *et al.,* 2003), relatively few have considered the mineralisation processes in the interior tooth regions (Lowenstam & Weiner 1985; Evans *et al.* 1992; Evans & Alvarez, 1999; Lee *et al.,* 2000, 2003a). This is, in part, due to difficulties associated with sectioning heterogenous material that is comprised of regions of varying hardness. As a consequence, the analysis of the biominerals

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present in chiton teeth has often been limited to *ex-situ* bulk analysis (by X-ray diffraction or infrared spectroscopy), of pooled material, gathered from many animals (Lowenstam, 1962; Lowenstam & Weiner, 1985). The use of these methods introduces serious limitations to the interpretation of results. For example, the extraction processes have the potential to induce significant chemical changes in the biominerals. In addition, the pooling of crushed material obscures any fine structural differences that exist between successive tooth rows. Finally, the separation of the inorganic component from the organic matrix compromises the chemical integrity of the organics, and destroys the interface between the two components of these composite biomaterials (Lee *et al.,* 2000).

A variety of phosphate minerals have been reported to comprise the anterior region, or internal core (depending on the extent of magnetite distribution), of the major lateral teeth of chitons (Lowenstam & Weiner, 1985; Evans *et al.,* 1992; Evans & Alvarez, 1999). In many species this region is suggested to be comprised primarily of calcium phosphate materials such as: a carbonated fluorapatite, francolite ($Ca_5(PO_4)_3F$), in *Acanthopleura echinata* (Lowenstam, 1967); a carbonated hydroxyapatite, dahllite ($Ca_5(PO_4,CO_3)_3OH$), in *A. haddoni* (Lowenstam & Weiner, 1985); a carbonate substituted apatite in *Chiton pelliserpentis* [= *Sypharochiton pelliserpentis*] (Evans & Alvarez, 1999); or a carbonate and fluoride substituted apatite material as in *A. hirtosa* (Evans *et al.,* 1992). In contrast, the tooth core of other species has been found to contain varying amounts of iron, in addition to phosphorous and calcium, and it has generally been accepted that they deposit only a hydrated ferric phosphate (Lowenstam, 1972; Lowenstam & Rossman, 1972; Brooker & Macey, 2001). Lowenstam & Weiner (1989) reported that the tooth core of *Cryptochiton stelleri* contains amorphous hydrous ferrous phosphate and opal. An iron (III) phosphate in association with small amounts of calcium was suggested as being present in the teeth of *Cryptoplax striata* (Macey and Brooker, 1996). Through *in situ* investigations into the biomineralisation in a number of chiton species, we have recently challenged the assertion that hydrated ferric phosphate is the primary component of the tooth core of species that do not possess an apatitic mineral. For example, in the tooth core of *Plaxiphora albida*, the hydrated iron (III) oxide limonite has been identified as the major constituent (Lee *et al.,* 2003a), a mineral that is also found in the teeth of *A. rehderi*, *A. curtisiana* and *Onithochiton quercinus* (Lee *et al.,* 2003b).

Clearly, there is considerable uncertainty regarding the composition of biominerals in the core region of chiton teeth. This study, which deals with some representative genera from the suborder Chitonina, is thus the first of a series that will investigate the variation in core composition of representative polyplacophoran taxa, with the ultimate aim of determining whether tooth core biomineralisation can be used as a viable taxonomic character.

The analytical techniques developed by our team using laser Raman microscopy provide the opportunity to undertake rapid *in situ* investigations into the biomaterial deposits in micro domains of individual chiton teeth that are rich in a wide variety of elements. Furthermore, they facilitate the characterisation of the complex composite biomaterials that are present (Lee *et al.,* 1998, 2000, 2003a, 2003b; Brooker & Macey, 2001; Brooker *et al.,* 2003). Hence, these studies also have the potential to answer additional fundamental questions concerning the formation of iron, phosphorus and calcium containing biomaterials in the natural world.

Materials and Methods

A single specimen from each of seven species of chiton, representing six genera and three families of the suborder Chitonina was utilised in this study (Table 1), with all animals collected from intertidal rocky habitats. Detailed descriptions of the general method of radula preparation and the specific preparation of the radulae examined in this study have been reported previously

Table 1. Specimen details of seven species of chiton (Polyplacophora: Chitonina) utilised in the study.

Family	Species	Collection site
Ischnochitonidae	*Ischnochiton australis*	Bathers Beach, Ottway Range, Victoria, Australia
Mopaliidae	*Plaxiphora albida*	Bathers Beach, Ottway Range, Victoria, Australia
Chitonidae	*Chiton mamoratus*	Florida Keys, USA
Chitonidae	*Sypharochiton pelliserpentis*	Botany Bay, Sydney, NSW, Australia
Chitonidae	*Acanthopleura echinata*	Quintay, Santiago, Chile
Chitonidae	*Acanthopleura miles*	Dampier Archipelago, Western Australia
Chitonidae	*Onithochiton quercinus*	Rottnest Id., W. Australia

(Evans *et al*., 1992; Macey & Brooker, 1996; Lee *et al.,* 1998). Briefly, radulae were dissected out of freshly sacrificed animals and rinsed in 5% w/v NaOCl prior to overnight fixation in a solution of 3% glutaraldehyde in filtered seawater. Straightened and immobilised radulae were examined under an Olympus ZH10 research microscope, prior to being dehydrated and embedded in Epon-Araldite. The resultant resin blocks were subsequently trimmed and re-embedded, such that radulae were presented in longitudinal section. Satisfactory blocks were then ground using progressively finer grades of silicon carbide paper, to P2000 for Raman spectroscopy and to P4000, with subsequent polishing (Dialux© jewellers paste), for energy dispersive spectroscopy (EDS) analysis. The blocks were cleaned between successive grades by sonication in fresh distilled water, and carbon coated prior to examination in a Philips XL30 scanning electron microscope (SEM).

Mature teeth, prior to those used in feeding, were examined in the SEM and X-ray spectra were acquired using a LINK/ISIS series 300 analytical system with a germanium window and lithium drifted silicon detector, attached to the SEM. Quantitative small square analyses were acquired from a number of regions in the core of several mature teeth, with acquisition conditions of 60 seconds at 20 KeV, a working distance of 11 mm, magnification of × 10,000 and specimen tilt of 15˚ using a spot size which produced an appropriate dead time of 35% (as recommended by the software manual). A polished cobalt standard was used to calibrate the equipment. Quantitative analysis was obtained for C, O, Na, Mg, Al, Si, P, S, Cl, K, Ca and Fe, with each element expressed as a percentage of total elements analysed.

For laser Raman spectroscopy radulae were placed on a calibrated motorised stage of a Dilor Labram spectrometer and excited with a diode (783.815 nm) laser. Samples were mounted horizontally in the spectrometer beam, which was focussed with an incident angle of 90˚, and a × 100 objective, using a confocal hole of 1100 μm, 150 μm slit and 600 grooves/mm grating. This gave a spot size of ~5 μm, a depth of focus of ~15 μm, and an average spectral resolution of 2.7 cm^{-1}. The source laser power used was 6 mW, with integration times typically of the order of 100 seconds and spectra the result of 10 co-additions. Survey spectra were measured on selected teeth in the radula at accurately located points along transects from the posterior cutting edge through the core to the anterior surface, at a point approximately half way between the tooth tip and junction zone (Lee *et al.,* 1998, 2000). In order to accurately localise minerals in an individual tooth, distances along individual transects were measured from a point on the posterior surface where the first recognisable iron oxide spectrum was obtained. Subsequently, longer duration spectra (for improved signal to noise ratio) were measured at points of interest along each transect. Dark current background spectra, obtained using the same conditions, were subtracted from sample spectra to remove CCD detector readout noise.

Raman spectra were also measured on well-characterised iron phosphate minerals and a mineral hydroxyapatite single crystal obtained on loan from the University of Western Australia

Table 2. Radula characteristics of seven chiton species, including tooth row numbers, major lateral tooth cusp numbers, and principal elements present in the central core region of mature major lateral teeth.

Species	Number of tooth rows	Number of tooth cusps	% of all elements analysed			
			iron	calcium	phosphorus	magnesium
Ischnochiton australis	123	2	2.7	16.2	13.6	6.0
Plaxiphora albida	58	3	26.7	6.5	11.2	2.4
Chiton mamoratus	84	1	0.8	33.2	16.7	0.5
Sypharochiton pelliserpentis	78	1	4.2	30.5	16.3	0.6
Acanthopleura echinata	82	1	1.3	27.3	14.2	0.4
Acanthopleura miles	60	1	7.1	28.4	13.6	0.6
Onithochiton quercinus	63	1	0.2	31.3	15.3	0.4

geology museum, and from the Simpson collection held at the WA Museum. In addition to the mineral hydroxyapatite, carbonate-containing hydroxyapatites, made by the method of Hayek & Newesley (1963) with the addition of stoichiometric quantities of carbonate, were also measured. Reference spectra from the hydrated iron (III) oxide mineral limonite, and a synthetic amorphous iron (III) phosphate, prepared by the method of Cate *et al*. (1959), were also measured, in addition to the biologically significant iron oxides examined previously (Lee *et al*., 1998; Brooker *et al*., 2003).

Results

Light microscopy and energy dispersive spectroscopy

Light microscopic examination of the radula of representatives from seven genera within the suborder Chitonina revealed that tooth row numbers range from 58 in *Plaxiphora albida*, to 123 in *Ischnochiton australis* (Table 2). The major lateral teeth of all members of the family Chitonida possess a single discoid cusp, while those of *I. australis* are biscuspid and those of *P. albida* are tricuspid.

EDS analyses of the central core region of mature radula teeth revealed substantial differences in the relative percentages of the major elements present. The core of all species belonging to the family Chitonidae was dominated by calcium (27.3–33.2%) with a ratio of calcium to phosphorus of approximately 2:1. Magnesium was consistently low in these species (< 1%), while iron varied from 0.2% in *Onithochiton quercinus* to 7.1% in *Acanthopleura miles*. In the tooth core of *I. australis*, the single representative of the family Ischnochitonidae, calcium was also the dominant element present (16.2%). However, it occurred in approximately equal proportions with phosphorus (13.6%), with smaller quantities of magnesium and iron at 6 and 2.7%, respectively. In contrast to all other species, the tooth core of *P. albida*, the single representative of the family Mopaliidae, was dominated by iron (26.7%), with a 2:1 ratio of phosphorus to calcium (11.2 and 6.5%, respectively) and a small amount of magnesium (2.4%).

Laser Raman spectroscopy

Raman spectra measured on standard samples of biologically relevant iron oxides and oxyhydroxides of iron, an apatitic calcium phosphate and the ferric iron phosphate mineral, strengite ($FePO_4.xH_2O$) are provided in Figure 1. All of the minerals present can be conclusively distinguished from one another by the identification of characteristic peaks (Lee *et al.,* 1998). A summary of the peak positions of the standard materials is listed in Table 3. Representative Raman spectra, from a fully mineralised tooth core, of each of the Chitonidae species listed in

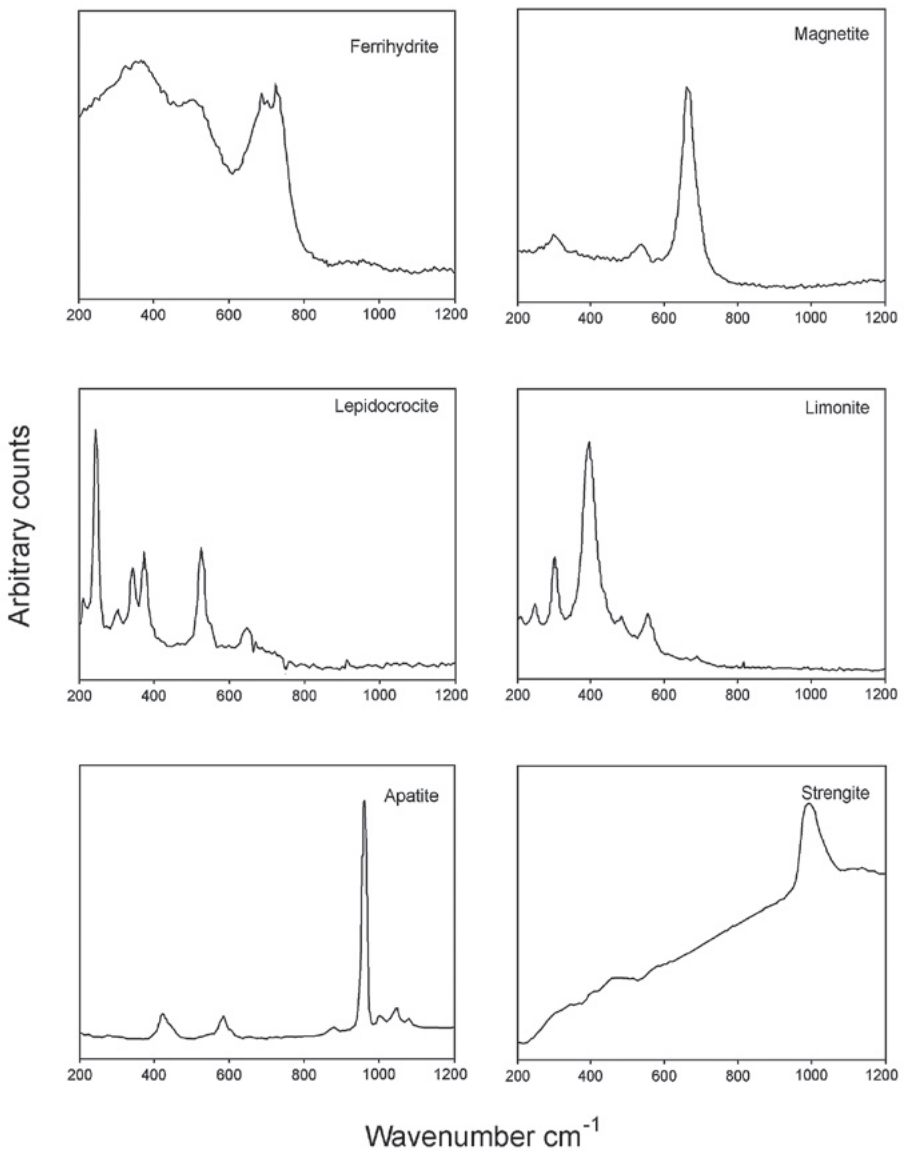

Fig. 1. Raman spectra obtained from standard samples of ferrihydrite, magnetite, lepidocrocite, limonite, hydroxyapatite and strengite.

Table 1 is given in Figure 2. With the exception of the *Onithochiton* species, spectra from the surface magnetite have been omitted. A spectrum from magnetite is recorded for *O. quercinus* since this species has teeth that are of approximately the same dimensions as the Raman laser footprint, and as such, it is not possible to discriminate individual micro-regions as easily as for other species. However, it can be seen, with reference to Figure 1, that the distinguishing peak 661 cm^{-1} from magnetite is very easily identified and that, with reference to Table 3, this mineral does not have peaks that overlap any other iron oxide, phosphate or calcium phosphate mineral.

Interestingly, the central core regions of all members of the Chitonidae, with the exception of *A. echinata*, contained large quantities of the oxyhydroxide pseudo-mineral limonite. This is

Table 3. Raman peaks for standard materials.

Compound	Formula	Band Position (cm^{-1}) and intensity*
Ferrihydrite	$5Fe_2O_3.9H_2O$	347, 493, 692, 725
Limonite	$2Fe_2O_3.3H_2O$	207w, 251ms, 304ms, 398s, 407m, 485w, 587w
Lepidocrocite	γ-FeOOH	214w, 245s, 301w, 345m, 373m, 521m, 644w
Magnetite	Fe_3O_4	297w, 535w, 661s
Strengite	$FePO_4$	419w, 947s, 1070vw
Hydroxyapatite**	$Ca_{10}(PO_4)_6(OH)_2$	425, 422, 560, 570, 583, 961vs, 1010, 1023, 1032, 1060,

* Band intensity: w = weak ; m = medium ; s = strong.
** The Raman Peaks for carbonate-containing apatites (data not shown) were identical to those of the mineral hydroxyapatite standard.

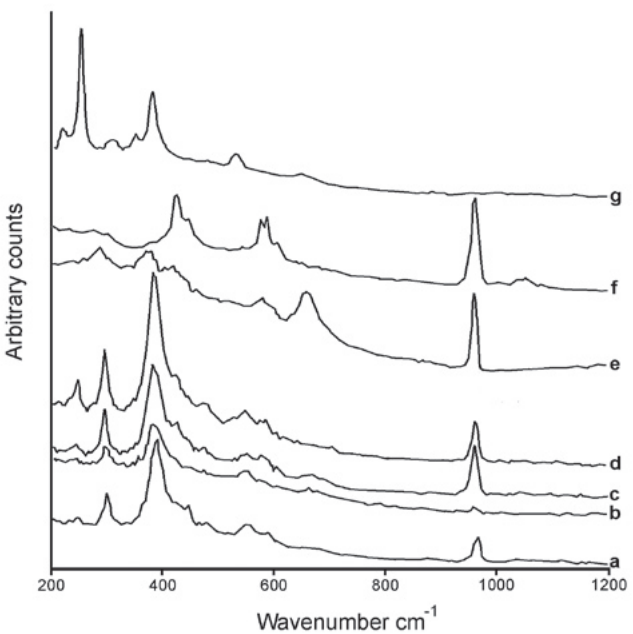

Fig. 2. Raman spectra obtained from the core region of the major lateral teeth of *Chiton mamoratus* (**a**); *Acanthopleura miles* (**b**); *Sypharochiton pelliserpentis* (**c**); *Ischnochiton australis* (**d**); *Onithochiton quercinus* (**e**); *Acanthopleura echinata* (**f**); *Plaxiphora albida* (**g**).

of particular interest since the tooth cores of all species in this study contained quantities of iron and phosphorus together with significant amounts of calcium and magnesium. It is also clear, by comparison of these spectra with those measured from a selection of iron phosphates and iron oxide minerals, that none of the species of chiton with high levels of iron and phosphorus in the tooth core, mineralises with an identifiable ferric phosphate.

Discussion

In situ laser Raman spectroscopy is providing new insights into the diversity of biomineralisation strategies employed by chitons, and the range of biominerals present in their teeth. This is particularly clear in the case of *Plaxiphora albida*, where the most abundant

Table 4. Major biominerals identified, using Raman spectroscopy, in the core of the major lateral teeth of seven species of chiton utilised in the study.

Family	Species	Biominerals in tooth core
Ischnochitonidae	*Ischnochiton australis*	limonite, apatite
Mopaliidae	*Plaxiphora albida*	limonite, lepidocrocite
Chitonidae	*Chiton mamoratus*	limonite, apatite
Chitonidae	*Sypharochiton pelliserpentis*	limonite, apatite
Chitonidae	*Acanthopleura echinata*	apatite
Chitonidae	*Acanthopleura miles*	limonite, apatite
Chitonidae	*Onithochiton quercinus*	limonite, apatite

elements of the core are iron and phosphorus, yet the mineralisation is exclusively lepidocrocite and limonite, two oxyhydroxides of iron. Laser Raman spectroscopy demonstrates that the core of this chiton species is not mineralised with a phosphate mineral as would be expected by the results obtained from EDS measurements of the elemental composition. This study challenges, on two counts, the long accepted notion that the presence of phosphorus in the central tooth core of chiton teeth implies the presence of a phosphate mineral (Towe & Bradley, 1967; Lowenstam 1972; Lowenstam & Rossman, 1975): firstly, by the conclusive identification of a mineral not previously associated with the central tooth core of any chiton species, and secondly, by the consistent failure to identify a separate phosphate phase in any chiton species studied.

If the phosphorus is not present as a discrete mineral phase it is possible that it is associated with the surface of the iron mineral, since iron (hydr)oxides are well known and active phosphorus sorbents, and studies concerning the sorption of phosphorus on these minerals are well represented in the literature (Parfitt *et al*., 1975; Couling & Mann, 1985; Nanzyo, 1986). The hypothesis of surface adsorption of phosphorus in chiton teeth draws parallels with the nature and location of phosphorus in soil systems, where phosphate species are routinely found in association with iron oxide materials (Nanzyo & Watanabe, 1982; Borggaard, 1983; Torrent *et al.,* 1994). The surface interaction between iron oxides and phosphorus is based on the replacement of surface hydroxyl ions (or water) by phosphorus. In soil systems, two of the oxygen atoms expected to form a phosphate ion are hypothesised to be co-ordinated to two iron (III) ions resulting in a binuclear surface complex of formula Fe-O-P(O_2)-O-Fe.

Laser Raman spectroscopy is also able to highlight the variety of possible mineralisation strategies that can be associated with the variable composition of the tooth cores of many chitons. For example, the tooth core of *Ischnochiton australis*, which, although not as rich in iron as other species studied, is also shown to contain limonite. In addition, the relatively larger quantities of calcium and phosphorus together with magnesium are combined in the co-deposition of an apatitic mineral along with the limonite.

The presence of an iron mineral in the core of *I. australis* is of particular note since there is very much less iron present, relative to other elements, than in other species. Therefore it is possible that the deposition of limonite in this species, and perhaps by extension to other species, has a much greater significance than its role as a structural component of the teeth. The presence of magnesium may also indicate that the phosphate mineral present is likely to be in the form of whitlockite, $Ca_9(Mg,Fe)(PO_4)_6[PO_3(OH)]$, the natural mineral analogue of β tricalcium phosphate, a material similar to apatite phosphate. However, since the peak at around 960 cm^{-1} in the Raman spectrum of *I. australis* is indistinguishable from that of other species and the standard materials, conclusive identification of the biomineral present is very difficult. The actual role of magnesium in these teeth can only be surmised, but it is has been shown to stabilise an

amorphous calcium phosphate mineral against crystallisation. (Dickens *et al.,* 1974; Blumenthal *et al.,* 1977; Okazaki, 1995).

The issue of tooth size can present a challenge to the use of laser Raman spectroscopy for the *in situ* study of chiton tooth biomineralisation. In a previous study, of *Onithochiton quercinus*, we reported that we were unable to distinguish a lepidocrocite layer intermediate between the magnetite of the cutting surface and the mineral of the central core (Brooker & Macey, 2001). However, Raman spectroscopy clearly shows that, in addition to the hydroxyapatite of the central tooth core and the magnetite of the cutting surface, there is a third mineral present. Due to the current magnification (× 100) limitations of Raman spectroscopy, the laser footprint covers a significant proportion of the total area of the tooth. Hence, in this species, it is not clear whether lepidocrocite is present as a layer intermediate between magnetite of the cutting surface and the hydroxyapatite of the bulk of the tooth, as is the case for this mineral in *Acanthopleura* species, represented here by *A. echinata*.

The results presented here, utilising data from a limited number of species, are both significant for the definition of the suborder Chitonina, and have important implications for the study of chiton tooth biomineralisation. It is apparent that all representatives of Chitonina do not exhibit a single biomineralisation strategy, with differences occurring even at the subfamilial level. The results of this study are inconclusive in determining whether the biominerals present in the tooth core are a true reflection of phylogenetic affinities in chitons. However, an extension of the study to include many more investigations of the biomineral deposition events, over a wider range of chiton taxa, could provide a basis for the utilisation of radula tooth biomineralisation as a systematic tool in the Polyplacophora.

The significance of tooth biomineralisation could also extend well beyond the systematics of the Polyplacophora. Many malacologists consider that radula mineralisation involving metallic ions is the plesiomorphic condition in molluscs, and that the evolutionary tendency has been to lose this feature (Ponder & Lindberg, 1997; Cruz *et al.,* 1998). Indeed, iron oxides have been found in the lateral teeth of the Monoplacophora (McLean, 1979; Lindberg, 1986) and are also present, along with hydroxyapatite, in the radular apparatus of the Caudofoveata (Aplacophora: Chaetodermomorpha) (Cruz *et al.,* 1998). Cruz *et al.* (1998) concluded that the presence of apatite, which is rarely found in invertebrates, in both the Caudofoveata and Polyplacophora, is indicative of the inheritance of this form of biomineralisation from a common molluscan ancestor. Hence it is possible that an understanding of the systematic implications of radula biomineralisation in chitons could also have a profound effect on our understanding of the evolutionary development of this important class.

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クサズリガイ亜目の歯舌の生体鉱物沈着に関する *in situ* 研究

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要　約

軟体動物門多板綱のヒザラガイ類の摂餌関連器官である歯舌には一列に 17 本の歯舌歯が並んでいる。そのうちの 2 本の大側歯の歯冠部は，有機基質の枠組みの中に様々な生体鉱物（バイオミネラル）が含まれる複合材料で形成されている。ヒザラガイ類はこの大側歯を主に用いて岩の表面などに付着した藻類などをかき取って食べている。これまで調べられたヒザラガイ類では，全ての種で大側歯歯冠部にはマグネタイト（磁鉄鉱）が存在することが報告されている。しかし，これらは藻類をかき取る先端や外側の部分（cutting surface）での分析の報告であり，中心部（central core）についてのものではない。そこで本研究では，クサズリガイ亜目の 3 科に属するヒザラガイ類 7 種の成熟歯（形成された後歯舌嚢の中に存在し，まだ摂餌には使用されていない歯）を材料に，エネルギー分散型元素分析装置（EDS）を装備した電子顕微鏡とラマン分光分析装置を用いて，前者では歯冠部の元素分布を，後者では存在する生体鉱物種の特定を in situ で行った。その結果，中心部は主なバイオミネラルとしてリモナイト，レピドクロサイトおよびハイドロキシアパタイトを含む，様々な元素で構成されていることがわかった。*Ischnochiton australis* に加えて，クサズリガイ科の 5 種はアパタイト鉱物を中心部に沈着していたのに対し，*Plaxiphora albida* はいかなるカルシウムを含む生体鉱物も沈着していなかった。中心部の鉄の量が比較的少ない *I. australis* を含め，*Acanthopleura echinata* を除いた全ての種から水酸化鉄（III）であるリモナイトが見つかった。中心部に高レベルでリンをもつ種においてリン酸塩鉱物が存在する証拠が見つからなかったことから，これまで長い間受け入れられてきたバイオミネラルとしてリン酸塩が存在するだろうという見解について本研究では異議を唱えることになる。本研究で用いた EDS とラマン分光を組み合わせたテクニックは，ヒザラガイが採用した生体鉱物化（バイオミネラリゼーション）の戦略を解析する上で，*in situ* で簡単にしかも効果的に評価する方法を提供してくれる。本研究の結果は，ヒザラガイの生体鉱物化の戦略が系統的類似性を反映するかどうかをはっきりさせるまでにはいたっていないが，ヒザラガイ分類群の広い範囲から種を集め本法による解析を拡大していけば，属や科レベルでの生体鉱物の類似性あるいは違いが明らかになり，多板綱においては歯舌歯のバイオミネラリゼーションが分類上のツールとして利用できるようになるかも知れない。