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Molluscan shell evolution with review of shell calcification hypothesis

Takeshi Furuhashi [a,*], Clemes Schwarzinger [b], Ivan Miksik [c], Miloslav Smrz [c], Anton Beran [d]

[a] Department of Evolutionary Biology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria
[b] Institute for Chemical Technology of Organic Materials, Johannes Kepler Universität Linz, Altenbergerstrasse 69, A-4040 Linz, Austria
[c] Institute of Physiology, Academy of Sciences of the Czech Republic, and Cardiovascular Research Centre, Prague, Czech Republic
[d] Institute of Mineralogy and Crystallography, University of Vienna, Althanstrasse. 14, A-1090 Vienna, Austria

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ABSTRACT

Biomineralization research on mollusc shells has mostly focused on nacre formation. Chitin, silk fibroin protein, and acidic macromolecules are important components for shell formation. Although the principle concept behind shell calcification was developed many years ago, the individual components have not been well scrutinized. Besides that, Mollusca are the second largest invertebrate phylum, but comprehensive biochemical research involving a comparison of different taxa is still rare. This study reconsiders the above three components with adding some biochemical data of aculiferans. The presence of chitin in polyplacophorans sclerites was confirmed by IR and pyrolysis GC/MS. DMMB staining data inferred that sulphated groups present in aplacophoran cuticle but not in polyplacophorans cuticle. These insight suggested importance of comparison between acuriferans and conchiferans.

1. Introduction

1.1. History of mollusc shell research

According to early encyclopedia Britannica in 1911, research on molluscs began quite early. George Cuvier himself erected the "Mollusca" as a designation around 1788–1800. The studies on molluscs initially dealt with systematics based on anatomy. Clearly, the shell is a hallmark of molluscs and has many functions including mechanical protection from predators and support for internal organs (Fishlyn and Phillips, 1980; Carefoot and Donovan, 1995). As Mayan Indians used nacre shells for dental implants 2000 years ago (Bobbio, 1972), the properties of shells were recognized quite early on. From the technical perspective, the more recent task has been to determine the crystal polymorphism of shells and their organic matrices.

It is uncertain when exactly the first attempts were made to describe shell structure. In 1930, Bøggild had already analyzed shell structure and distinguished aragonite and calcite. Some researchers cited a de Waele article from the 1930s that published the discovery that the crystals and shell organics are formed from 'extrapallial fluid' (Stolkowski, 1951; Kobayashi, 1964).

The first report about shell organic matrices was published by the French researcher Frémy (1855). He decalcified shells with HCl, found insoluble shell organics, and named this conchiolin. At that time, the analytical methods were clearly insufficient to investigate shell organics in detail, and no further investigations were conducted until the 1950s. The mid-1950s were the golden age of amino acid analysis, and such analyses were applied to insoluble shell organic matrices (Ghiselin et al., 1967). In that era, the analyses were done from a taxonomic point of view and many different molluscs were compared. At the same time, transmission electron microscopy (TEM) confirmed the presence of interlamellar sheet (Grégoire et al., 1955; Mutvei, 1969) and revealed compartments where crystals are disrupted by an interlamellar sheet (Bevelander and Nakahara, 1969). Nevertheless, these studies were only about nacre.

Since shell organics were initially found as insoluble shell organics named 'conchiolin', the focus was on such organics. Wada (1961) pointed out the importance of sulphated sugar group. In the 1970s, soluble shell organic matrices were discovered (Crenshaw, 1972) and the presence of sulphated polysaccharides as a nucleation factor was postulated. Crenshaw and Ristedt (1976) stained fixed insoluble shell organic matrices and found sulphated groups located at the polygon center. They envisaged that polygon center as a potential initial nucleation point and postulated that sulphated groups would play an important role in this process. Acidic amino acid analysis on soluble shell organics was done by Weiner and Hood (1975), and the result showed that Asx (Asp + Asn) was present in relatively high proportions in the investigated mollusc shells. The original idea of an aspartic acid-rich protein came from this research. Subsequent electron microscope observations by Nakahara (1972) indicated that the interlamellar sheet might be composed of various layers, based on the different electron density of the sheet's inner and outer organic layer. In the 1980s, X-ray/electron diffraction was used to study insoluble shell organic matrices. This approach yielded information on matrix–crystal spatial relations and on protein structure (β-sheet), revealing the presence of chitin (Weiner and Traub, 1980; Weiner et al., 1983).

* Corresponding author. Tel.: +43 1 4277 57702; fax: +43 1 4277 9544.
E-mail address: Takeshi_FR@hotmail.com (T. Furuhashi).

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In 1984, Weiner and Traub proposed a new model involving β-chitin–silk fibroin–acidic macromolecules (*e.g.*, aspartic acid-rich proteins) based on data from nacre shells (Fig. 1A–C). Superficially, research on shell calcification is well advanced, but problematic aspects remain. Firstly, overemphasis on shell proteins diverted researchers from more carefully investigating other factors in the calcification process. Although the calcification model has been updated (Addadi et al., 2006), major components (chitin–silk fibroin–acidic macromolecules) have not been reconsidered since 1984. Each component of this hypothetical concept contains ambiguous points, but most researchers on biomineralization agree with the idea of "chitin–silk fibroin gel proteins–acidic macromolecules (mainly aspartic acid-rich proteins)". Factors beyond these three components have received less attention.

The theory of mollusc shell biomineralization was based on histochemical data from nacre shells and biochemical analyses of nacre shell organic matrices. This integration of histochemical and biochemical data has been a major aim in the field. Histochemical data confirmed the presence of a shell organic layer provided information on its structure. Indeed, localization of particular substances (*e.g.*, sulphated group) in this layer was studied by similar technique (Nudelman et al., 2006, 2007). Mollusc shells are calcified, precluding direct observation of organics without decalcification. For instance, although intra-crystal proteins/glycosaminoglycans/polysaccharides are present, it is still uncertain whether previous histochemical investigation could have observed them inside crystals after decalcification. Biochemical analyses of shell organic matrices typically focused on shell proteins (Marin and Luquet, 2004; Matsushiro and Miyashita, 2004; Zhang and Zhang, 2006; Marin and Luquet, 2007; Evens, 2008; Marin et al., 2008). Although recent studies have made remarkable progress, the emphasis on shell proteins as main components for mineralization have diverted research on other components (*e.g.*, neutral polysaccharides, lipids).

1.2. Reconsideration of current hypothetical model

The current chitin–silk fibroin gel proteins–acidic macromolecules model itself is relatively simple, but the individual components have been studied on different mollusc species. Hence, we firstly review previous studies and scrutinize each component.

1.2.1. Chitin

Crystallographic investigation of chitin itself has been conducted since the 1920s, and polymorphism was demonstrated (Minke and Blackwell, 1978). Later research on chitin in mollusc shells was initiated around the mid-1900s and involved chemical analyses, *e.g.*, Jeniaux (1965) and Peters (1972). They decalcified shells and estimated the abundance of chitin in insoluble shell organics by calculating NAc-glucosamine or using a chitosan assay. There are some recent more reliable studies that detected chitosan by NMR (Weiss et al., 2002), while some classical assays might have been insufficiently sensitive and they yielded contradictory results. In fact, in a well-cited article (Weiner and Traub, 1980), the researchers admitted the possibility of different chitin abundance (or the absence of chitin) and stated that chitin is present in some mollusk shells, but absent in others, as determined by the chitosan test and confirmed with chitinases. Since then, almost no comprehensive research had been done to confirm the occurrence of chitin in all mollusc shells. In contrast, biomineralization researchers generalized the mollusc shell (prismato-nacreous shells) and created hypotheses based on the assumption of no significant differences in chitin abundance even between nacre shelled molluscs (Addadi et al., 2006).

Even relatively recent reports on chitin still contain ambiguous points. For example, Levi-Kalisman et al. (2001) concluded the presence of chitin in a nacreous shell fragment of *Atrina rigida* because the nacreous shell insoluble organics were not destroyed by 1 M NaOH treatment. Although they stated that interlamellar sheets (*Atrina*) are composed mainly of highly ordered and aligned β-chitin fibrils, they were unable to provide a electron diffraction pattern for *Atrina* nacre organics. Moreover, the X-ray diffraction pattern of β-chitin they showed was obtained from a non-calcified chitinous *Loligo* shell, concluding that it would be most surprising if the matrix of *Atrina* contains α-chitin, whereas all other mollusks examined to date contained β-chitin.

Contradictions on the presence of chitin are evident in several more recent articles. Nudelman et al. (2007) partially decalcified *A. rigida*

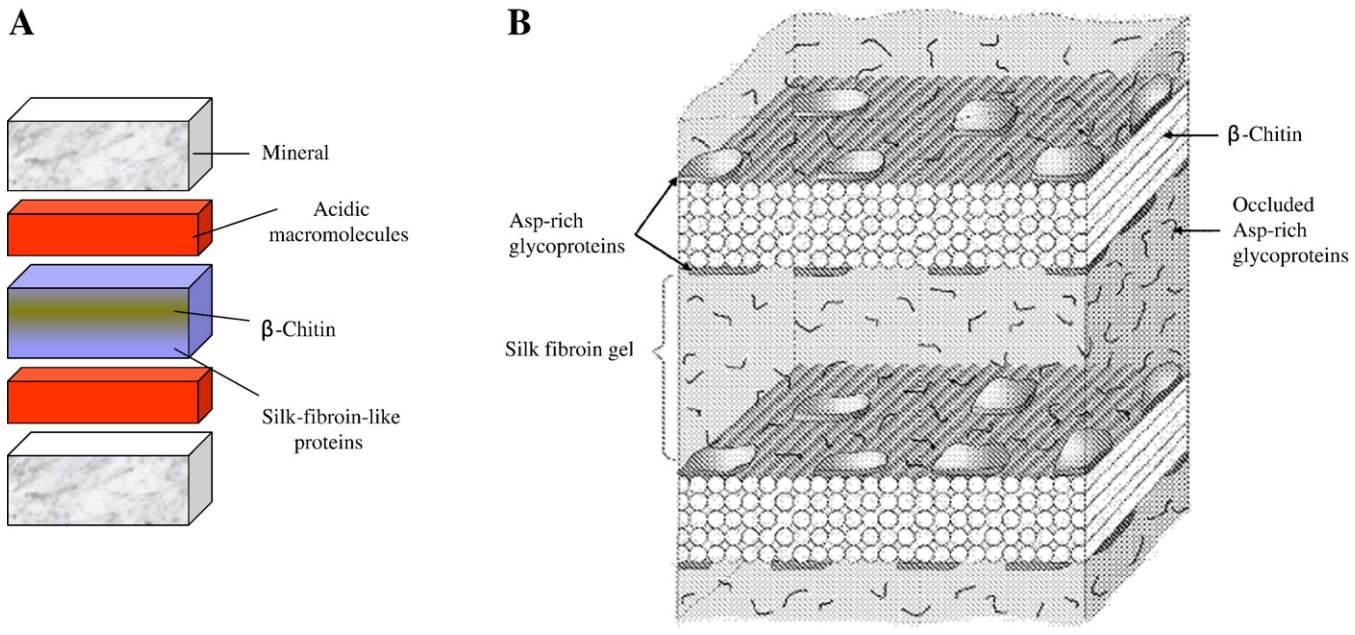


Fig. 1. A) Model of shell calcification in 1984 (modified after Weiner and Traub, 1984). B) Model of shell calcification in 2001 (Levi-Kalismann et al., 2001). C) Model of shell calcification in 2007 (Nudelman et al., 2007).

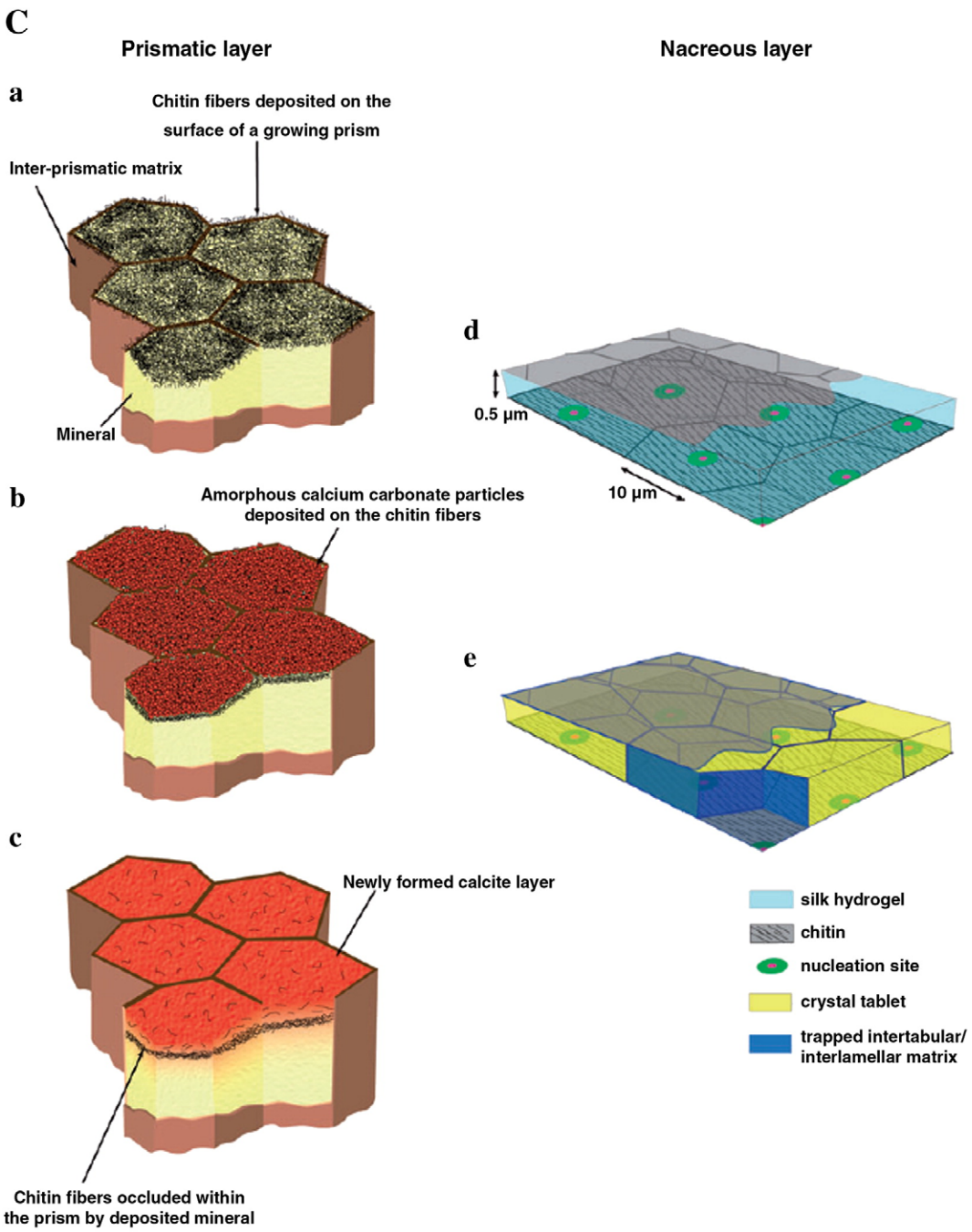


Fig. 1 (*continued*).

single prisms and found fiber network structures with SEM. This network was degraded by chitinase but not by NaOCl. They concluded that the fiber network structure was apparently chitin. In 2008, Nudelman et al. applied the same treatment to *A. rigida* and *Pinctada margaritifera* nacre layer pieces. In those experiments, the similar fiber network structures were degraded by both chitinase and NaOCl, and the authors concluded that the fiber network structures should be chitin. Only proteinase-K-treated nacre tablets showed fiber network structures, but such a structure was not shown at the growth front of nacre without this treatment. Besides that, the intensity of the epifluorescence micrograph stained with GFP-tagged chitin-binding proteins was much weaker than those labeled with antibodies against Asprich. Chitin should have been more abundant than Asprich, because the network considered as chitin was clearly visible under SEM and Asprich is supposed to be water soluble and hence absent in insoluble organics. Moreover, only the marginal region of single prism was stained the epifluorescence micrograph. The micrograph was not of a transverse section of a single prism; therefore, fluorescence should not have been limited to the marginal region, due to the 3D crystal structure.

In 2008, Bezares et al. applied similar methods to *Haliotis rufescens* nacre and observed a similar organic network. They stained with WGA or Calcofluour White, concluding that the network must be chitin. Their epifluorescence figure did not appear to show consistent staining and even stained parts were limited to certain parts of the network. This figure indicated that the network might contain more than chitin alone. In addition, they used WGA with PNGaseF. WGA is not always so specific and can react with the GlcNAcβ1,6 Gal sequence (Muraki et al., 2002).

This calls for scrutinizing the probe for chitin detection. Calcofluor White has been used by some researchers (Suzuki and Nagasawa 2007; Bezares et al., 2008) to detect chitin and identify the localization. As stated on the Fluka Company web site, Calcofluor White binds non-specifically to chitin and cellulose. Albani and Plancke (1999) reported that acid glycoproteins interact with Calcofluor White. In fact, acidic proteins in insoluble shell organic matrices are present as MSI31 and Pearlin (Marin et al., 2008). In particular, Pearlin is bound to sulphated sugars and this is certainly an acidic glycoprotein as well (Miyashita et al., 2001). With regard to the specificity to chitin detection, fluorescence probes with chitin-binding proteins (*e.g.*, GFP-tagged chitin binding protein) probably have a higher specificity because such proteins (or domain from chitinase) recognize the 4–5 tandem repeat of β1,4-NAcGlu specifically (Watanabe et al., 2003).

Certain inconsistencies remain regarding the chitin network structure. The diameters of the chitin fibers observed by Nudelman et al. (2007) and Bezares et al. (2008) were indeed similar to those of nonwoven chitin nanofibers (Min et al., 2004). Nevertheless, some protein nanofibers, *e.g.*, collagen and silk fibroin gel could form similar fibers (Stuart and Panitch, 2008), and identification of chitin based solely on fiber structure might be unreliable.

This chitin network structure does not appear to fit to the structure of highly ordered and aligned β-chitin fibrils in Levi-Kalismann et al. (2001) or to film-like and fibrous squid β-chitin (Fan et al., 2008). Normal chitin nanofibers are prepared in vitro by a special procedure (*e.g.*, via elecrospinning in HFIP or cationization of the C2 amino groups). It would therefore be necessary to reconsider the interpretation of chitin observation by Nudelman et al. (2007, 2008) and Bezares et al. (2008), who found nonwoven but spider net like chitin nanofiber network structures as a natural product in etched single prisms and in the nacres of *A. rigida* and of *H. rufescens*. The globular structure on the network in Nudelman et al. (2007) was also observed in the silk fibroin nanofiber prepared by elecrospinning (Min et al., 2004).

The silk fibroin gel protein structures have not been figured. Although Nudelman et al. (2008) emphasized the importance of such gels, the etched single prisms failed to show any protein fibers even after chitinase treatment. Park et al. (2006) reported that the silk fibroin gel proteins showed different fiber shapes depending on the chitin content. In particular, the fibers were much thicker than chitin nanofibers and took on a membrane shape when no water vapor was applied. Furthermore, all other previous chemical analysis (Jeniaux, 1965; Peters 1972; Goffinet and Jeniaux, 1979) showed the chitin abundance was lower in sheet nacre bivalves as well (e.g., *Pinctada*, *Pinna*, *Mytilus*). This suggests that *Atrina* shell organics would be rather proteinous, and that silk fibroin fibers could be found with chitin fibers. There might be some fuzzy points about only purified chitin nanofibers observed by Nudelman et al. (2007). This points to inherent problems with confirmations based only on the observation of chitin fiber structure.

Some researchers attempted to demonstrate chitin by cloning chitin synthase or by using fluorescence probes to detect chitin. Chitin synthase has been indeed been cloned from *Atrina* and *Pinctada*, but the presence of enzyme would not always be a definitive proof of the presence of the final product in calcified shells. In recent work on chitin synthase of *Mytilus*, Schönitzer and Weiss (2007) used Nikkomycin as a chitin synthase specific inhibitor because it is an analogue of NAc-Glucosamine. Nikkomycin caused some morphological change in larval *Mytilus* shells, *e.g.*, one valve became larger than the other. They concluded that chitin deficiency might have caused these morphological changes. Contrary to this, shell shape and size are normally determined by the morphology of mantle tissues, and chitin as a structural component would not affect shell sizes. It appears unclear that Nikkomycin is chitin synthase specific in molluscs, as the specificity to chitin synthase was demonstrated mostly in *Candida* research.

Some other studies have detected chitin in mollusc shells chemically. For instance, Dauphin and Marin (1995) and Treves et al. (2003) used IR spectroscopy and compared spectra with standard chitin. If the material was mostly chitin, it is no problem to compare IR spectra data with standard chitin. Most shell organics, however, are a mixture of insoluble proteins and polysaccharides, and it is necessary to distinguish these compounds from purified chitin data (Furuhashi et al., 2009). For example, the relative height of the Amide II peak to hexose peaks indicates a degree of N-acetylation in purified chitin (Fan et al., 2008), but it would also indicate a different ratio of protein:chitin:polysaccharide in shell organic matrices (Furuhashi et al., 2009).

Chitin polymorphism (α- versus β-chitin) is another important issue. β-chitin in mollusc shells was identified only from cephalopods by using X-ray diffraction (Weiner and Traub, 1980) and solid NMR (Kono, 2004). In fact, most chitin research on molluscs was on non-calcified squid chitinous shell; the chitin polymorphism in other calcified conchiferan shells remains unknown, although many biomineralization researchers believe that all mollusc shells contain β-chitin (Levi-Kalismann et al., 2001; Addadi et al., 2006). There is still a possibility that some mollusc shells have α-chitin, as the radula sheath of polyplacophorans (Evans et al., 1990), and the non-mineralized portion of *Nautilus* siphuncle (Lowenstam et al., 1984) is composed α-chitin.

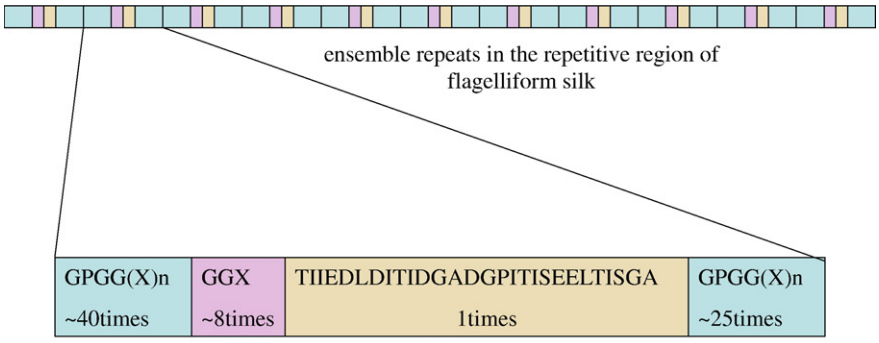
The mechanical characteristics of chitin are influenced by polymorphism. Fan et al. (2008) determined that squid β-chitin as well as tubeworm β-chitin had a heterogeneous, film-like fibrous shape. Conversely, α-chitin in crab shells and tendons had a particle-like, flake form. Tubeworm and squid β-chitin also differed. Only squid β-chitin was well dispersed and converted to transparent at pH 3–4 with sonication. This was due to the very low degree of N-acetylation squid β-chitin. The film-like shape and easy dispersibility would be suitable for the interlamellar sheet of shell organics. Further confirmation of chitin polymorphism as well as degree of N-acetylation rate in shell organics is required by comparing different shell structures, *e.g.*, sheet nacre and columnar nacre.

1.2.2. Silk fibroin gel protein

Silk fibroin protein was originally studied in arthropods. Two types of silk were distinguished: *Bombyx mori* (cocoon) silk and spider silk (Macintosh et al., 2008). In general, the former contains sericin (serin-rich protein glue), the latter not. All silk fibroin proteins are considered to be members of a common gene family, having the same set of motifs, *i.e.* β-spirals, β-sheet, helical structure, and spacer region (Craig and Riekel, 2002; Foo and Kaplan, 2002). These motifs are normally internally repeated and are known as the ensemble repeat region (Hayashi and Lewis, 2001) (Fig. 2A). For example, $GQGGYGGLGGQGAGRGGLGGQGAGA(A)_nGGA$ of *Nephila* dragline silk is a consensus sequence in each ensemble repeat. Most silk fibroins contain crystalline and non-crystalline regions (Craig and Riekel, 2002). Crystalline regions are normally a β-sheet domain made from poly (Gly/Ala) or poly (Ala). Flagelliform (Flag) silk is a structurally unique form. This spider silk possesses neither polyalanine nor β-pleated sheet, but has an internal ensemble repeat (Hayashi and Lewis, 2001).

In mollusk research, as recently reviewed by Addadi et al. (2006), molluscs shell silk fibroin proteins have been defined as Gly- and Ala-rich insoluble proteins with a β-sheet domain; such proteins have been considered to be similar to silk proteins, *e.g.*, spider dragline silk and cocoon silk. This definition was originally deduced from amino acid analysis of shell organic matrices, based on the higher proportion of Gly and Ala in certain insoluble shell organic matrices. Pereira-Mouries et al. (2002), for example, reported a silk fibroin-like protein in *Pinctada* nacre due to the high proportion of Gly and Ala. Contrary to this, the proportions of Gly and Ala were not always high, and they are not always the same in the insoluble shell organic matrices of other molluscs

A



B

MKLLVVL TTLVGFSSALSFGCNYRPVLGFNSQ YMLGGLRLFCMPAMVYDPWACGCVS
AWSSAGL YGVGGGGGAWGAGGAGGADGGRGGGGGDWEYDYDDDSDDDDEWDWD
DDGGMGAGAGGGAGGGAGGGAGAGAGAGAGAGAGAGLGLGLGGGLGGGLGGLGG
LGGLGGGDDLFDLDFDDLGAALALGGAGGAGG
AAAAAAAAAAAAGGGVGG
AAAAAAAAAAAAGGGAGRLGG
AAAAAAAAAAAAGGAGGLGGLGGGLGGLGGGLGGLGGLGGYGGS
AAAAAAAAAAAAGGGGLGGVGFYGGRGGRRGRGRGGRRR
AAAAAAAAAAAAAGGGGGGGGGGGGGGGAG
AAAAAAAAAASASASRQMSGIRDALGDIKDLLRSNGASAKASAKASAVASTKS QIDDL
KDVLKDLAGLLKSSASASASASASASAGGGGGGGNGGGNGGGGGGGAG
ALAAALAAAGAGGGLGGGGGGG
ALAAALAAAGAGGGGFGGLGGLGGLGGGS
AAAAAAAAAAAASGGGGRALRRALRRQMRGGGS
AAAAAAAAAAAAGGGWGGGMGGGFGVGLGGGFGGGFGGGSS
AAAAAAAAAAAGFGGGGRRGRGRGRGGDGDGNGASAV
AAAAAAAAAAGGSAADV
AAAAAAAAAMYGDGADGPDFDNGFGGGNGNGGGGSGGGGSGGGGSGGGSGGGGGS
GGSGGGGGSGGSGGGGSGGGGNNGWGNNGNNKYDDDDCDE YGNPIRRG

C

GPGGYGPGQQGPGGYGPGQQGP----SGPGSAAAAAAAAAA-------GPGGYGPGQQ
GPGCYCPCQQCPGRYGPGQQGP-----SGPGSAAAAAA----------GSGQQ
GPGGYGPGQQGPGGYGQGQQGP----SGPGSAAAASAAASAESQQGPGGYGPGQQ
GPGGYGPGQQGPGGYGPGQQGP----SGPGSAAAAAAAAS----------GPGQQ
GPGGYGPGQQGPGGYGPGQQGL----SGPGSAAAAAAA----------
----------GPGQQGPGGYGPGQQGP----SGPGSAAAAAAAAA----------
GPGGYGPGQQGPGGYGPGQQGP----SGAGSAAAAAAA-------GPGQQ
GLGGYGPGQQGPGGYGPGQQGPGGYGPGSASAAAAAA----------
----------GPGQQGPGGYGPGQQGP-----SGPGSASAAAAAAAA----------
GPGGYGPGQQGPGGYAPGQQGP-----SGPGSASAAAAAAAA----------
GPGGYGPGQQGPGGYAPGQQGP-----SGPGSAAAAAAAAA----------
----------GPGGYGPAQQGP-----SGPGIAASAASA----------
GPGGYGPGQQGPAGY----------GPGSAVAASAGA----------
----------GSAGY----------GPGSQASAAA----------

Fig. 2. A) Ensemble repeat of flagelliform silk protein (modified from Hayashi and Lewis, 2001). Repeat structure is clearly visible. B) Protein sequence of mollusc shell protein MSI60 (modified from Sudo et al., 1997). C) Protein sequence of silk fibroin protein NCMAG2 (modified from Foo and Kaplan, 2002).

(Ghiselin et al., 1967). No clear tendency was determined on the taxon level. Furthermore, there is no evidence of internal ensemble repeats in mollusc shell proteins, nor is there evidence of a clear crystalline region and other motifs (*e.g.*, helical structure and β-spirals) that are important characteristics of silk fibroin. Indeed, Weiner and Traub (1980) reported only β-sheets in insoluble shell organic matrices (*e.g.*, *Nautilus*) but no helical or β-spirals as seen in silk fibroin proteins.

A well-known publication that has been considered to support the definition of silk in mollusc shells deal with the MSI family (Sudo et al., 1997). Those authors identified the sequences of MSI obtained from insoluble shell organic matrices of the *Pinctada fucata* nacre layer. Surperficially, MSI60 fits the definition relatively well. It contains many glycine repeats and 11 alanine blocks as well as GA/GS repeats. MSI31, however, lacks any alanine blocks or GA/GS repeats, and in situ hybridization data showed that the MSI31 expression pattern appeared to be rather specific to the prismatic layer. MSI31 is rather acidic (pI 3.8), although silk fibroin proteins are supposed to be basic. The presence of MSI has not been demonstrated in other taxa, *e.g.*, *Haliotis*, and only immunohistochemistry data has pointed to a potential presence (Jolly et al., 2004). In all MSI, there was no internal ensemble repeat, and the Ala repeat appeared to be inserted rather randomly (Fig. 2B, C). The absence of an ensemble internal repeat would have indicated that MSI families are not a homologue of silk fibroin proteins. It is necessary to reconsider the comparison between mollusc shell insoluble proteins and silk fibroin proteins, and the naming of mollusc shell protein as silk fibroin proteins or nacre silk.

All other reported insoluble shell proteins were not similar to silk fibroin. Furthermore, the definition of insoluble proteins is not always clear due to different solubilities. Shell proteins previously reported to be insoluble were normally obtained by denaturing proteins with detergent. For example, Lustrin A (*H. rufescens* nacre) has been extracted with urea and SDS (Belcher et al., 1996), N14 (*P. fucata* nacre) with NH_4OH alkaline extraction (Kono et al., 2000), and Schematrin (*P. fucata* non-intra crystal protein from prismatic layer) with urea. The characteristics of insoluble shell proteins differed from one another. Thus, N14 is rather acidic, LustrinA is a large and multi-functional protein, Schematrin families (1–7) possess Gly repeats and a lower proportion of Ala. In particular, Yano et al. (2006) compared Schematrin with glycine-rich proteins in plants with respect to a G_nY or G_nX repeat. As Marin et al. (2008) pointed out; only the Schematrin2 glycine-rich part was similar to MSI31.

Finally, our current data showed that proteins in insoluble shell organic matrices also contained some soluble chitin-binding proteins that were solubilized after chitin digestion by chitinase treatment (unpublished data). This underlines that proteins in insoluble organics do not always refer to water-insoluble proteins like silk fibroin. On the other hand, some proteins are more insoluble and cannot be dissolved by detergent or any other alkaline solution. After all, 'silk clothes' made from cocoon silk can be washed with detergent or alkaline solution.

Protein solubility is also critically linked to protein structural transition, as is evident in β-amyloid (Goldsbury et al., 1997). In the case of spider silk, silk fibroin proteins are secreted as silk-I (helical structure) and converted into silk-II (β-sheet), possibly by external pressure (Knight et al., 2000). This involves polymorphic conversion of silk proteins, and an insoluble crystallizable block and a soluble amorphous block are important for the solubility. Wilson et al. (2000) applied FT-IR, CD, and electron diffraction to the crystallizable sequence (GAGAGS) and the amorphous sequence (GAGAGY) under various conditions (*e.g.*, with ZnSe substrate and methanol treatment) in order to clarify the structural transition of silk protein. Among the interesting results was that conformational diversity depends on the presence of solid substrate, and β-turn-rich might be intermediate between helical structure and a β-sheet.

The solubility of mollusc insoluble shell proteins may involve a mechanism similar to that in the transition of As spider silk from silk I (α-helix) to silk II (β-sheet). Using cryo-TEM, Levi-Kalismann et al. (2001) concluded that disordered gel in *Atrina* was insoluble proteins in a final state. Bedouet et al. (2007) measured an oxidative activity of *Pinctada* shell proteins and concluded that this activity would be important to transform soluble proteins into an insoluble network during nacre growth. In their opinion, covalent bond formation is required to mature soluble proteins into insoluble network, and oxidative activity would be important for this.

There is another recent study dealing with the relationship between chitin and proteins in insoluble shell organic matrices. The study proposed that amino-acyl group was covalently attached to chitin in *Mytilus galloprovinciallis* and named this as silk modification (Weiss et al., 2009). The hypothetical scheme based on IR spectra and MS spectra data. This idea appears to be contradictory to previous results. Weiss et al. (2002) analyzed chitosan by using NMR, which was originally prepared from *H. rufescens* chitin in insoluble shell organics. In this study, amino-acyl groups or any other modification has not been described. Given that NMR spectra data is generally more precise for chemical structure identification, silk modification speculated from MS spectra (only from m/z) might be necessary to be reconsidered carefully.

Furthermore, previous studies on monosaccharides analysis of shell organic matrices showed the presence of N-Acetyl glucosamines (or glucosamines) but not amino-acyled glucosamines (Dauphin and Marin, 1995). In mild hydrolysis condition (*e.g.*, methanolysis), amino-acyled could have been separated by GC/LC column and been identified. Indeed, Marxen et al. (1998) did GC monosaccharides analysis with methanolysis to insoluble shell organic matrices of *Biomphalaria glabrata*. They successfully identified N-acetyl glucosamines but not amino-acyl groups.

Besides that, amino-acylation should be enzymatic reaction (it is more difficult to explain in view of organic sysnthesis). Not only the enzyme has not been found in molluscs, but also protein and/or amino acid is not suitable as the substrate for amino-acylation.

Covalently bond cannot allow solubilization of proteins and previously cloned proteins from insoluble shell organic matrices with detergent. Therefore, this covalent bond modification does not appear to fit to previously report.

This idea harbors an inherent problem. Many insoluble proteins, including those identified in the proteomics analysis, were extracted with alkaline substances or detergents. Proteins, however, would not be always extracted (with conformation change) by detergent and alkaline treatment if covalent bonds were formed. Since both oxidative activity in mollusc shells and protein structural transition (similar to that in silk) were not completely proved, more data are required.

1.2.3. Acidic macromolecules

To date, all researchers assume that acidic macromolecules with a negative charge are important for the calcium carbonate crystallization process. The first hypothesis on the calcium nucleation process was based on amino acid analysis of soluble shell proteins and the distance between Ca ions in the crystal (Weiner and Hood, 1975). They estimated a distance between repeating carboxyl residues of aspartic acid in protein with β-sheet structure, as well as the calcium ion distance in calcium carbonate crystals. The authors stated that the distances would be 6.95 Å (aspartic acid) and 5 ± 1.5 Å (calcium ion), respectively. In their opinion, these similar distances would be effective for shell protein to provide negative charge bonds (carboxyl residue of aspartic acid) for effective calcium carbonate nucleation. Although the mean calcium ion distance in their model was 5 ± 1.5 Å, calcite and aragonite are crystals with 3 axes. This requires considering which plane is optimal for crystallization. For instance, aragonite has 3 different calcium ion distances which are 4.96 Å (a axis), 7.97 Å (b axis), and 5.74 Å (c axis) (Weiner and Traub, 1980; Litvin et al., 1997). Previously, Litvin et al. (1997) found that 5-hexadecyloxyisophthalic acid (C_{16}ISA monolayer) can form aragonite in vitro and reported a similarity in the carboxylic acid distance (4.4 Å and 5.5 Å) and the aragonite a–c plane (a, 4.96 Å; c, 5.74 Å). Accordingly, the distance between calcium ions and negative

charged bonds of acidic organics would not be so different (not>1 Å) if the template theory is correct.

A figure of the β-sheet of silk fibroin-like proteins in Weiner and Traub (1980) showed a distance of 6.9 Å between carbon atoms that were not aspartic acid residues. The distance between aspartic acid residues (6.95 Å) calculated by Weiner and Hood (1975) is quite similar to the above, and it appears that they simply used the same value. In 1985, Addadi and Weiner conducted an in vitro crystallization assay and discussed a distance between calcium ions and negatively charged bonds. In the case of calcium malonate, the Ca–Ca distance was 6.8 Å, which was quite similar to the carboxylate repeating distance of the β-sheet (6.7–7.0 Å). Although they concluded that the calcite (001) plane should be the crystal growing plane, they did not discuss the Ca–Ca distance in the case of calcite. In the calculation of the repeating Ca–Ca distance, all Ca–Ca distances differ from the distance between carboxyl residues (Fig. 3A, B). Although the β-sheet aspartic acid-rich protein template theory model was attractive, the evidence was not always reliable. Since the 1980s, Weiner's group started to emphasize the importance of the (001) plane where acidic macromolecules would interact without arguing distance so much. In relation to this, Nassif et al. (2005) proposed another interesting idea that charge interaction between (001) aragonite face and negatively charged organic surface is important for nucleation process.

In 1987, Addadi et al. performed in vitro crystallization using sulphonated polystyrene and polyaspartate as an ideal analogy to acidic macromolecules. They explained that specific chelation of calcium between groups of closely spaced sulphonate moieties and (001) oriented calcite was nucleated by sulphonated polystyrene. Although they urged caution in interpreting the results and noted the difference between sulphonate and sulphate, many researchers have cited the article as a good example of the sulphated polysaccharide crystallization model. Sulphated polysaccharides/glycosaminoglycans can change their structure much more flexibly than a protein β-sheet (Questel et al., 1995). Therefore, the distances between two sulphates cannot be simply determined and generalized. The chelation model involving sulphate bonds still contains some speculation.

Crystallization research has become more sophisticated since 2000, but determining the crystal structure in vitro is still quite problematic because the crystal structure is influenced by temperature, calcium ion concentration, the concentration of negative charged acidic macromolecules (Wang et al., 2006, 2008), carbonate ions, water (Li and Mann, 2002), and other cations (*e.g.*, Mg ion) (Takeuchi et al., 2008).

Indeed, a discrepancy was evident in recent in vitro crystallization studies. Addadi and Weiner (1985) found that the (001) surface was important for crystal growth by aspartic acid-rich proteins. The crystal structure in their article was, however, completely different from crystals grown with aspartic acid-rich proteins in recent articles, *e.g.*, Asprich (Politi et al., 2007) and Aspein (Takeuchi et al., 2008), but similar to the crystals formed by compounds other than proteins. One plausible explanation for the discrepancy is that the concentration of acidic proteins in Addadi and Weiner (1985) might not have been sufficient. The focus should be on further determination of acidic macromolecules or nucleation factors rather than developing hypothetical models.

The main acidic macromolecules are thought to be aspartic acid-rich proteins (Marin and Luquet, 2007) and sulphated GAG (Dauphin, 2003). Acidic proteins have long been considered to be aspartic acid-rich proteins due to higher proportions in amino acid analyses (Weiner and Hood, 1975). This idea was originally developed for the nacre formation model. Recent research revealed that all reported aspartic acid-rich proteins (*e.g.*, Aspein (Tsukamoto et al., 2004) and Asprich (Gotliv et al., 2005)) were present in the calcitic prismatic layer (Endo and Sarashina, 2007), although some researchers generalized this to mollusc shells in general (Addadi et al., 2006). It is well confirmed that these acidic proteins are expressed in the mantle epithelium for the prismatic layer (Takeuchi and Endo, 2006). Accordingly, the variation of shell microstructure (*e.g.*, columnar nacre, cross lamellar structure, foliated structure), the localization of each acidic protein in each of these, as well as taxonomic differences might need to be considered as well. Variation of acidity in shell protein were suggested by some researchers previously (Dauphin, 2003, 2004; Marie et al., 2009). In fact, the acidity of MPP1 in *Crassostrea* foliated structure was due to the phosphorylation of serine and not due to aspartic acid (Samata et al., 2008); MSP1 in *Pecten* foliated structure, on the other hand, was aspartic acid rich (Sarashina and Endo, 2001). Secondly, the acidity of proteins in soluble organic matrices can differ. In *P. fucata* nacre, for example, the acidity of these matrices varied, due to glycosylation (Takakura et al., 2008), and in *Haliotis gigantea* the soluble proteins in the nacre are rather basic (unpublished data). These data were consistent with the information that there were only few homologues of acidic shell proteins as well as of other non-acidic shell proteins (especially between gastropod and bivalves). Nacrein (carbonic anhydrase) is reported to be present in different mollusc classes, for example in bivalves (*Pinctada*, *Pecten*, *Crassostrea*, and *Unio*), cephalopod (*Nautilus*) and a gastropod (*Turbo*) (Marie et al., 2008, 2009; Marin et al., 2008). On the other hands, the homology of N14, Tyrosinkinase-like1 and 2, Mucoperline, MSI60, MSI31 between bivalve nacre and cephalopod nacre were recently confirmed, but these proteins still have not been found in gastropods (Marie et al., 2009). These reported proteins are not Asp-rich proteins, and there is no clear tendency associated with the difference in sheet nacre and columnar nacre. In addition, data of proteins in other shell structures, such as cross lamellar, is relatively little. As such, it is still uncertain whether reported proteins in nacre present only in nacre shells or also present in other shell structures. Thirdly, the proteins: sulphated GAG ratio could also differ between soluble shell organics, even between shells with the same structures (unpublished data). Conspicuously, the abundance of both acidic proteins and sulphated GAG in soluble shell organics varied between taxa as well as between shell structures. As described above, acidic proteins bind to the (001) plane. Earlier research (Weiner and Hood, 1975) underlined the importance of the β-sheet structure of aspartic acid-rich proteins, but there is still not enough evidence that poly-aspartic acid is a β-sheet rather than an α-helix structure. The shell calcification model involving acidic proteins contains some ambiguous points.

Although in vitro crystallization assays have been done a long time ago (Kitano and Hood, 1965), still there is no clear conclusion. Recently, Takeuchi et al. (2008) found that low concentrations of Aspein (in 50 mM Mg: 10 mM Ca) formed spherulithic aragonite, whereas higher concentrations formed dumbbell-like calcites. Politi et al. (2007) used Asprich, yielding some spherulites. They did not identify the spherulites as being composed of calcite or aragonite, but they could be aragonite due to their morphological similarity to the aragonitic spherulites made by Aspein. Note that dumbbell-like crystals were not formed by Asprich.

Not only aspartic acid-rich proteins but also other proteins form crystals in vitro. Collagen might play a role similar to that of aspartic acid-rich proteins in view of crystal morphology (Jiao et al., 2006). Moreover, silk fibroin (Cheng et al., 2008) and free amino acids (*e.g.*, glycine and aspartic acid) (Hou and Feng, 2006) regulated the crystallization. Thus, the crystal morphology data obtained with acidic proteins were not always conclusive.

With regard to sulphated GAG and glycoproteins as a nucleation factor, Wada (1980) emphasized the potential importance of sulphated sugars for mollusc shell formation. Dauphin (2003) conducted XANES analyses on *Pinna* and *Pinctada* prism and found a specific distribution pattern of sulphate (SO_4^-) and protein S: sulphate, for example, was localized at both the outer wall and central part of prisms in both species, although its function is still speculative. Nudelman et al. (2006) proposed two different aragonitic crystal formation schemes (in *Atrina* and *Nautilus*) governing the calcium carbonate nucleation in each nacre tablet. In that interesting article, a center of tablet would be an independent nucleation point, and sulphates were detected only in *Nautilus* aragonitic crystals and not in *Atrina*. Bezares et al. (2008)

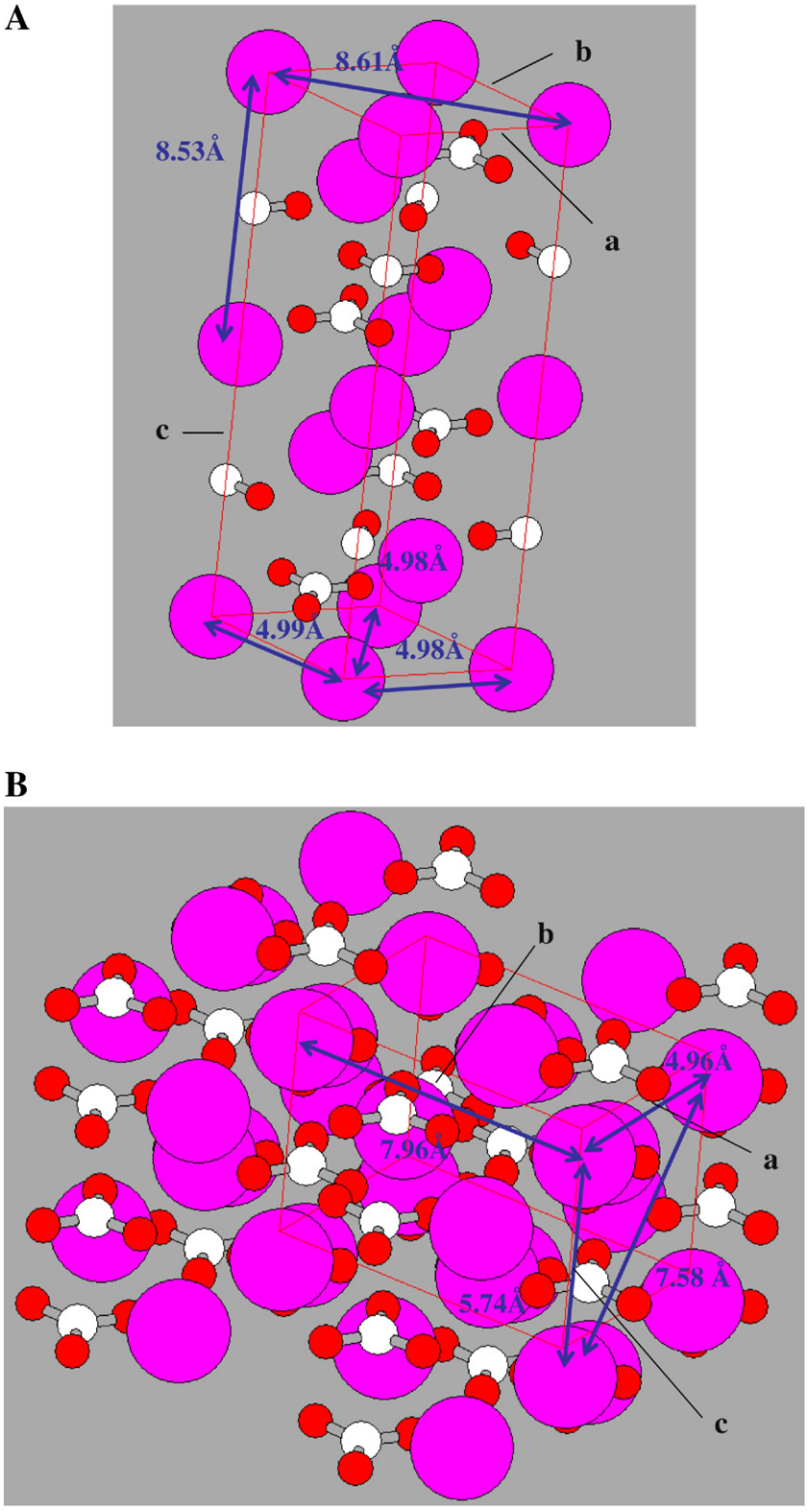


Fig. 3. A) Crystal structure of calcite. Pink, calcium ion; red, oxygen; white, carbon. B) Crystal structure of aragonite. Pink, calcium ion; red, oxygen; white, carbon. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

repeated this experiment with *Haliotis* and obtained the same result. On the other hand, our DMMB colorimetric assay (unpublished data) detected sulphate in both *Atrina* and *Nautilus*. The *Atrina* data initially appears to be contradictory to Nudelman et al. In principle, sulphate groups for calcification mostly involve sulphated GAG (and some sulphated glycoproteins), and these are certainly water soluble. Our DMMB colorimetric assay was applied to soluble organic matrices, and Marie et al. (2007) also found that sulphate rather than insoluble shell organics dominated in *Unio* soluble shell organics. Nudelman et al. (2006) and Bezares et al. (2008), on the other hand, used

cetylpyridinium chloride (CPC) for sulphate precipitation and stained decalcified insoluble shell fragments with colloidal irons. Loss of sulphate in *Atrina* insoluble shell fragments in Nudelman et al.'s experiment might be due to this final washing step and the dissolution of some sulphate groups into water. It remains uncertain whether all or only some sulphated groups in shell organic matrices were stained histochemically. In addition, Dauphin et al. (2003) detected sulphate by XANES in prismatic layer of *Pinna* and *Pinctada* without using CPC fixation. In 2008, Dauphin et al. also confirmed the presence of sulphate groups in both prismatic and nacre layer, and the abundance was larger in prismatic layer than nacre. Considering the taxonomically close relationship between *Atrina*, *Pinna*, and *Pinctada*, the lack of sulphate in the central part of each nacre tablet in *A. rigida* might need to be confirmed by further experimental data.

Sulphate presence in insoluble shell organics might be due to sulphated sugars attached to insoluble proteins as well. A case in point is the protein (Pearlin) extracted from insoluble organics in *Pinctada funca* nacre: it contains sulphate, which was confirmed by Alcian Blue (pH1) staining (Miyashita et al., 2001). In other words, sulphate sugars detected by Nudelman et al. (2006) and Bezares et al. (2008) could also be insoluble sulphated glycoprotein in *Nautilus*, *Haliotis*, and *Pinctada,* which was not in *Atrina*. The occurrence of these insoluble sulphated glycoproteins might be problematic for establishing a nucleation model. Compared with acidic shell proteins, there is still no structural information available about sulphated GAGs as related to shell formation. Further investigation will be required to clarify their role.

As a whole, the current chitin-silk fibroin gel proteins-acidic macromolecule framework/approach was a milestone as the first modern hypothetical model to grasp the mollusc shell biomineralization mechanism. Additional effort is required to clarify some of the model's fuzzy points addressed above. In particular, many of previous research focus on nacre. It is not amenable to develop hypothetical scheme or evolutional concept of molluscs shell without data on aculiferans and other conchiferans groups. For this reason, it would be useful to investigate molluscs with broad taxomonical point of view.

1.3. Evolution of Mollusca, shell formation, and shell structure

In order to elucidate mollusc shell evolution, it is first necessary to examine the classification of the group. The Mollusca is one of the best-known and most successful invertebrate phyla, with the second largest diversity among invertebrates. Generalized mollusc characteristics are a radula, 4 nerve cords (ventral and lateral side) as well as calcareous secretions (Ruppert and Barnes, 1996). In particular, as seen in many conchiferans, the shell is one of the most outstanding characteristics. Such shells serve as a mechanical defense and structural support.

Calcareous shells are normally made of calcitic or aragonitic, but there are some reports indicating the presence of vaterite (Qiao et al., 2007) and ACC (Addadi et al., 2003). Crystals of calcium carbonate are formed between organic matrix layers, which are secreted from mantle epithelium (Wilbur, 1972). Shell shape varies considerably between conchiferans, from coiled snail shells to the two valves of bivalves to the internal shell of squids. The shape of the shell corresponds to the morphology of the mantle tissue (Beesley et al., 1998).

1.3.1. Classification of Mollusca

The phylum Mollusca contains eight classes, Caudofoveata, Solenogastres, Polyplacophora, Tergomya (within which the order Tryblidiida contains present day "monoplacophorans"), Bivalvia, Scaphopoda, Cephalopoda, and Gastropoda (Salvini-Plawen, 2007). In addition, there are two Paleozoic fossil classes Helcionelloida and Rostroconchia (Runnegar and Pojeta, 1974). The conchiferan status of Tergomya, Bivalvia, Scaphopoda, Cephalopoda, and Gastropoda is clear. The classification of Caudofoveata, Solenogastres, and Polyplacophora, however, continues to be argued. To date, 2 categorization models — Aculifera and Testaria — have been forwarded. In the aculiferan model, Caudofoveata, Solenogastres, and Polyplacophora are classified into same group Aculifera (Scheltema, 1993). In the Testaria model, polyplacophorans are classified in Testaria with other conchiferans (Salvini-Plawen, 2007). The idea of aculiferans is mainly based on the fossil record with dorsal valve, foot, and serial repetition (Sigward and Sutton, 2007). Salvini-Plawen (2007) pointed out some problems with this idea, mainly ignorance of the foot in Solenogastres and the misinterpretation of *Acaenoplax*, while Silurian fossil *Acaenoplax* with serialized morphology and spines would be still one of the attractive candidates for the primitive state of molluscs (Sutton et al., 2004). Some anatomical data support the Testaria model as well (Salvini-Plawen, 2007). For example, the midgut and intestine structures and the presence of midgut glands show homology to conchiferans. There are some homologies between Polyplacophora and Tergomya, which are recognized as the basal taxa of conchiferans. These include the paired esophagus with longitudinal folds and ciliary tracts, paired glandular esophagus pouch, radula apparatus configuration, dorso-ventral muscle bundles, and differentiation of pericardioducts into excretory organs. Current molecular phylogenetic approaches also support the closer relationship between Polyplacophora and Tryblidiida (Giribet et al., 2006). For this reason, there is the idea that polyplacophoran shell plates and sclerites as precursors of conchiferan shells. Nevertheless, polyplacophorans first appear in the Upper Cambrian, whereas univalved moluscs, such as the fossil helcionelloideans and rostroconchs, gastropods and bivalves have all already been reported from the Lower Cambrian (Runnegar and Pojeta, 1974). The fossil record then tells a different history and still phylogenetical relationship is uncertain.

1.3.2. Shell formation and calcium carbonates

In mollusc (conchiferan) shell formation, shell is made from extrapallial fluid secreted from outer mantle epithelium (OME) cells between haemolymph and extrapallial fluids. This process is classified as the extracellular calcification process (Mann, 2001). The mantle first secretes an organic membrane (periostracum), and the calcification process occurs at the fluid-filled space (extrapallal space) between the periostracum and the mantle.

For shell formation, calcium and carbonate ions are the most important components because shell mainly consists of calcium carbonate. The necessary calcium ions have been considered to be taken up from sea water and from the diet, although this is not yet clarified. Wilbur and Saleuddin (1983) and Coimbra et al. (1988) investigated inorganic ion concentrations in extrapallial fluids. The calcium ion concentrations in the extrapallial fluid of marine molluscs (10.7–11.8 mM) are quite similar to sea water (9.3 mM), but the values are lower in freshwater species (3.9–6.1 mM). The concentration of Ca in freshwater is only 0.2 mM. The calcium ion concentrations in haemolymph and extrapallial fluids are about same. Carbonate ions are higher in extrapallial fluid in all cases.

The supply system of calcium and carbonate ions to shell has been relatively well studied in freshwater bivalves, e.g. *Anodonta* (Lopes-Lima et al., 2008).

Considering the importance of calcium homeostasis in eukaryotic cells, the regulation of calcium content in the cell is crucial. The outer mantle epithelium has been assumed to play some role here. Although the presence of an ion channel/transporter for extrusion would be quite reasonable, no calcium pump has been detected to date at the basal/apical area in OME, but PMCA-like proteins (human plasma membrane Ca^{2+}-ATPase 1) appear to localize in the cytosol (Lopes-Lima et al., 2008).

Almost nothing is known about calcium metabolism in molluscs. In vertebrates, a peptide hormone named calcitonin (CT) and calcitonin gene-related peptides (CGRP) are important for calcium homeostasis. CT, however, has not been found molluscs and other protostomes, and only a CGRP-like peptides receptor has been reported (Lafont et al., 2007). In molluscs, cDNA of CGRP-like peptides receptor was cloned only from *Crassostrea gigas* (Dubos et al., 2003). The presence of CGRP-

like peptide as a ligand has not been confirmed, and only immunohistochemical detection has been reported (Lafont et al., 2007). Mollusc calcium homeostasis and a recruit of calcium ions to extrapallial fluids remain an enigma.

1.3.3. Shell layers, shell microstructures and crystal polymorphism

In principle, the mollusc shell is made up of several layers. For instance, typical nacre shells are made from organic periostracum, outer calcified (prismatic) layer, and inner calcified (nacre) layer. Calcified shell layers consist of calcite or aragonite and possess a special microstructure that is identifiable and characteristic for each shell. Publications on the classification of shell microstructure are available (Taylor et al., 1969; Carter and Clark, 1985; Chateigner et al., 2000). In general, shell microstructures are categorized into nacre, foliate, prismatic, cross lamellar, and homogeneous microstructure. This classification is based on SEM observations. Nacre is classified into sheet nacre and columnar nacre, and all nacres are aragonitic (Wise, 1970; Erben, 1972). In sheet nacres present in bivalves, the tablets grow laterally, and the extension of crystals is relatively quick. Columnar nacres present in cephalopods and gastropods have a "stack of coins" structure, as seen in *Haliotis* (Gastropoda) (Nakahara, 1991). In this type of nacre, the crystal between successive sheets appears to nucleate to the center of pre-existing tablets. Foliated structures are normally calcitic (Checa et al., 2007) but some of them are aragonitic (Checa et al., 2009a). There are prismatic calcitic and aragonitic, and fibrous prismatic aragonitic microstructures. Cross lamellar microstructures comprise simple crossed lamellar microstructures, complex crossed lamellar microstructures, and intersected crossed platy microstructures. These are normally aragonitic.

Crystal polymorphism is not always same between similar taxonomic groups. Sclerites and shell plates of aplacophorans and polyplacophorans were reported as being aragonitic (Treves et al., 2003). Scaphopods and cephalopods with calcified shells have entirely aragonitic shells (Falini et al., 1996; Reynolds and Okusu, 1999). For instance, scaphopod shells are made up of 3 calcified layers: outer thin prismatic shell layer, middle cross lamellar structure, inner calcified shell layers.

The calcitic prismatic layer and aragonitic nacre layer are present in only a few bivalve and gastropod taxa (Mutvei, 1970, 1979). While, *Nautilus* and freshwater nacre shelled bivalves possess aragonitic prismatic layers, *e.g.*, *Unio pictorum* (Marie et al., 2007). Qiao et al. (2007) found vaterite in freshwater pearls.

Patellogastropod limpets (Gastropoda) have 4–6 calcified shell layers (Fuchigami and Sasaki, 2005) and these contain different types of shell structures, *e.g.*, calcitic/aragonitic homogeneous structure, calcitic/aragonitic cross lamellar structure, and foliated structure (Hedegaard et al., 1997). On the other hand, other bivalves (*e.g.*, Heterodonta and Anomalodesmata) and most marine gastropods tend to possess entirely aragonitic shells. Entirely calcitic shells are quite uncommon in molluscs, and are limited to some Ostreoida with calcitic foliated and prismatic shell structures, *e.g.*, *Crassostrea gigas* (Bivalvia), although they have an aragonitic myostracum, as all bivalves do (Taylor et al., 1969).

Shell organic matrices are essential to form any shell structures. The composition of shell organic matrices can differ in the same shell structure. Nacre research has confirmed the different organic matrices in different positions (Bevelander and Nakahara, 1969; Bezares et al., 2008). For instance, interlamellar sheets are located between the nacre tablet layers, the intertabular/intercrystal sheet is located between each nacre tablet in the same layer, and intra-crystal organic matrices are located inside crystals. Eventually, it is required to investigate correlation between shell organic matrices with each shell structure and the position of shell organic matrices in each shell layer.

In this study, we investigated chitin and sulphated groups in some aculiferans and shell organic matrices of conchiferans. As stated above, biochemical research about aculiferans is little and need to be investigated with some taxonomic or evolutional point of view. Our new data will give some new useful insight to consider the evolution of shell and shell plate.

2. Methods

2.1. Shell organic matrices preparation

Calcified conchiferan shells and shell plates or sclerites of *Acanthopleura villantii* and *Acanthopleura japonica*, polyplacophorans, were incubated with 3% NaOCl overnight in order to remove organic contaminants. Air-dried samples were ground into powder (only shell plates were incubated with 3% NaOCl overnight again to remove neural tissues of photoreceptors). After lyophilization, samples were demineralized completely with 0.5 M EDTA (pH 7.8). The solution was centrifuged at 13,000 rpm for 3 min at room temperature and the supernatant (soluble organic matrices) and insoluble organic matrices were separated. Insoluble organic matrices were washed with water and lyophilized.

2.2. Infrared spectroscopy (IR)

IR requires relatively small amounts of organics and hence is useful in analyzing small shells. Crude shell organic matrices are a mixture of protein, chitin, neutral polysaccharides (*e.g.*, glucan) and lipids. IR spectra of crude shell organic matrices thus are not conclusive, even if the spectra look identical to the chitin standards. Accordingly, comparing IR spectra before and after protein and chitin removal by enzyme treatment is better suited in order to infer the presence of chitin. A washing step after each enzyme treatment removes any solubilized peptides (from insoluble proteins) and oligosaccharides (digested from the chitin) as well as the enzymes themselves. The remaining insoluble materials were subjected to IR spectroscopy.

Pepsin was used before chitinase treatment. Chitin, proteins, and some lipids form complexes in the insoluble fraction. Because amide peaks derive from both protein and chitin, removing as much protein as possible before IR is necessary in order to restrict the source of the amide peaks to chitin. In addition, breaking up the chitin–protein complexes is necessary in order to expose and digest chitin efficiently. Since we used several enzymes, we had to consider the pH for the enzyme reactions. The optimal pH for chitinase is about 6–8, and that for pepsin about 2–4. Pepsin is irreversibly inactivated at pH 6, while most other proteases, such as proteinase K (from *Tritirachium album*; Sigma-Aldrich P6556, St. Louis, MO) and trypsin, remain active at neutral and basic pH. Therefore, even if pepsin cannot be removed completely and remains during chitinase treatment, it cannot digest chitinase or any other proteins. Chitinase is specific to chitin. The changes in the amide and hexose peaks after chitinase addition were clearly due to degradation of the chitin into soluble oligosaccharides.

The crude insoluble organic matrix was washed with distilled water and dried by lyophilizer. To remove the protein amide peaks in the infrared absorption spectra, 1% (w/v) pepsin A (from porcine gastric mucosa; Sigma-Aldrich P7125, St. Louis, MO) in acetic acid solution (pH 3.0) was added and the solution with matrices was incubated at 37 °C overnight. Chitin was removed from the pepsin-treated organic matrix with 0.1U/80 µl chitinase (from *Streptomyces griseus*; Sigma-Aldrich, C6137, St. Louis, MO) in a phosphate buffer (pH 6.8) at 37 °C for 3–5 days.

KBr micropellets with a sample/KBr weight ratio of about 0.0025 were prepared from the insoluble organic matrix for IR powder measurements. A mixture of powdered samples and KBr was homogenized and dry-powdered by hand-grinding in an agate mortar. Spectra from 2,000 to 400 cm^{-1} were recorded using a Perkin-Elmer FTIR spectrometer 1760X equipped with a TGS detector and a CsI microfocus accessory (Perkin-Elmer, Tokyo). Background and sample spectra were obtained from 24 scans each with a nominal resolution of 4 cm^{-1} (Furuhashi et al., 2009).

Previous IR research on bacteria cell walls (Marcotte et al., 2007) revealed that it is necessary to compare amide II peaks and hexose peaks in order to estimate insoluble polysaccharides and proteins. Glucan and mannan, as insoluble neutral polysaccharides, have no *N*-acetyl bond. The IR spectra are therefore mainly hexose peaks. Hence we focused on amide II and hexose peaks for comparison.

2.3. Pyrolysis GC-MS

Since intact insoluble organics are difficult to analyze by traditional chromatography techniques, pyrolysis GC-MS was used. Pyrolysis of insoluble samples decomposes them into volatile molecules and makes it possible to separate and identify the decomposition fragments by gas chromatography and subsequent mass spectrometry. In addition, as compared with the usual GC/MS methods, this approach yields the relative ratio of chitin:protein: neutral polysaccharides in a single experiment, with no derivatization steps or special modifications. *N*-acetyl glucosamine is the only *N*-acetyl hexosamine found in insoluble shell organics, and it is derived mainly from chitin. Chitin is a polysaccharide with *N*-acetyl bonds, and its thermal decomposition products are very characteristic for chitin (*e.g.*, acetylpyridone); such products therefore cannot be found in any other polysaccharides and can be used as markers of chitin. Several putative protein-derived pyrolysis fragment compounds were abundant enough to appear in GC/MS chromatograms because not all insoluble proteins were digested by pepsin. To ensure that these were protein-derived and not humic acid-derived compounds, we verified their identity by MS.

Analysis of the insoluble material was generally performed after pepsin treatment in order to remove some insoluble peptides. Py-GC/MS measurement was done in a Pyroprobe 2000 (Chemical Data Systems, CDS Analytical Inc, Oxford, PA) pyrolyser equipped with a platinum coil and a quartz sample tube, coupled to an Finnigan GC 8000/MD800 GC/MS system (now Thermo Fisher Scientific, San Jose, CA) Samples were heated to 615 °C at 400 °C s^{-1} and held at this temperature for 15 s. The volatile pyrolysis products were separated on a Chrompack CP-Wax 52 CB column (30 m × 0.25 mm × 0.25 μm) (Varian, Boston, MA) with helium 4.6 as carrier gas (100 kPa). The pyrolysis chamber was held at 250 °C and flushed with helium 4.6. The GC was programmed from 50 °C (2 min) to 260 °C (30 min) at 10 °C min^{-1}. The mass spectrometer was operated in electron impact mode with a scan range of *m/z* 15–400 and the products were identified by comparison with NIST and Wiley mass spectral libraries as well as data in the literature.

To compare the relative contents of chitin, carbohydrates, and proteins, three to four selected marker compounds for each class were quantified using reconstructed ion chromatograms with the base ion of each mass spectrum and the sum of the peak areas set into relation. Pyrolysis products were identified based on their mass spectral characteristics and GC retention times, by comparison with the NIST mass spectral library and published GC and MS data. The relative standard deviation of the peak areas of the pyrolysis-GC/MS total ion chromatograms ranged from 5 to 25% when replica experiments were carried out within a few days. To distinguish chitin from other polysaccharides, we use the term neutral polysaccharides, meaning polysaccharides with no acidic or amine bond, such as glucan and mannan with no *N*-acetyl or acidic bond.

2.4. DMMB staining (histology)

Specimens (*Prochaetoderma boucheti*, (Caudofoveata); *Wirenia argentea*, (Solenogastres); *Lepidochitona cinerea* (Polyplacophora)) were fixed in 10% formalin containing 1% cetylpyridinium chloride for several days. Cetylpyridinium chloride was added to inhibit diffusion of glycosaminoglycan, and to facilitate its precipitation in the tissue.

Fixed tissues were dehydrated through a graded series of ethanol into xylene (70% ethanol for 45 min, 90% ethanol for 45 min, 100% ethanol 45 min (two changes), and 100% xylene 45 min (two changes)). Tissues were then paraffin embedded and 5–8μm sections cut using a microtome. Sections were deparaffinised through xylene and a graded series of ethanol (100% xylene for 10 min (two changes), ethanol:xylene (1:1) for 5 min, then 100%, 90% and 70% ethanol for 10 min each). The sections were then washed in water for 10 min and stained with DMMB solution (100 mL aqueous colour reagent contains 1.6 mg DMMB, 237 mg NaCl, 304 mg glycine and 95 μL of 32% HCl) for 30 min at room temperature (Farndale et al., 1986). Sections were washed with water prior to observation.

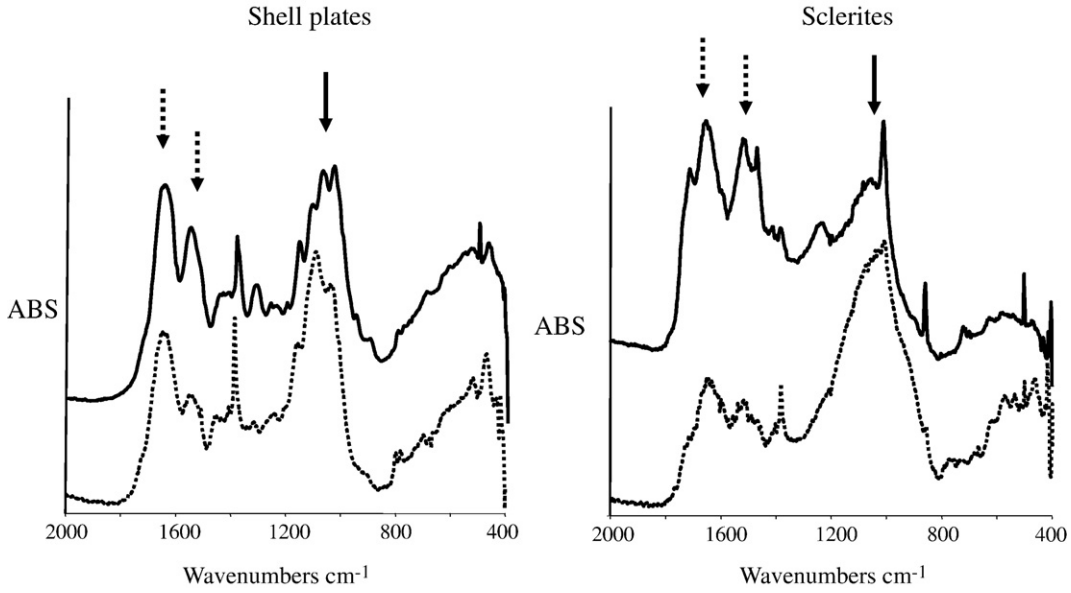


Fig. 4. IR spectra of insoluble shell organic matrices of *Acanthopleura villantii* shell plate and sclerites; Solid and dotted lines indicate pepsin-treated (before chitinase) and chitinase-treated organic matrices, respectively. After chitinase treatment, relative height of amide peaks to sugar peaks was reduced. Dotted and solid arrows indicate amide peaks (amide I; 1650 and amide II; 1550 cm^{-1}) and hexoses peaks (at about 1070 cm^{-1}), respectively. Spectra are offset for clarity.

2.5. Amino acid analysis

Amino acid analyses were carried out on soluble shell organic matrices using the PICO-TAG Amino Acid Analysis System (Waters, Milford, MA, USA). This method uses pre-column derivatization with phenylisothiocyanate, followed by separation of the arising products by HPLC in a reversed-phase column (C18; Pico-Tag column 25 cm × 4.6 mm I.D.; Waters) using an acetate (pH 6.4)–acetonitrile gradient. Protein hydrolysis was done in HCl vapour (6 M HCl with 2% phenol) for 20 h at 110 °C in a vial with an inert atmosphere of nitrogen under vacuum.

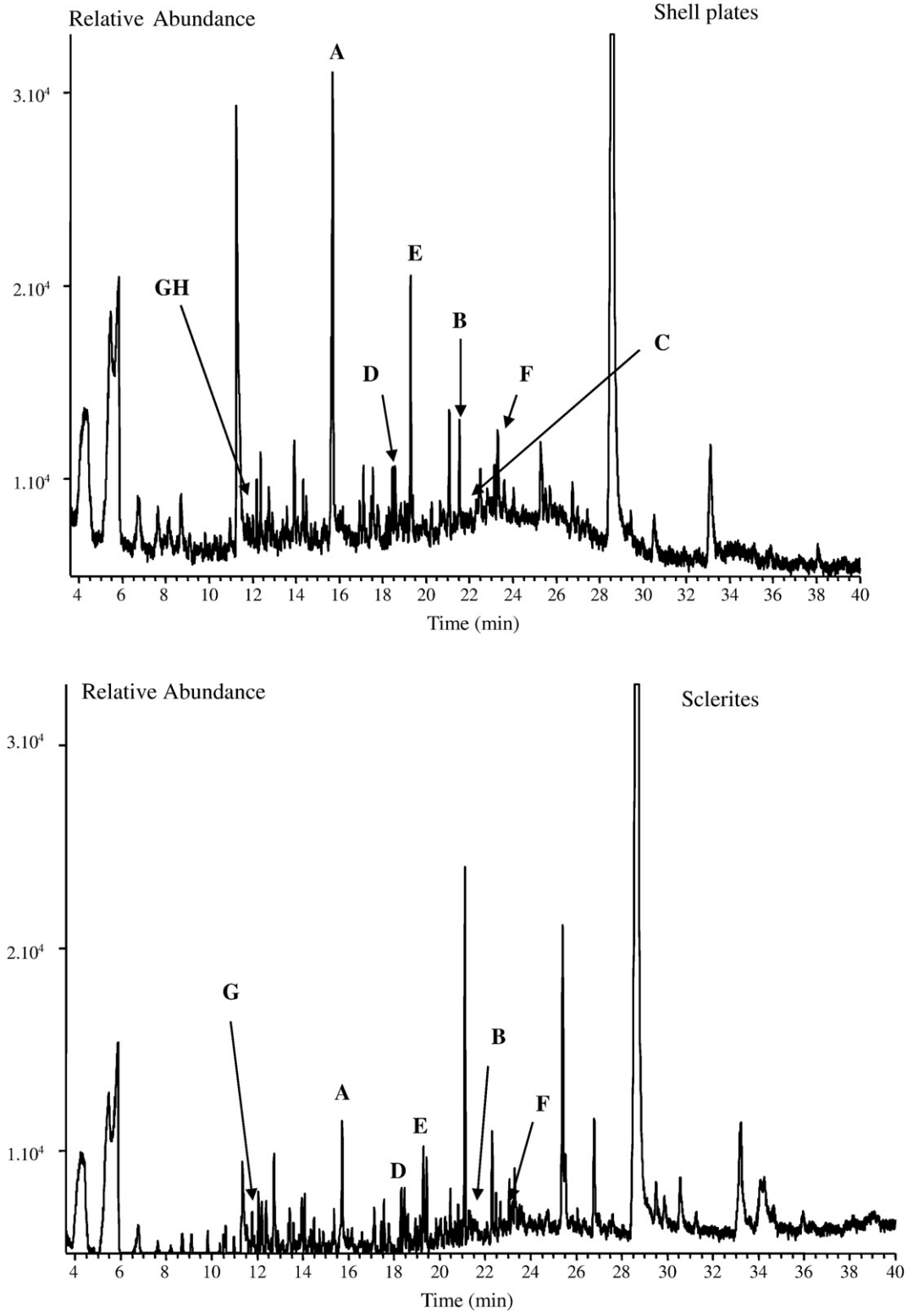


Fig. 5. Pyrolysis GC/MS total ion chromatograms of polyplacophoran shell plates and sclerites insoluble organic matrices (pepsin treated). A, B, C are chitin decomposition peaks; D, E, F are protein decomposition peaks; G, H are neutral polysaccharide decomposition peaks. A, acetamide (15.8 min); B, acetamido pyrone (21.3 min); C, acetamidomethylfurane (21.7 min); D, phenol (18.5 min); E, cresol (19.3 min); F, indole (23.2 min); G, furfural (11.8 min); H, furanon (11.9 min).

Table 1
Ratio of neutral polysaccharides/chitin in cuticle, sclerites, shell plate, and shell organic matrices assessed by pyrolysis GC/MS analysis.

Specimens	Neutral polysaccharide/Chitin
Neomenimorpha sp. (cuticle)	0.068
Acanthopleura japonica (shell plates)	0.042
Acanthopleura japonica (sclerites)	0.449
Nacre	
Pinctada fucata	0.025
Atrina japonica	0.000
Nautilus sp	0.006
Haliotis discus	0.035
Prismatic	
Pinctada fucata	0.407
Atrina japonica	0.038
Haliotis discus	0.019

All samples were pepsin treated to see clear chitin and neutral polysaccharide decomposition peaks.

3. Results

We detected both amide peaks (amide I;1,650 and amide II;1,550 cm^{-1}) and hexose peaks (at about 1,070 cm^{-1}) in IR spectra of both *Acanthopleura japonica* shell plates and sclerites. Chitinase treatment after pepsin treatment reduced the 2 amide peaks of the polyplacophoran shell plates (Fig. 4) and of the sclerites of *Acanthopleura japonica*. This change after chitinase treatment indicates the presence of chitin as well as proteins binding to chitin in insoluble shell organics.

In all insoluble organics analysed here, the presence of chitin, protein, and neutral polysaccharides was confirmed. Acetamide and acetamidopyrone, as chitin decomposition markers, were identified from pepsin-treated solenogastres cuticle, and from polyplacohoran (*Acanthopleura japonica*) sclerites and shell plates (Fig. 5). Additionally, acetamidemethylfuran was recorded in shell plate insoluble organics. Phenol, cresol, and indole as protein decomposition markers were clearly identified from all samples. Furfural and furanon, as neutral polysaccharide decomposition markers, were identified in solenogastres cuticle and polyplacophoran shell plates. Only furfural was found in the polyplacophoran sclerites. The chitin/protein ratio of the polyplacophoran shell plates (2.407) was higher than in the sclerites (0.373), whereas the ratio of neutral polysaccharides to chitin was higher in sclerites (Table 1). The chitin/protein ratio of the cuticle of solenogastres (0.674) was intermediate between shell plates and sclerites, but the neutral polysaccharides/protein ratio was lower than in both.

Cuticle of *Prochaetoderma boucheti* (Caudofoveata) and *Wirenia argentea* (Solenogastres) and musculature under cuticle were stained by DMMB (Fig. 6A,B). In *Wirenia argentea*, foot mucus glands at the ventral side stained as well. Data confirmed the presence of sulphated groups in these aplacophorans cuticle. On the other hand, Polyplacophora (*Lepidochitona cinerea*) cuticle did not have sulphated groups, although connective tissues inside of girdle stained faintly (Fig. 6C). Sclerites were not visible in all specimens here, possibly because of decalcification at fixative preparation.

3.1. Amino acid analysis

In principle, all tested sample showed large proportion of Gly (23.7–48.1) but lower proportion of Ala (1.2–4.4). *Haliotis discus*

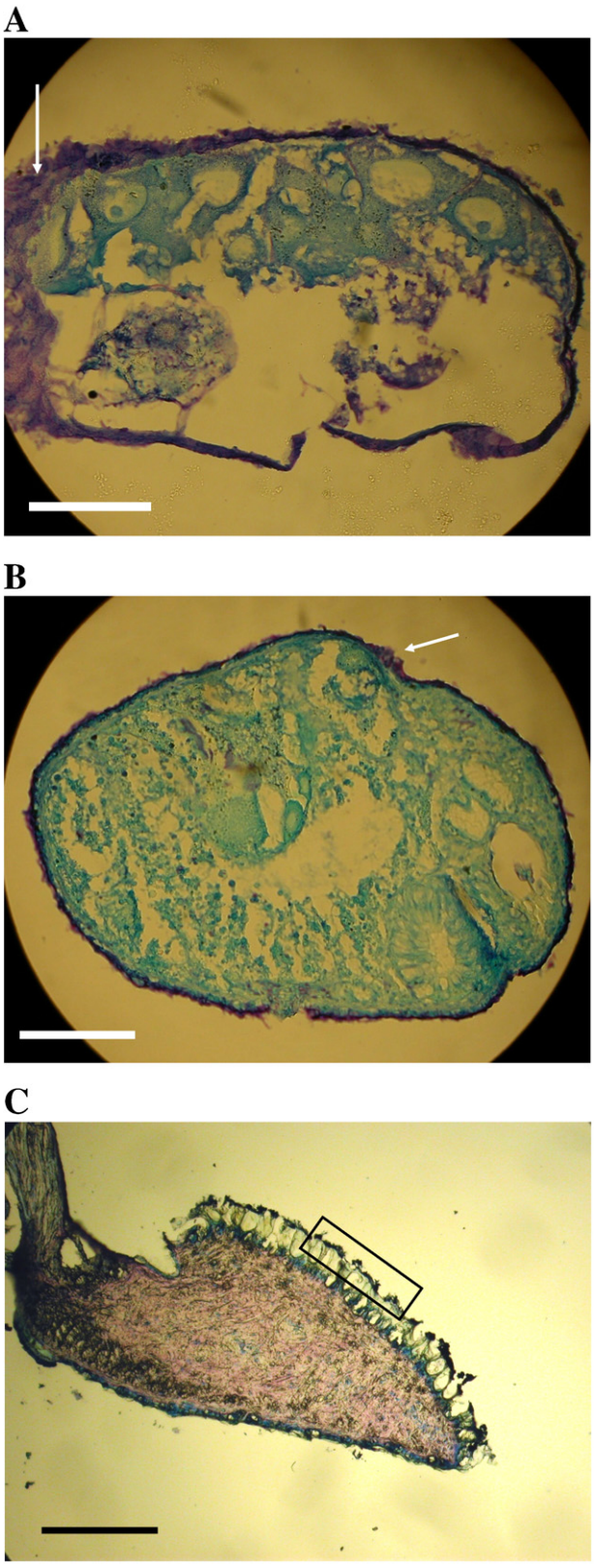


Fig. 6. A) Transverse section of *Prochaetoderma boucheti* (Caudofoveata). Section was stained with Dimethyl methylene blue (DMMB). Purple color indicated the presence of sulphated sugars. Arrow indicates cuticle part. Scale bar is 100 μm. B) Transverse section of *Wirenia argentea* (Solenogastres). Section was stained with Dimethyl methylene blue (DMMB). Purple color indicated the presence of sulphated sugars. Arrow indicates cuticle part. Scale bar is 100 μm. C) Transverse section of *Lepidochitona cinerea* (Polyplacophora). Section was stained with Dimethyl methylene blue (DMMB). Purple color indicated the presence of sulphated sugars. Black box part indicates cuticle part, which was not stained as purple color. Scale bar is 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prismatic soluble shell organics and *Patella* sp soluble shell organics contained lower Asx (6 and 6.4) and Glx (2.8 and 2.4). On the other hands, *Haliotis gigantea* nacre soluble shell organics showed larger Asx (39.0). With regard to basic amino acid, *Patella* sp showed higher proportion of Lys (5.4), but all other basic amino acid in other samples were not significant. Another characteristic was high proportion of Pro (16.2) in *Patella* sp.

4. Discussion

4.1. From sclerites to shell plates

Aculiferans comprise the worm-like Solenogastres and Caudofoveata, and the Polyplacophora. The former two are covered by a cuticle with embedded calcareous sclerites, whereas the latter also possess

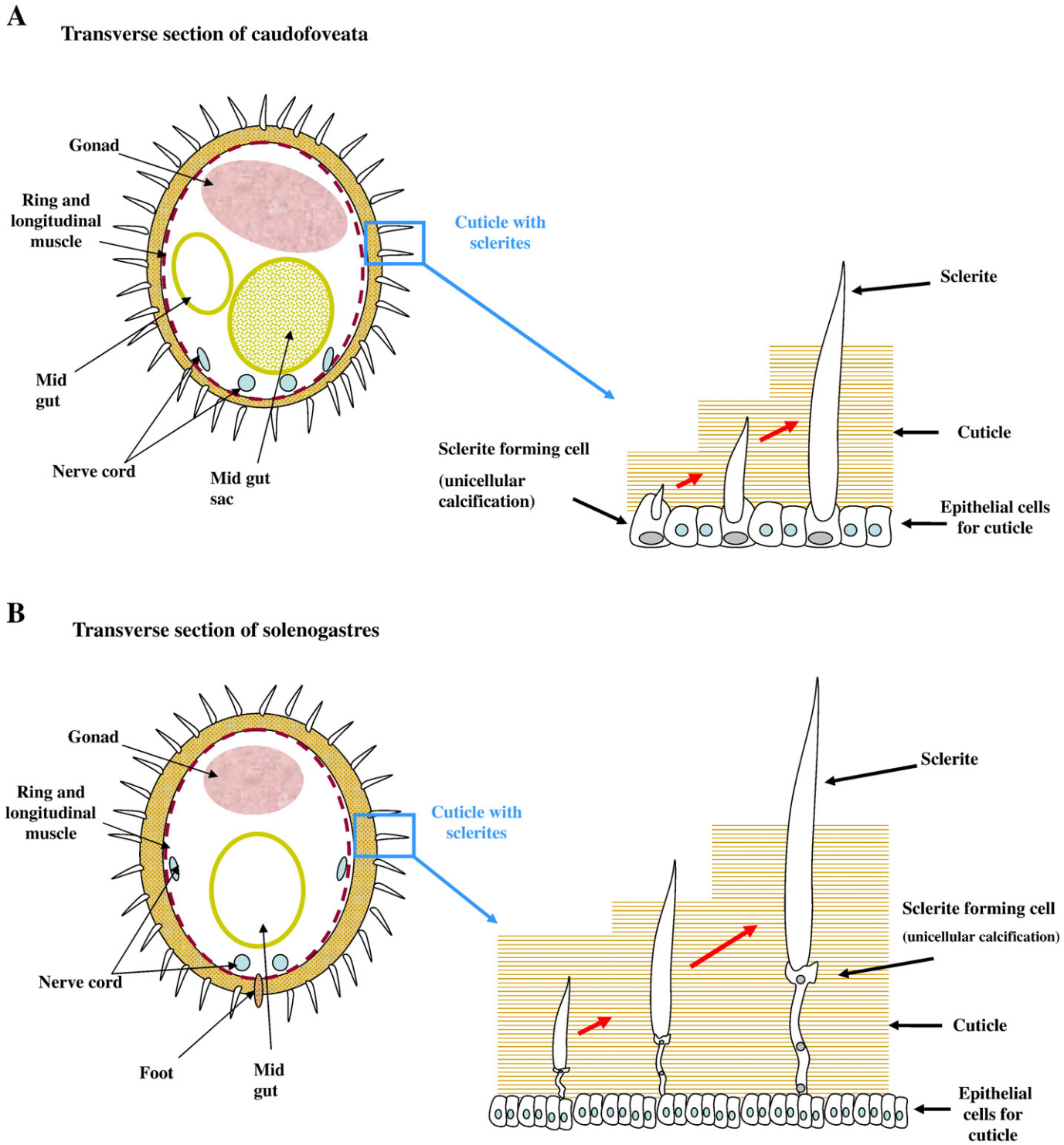


Fig. 7. A) Scheme of caudofoveata sclerite formation. Sclerites are formed unicellulary. B) Scheme of solenogastres sclerite formation. Sclerites are formed unicellulary. C) Scheme of polyplacophoran sclerite and shell plate formation. Sclerites are formed multicellulary. Shell plates are formed extracellulary.

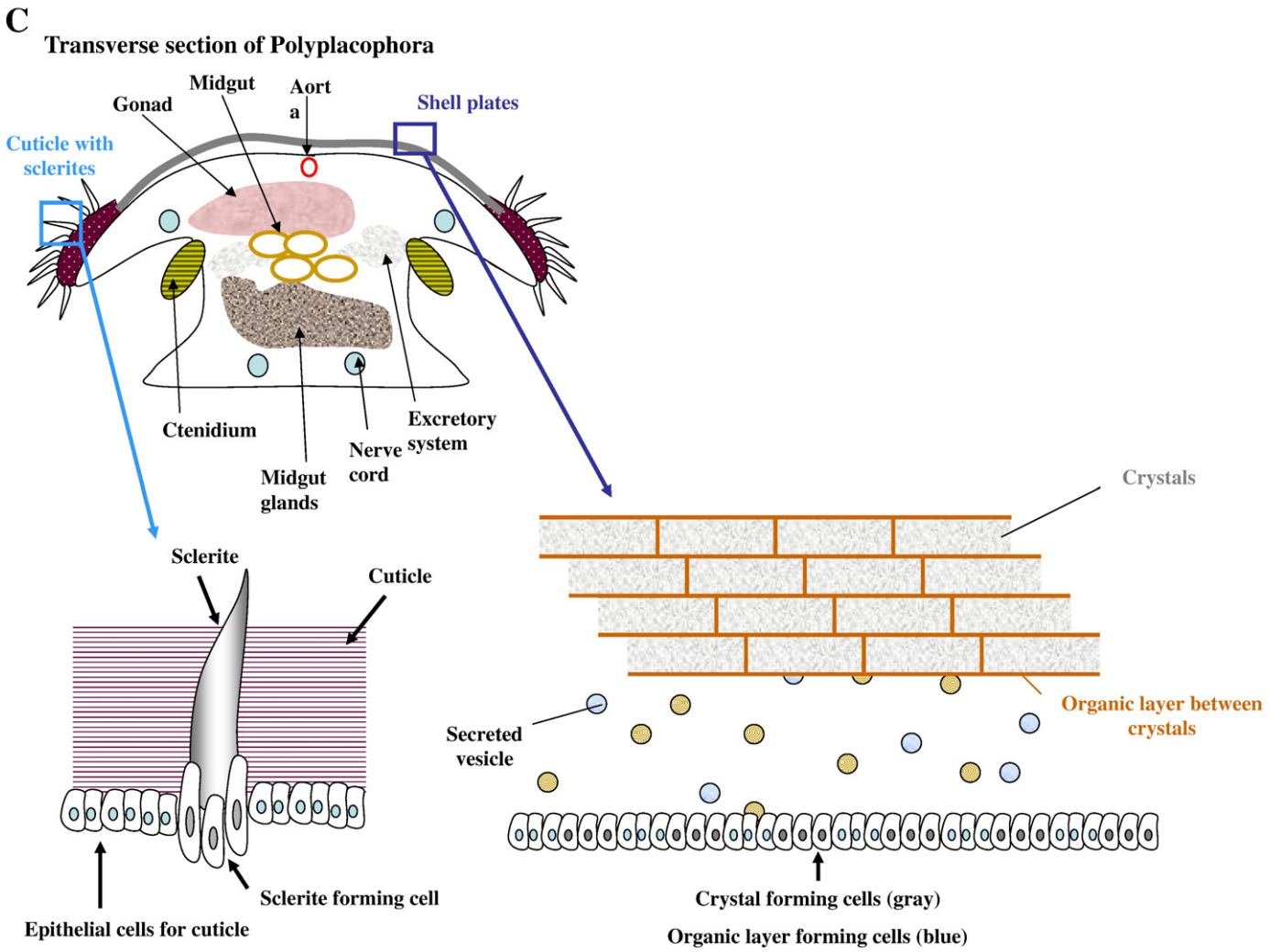


Fig. 7 (*continued*).

eight shell plates and sclerites instead of a single shell. These structures are considered precursors of the single conchiferan mollusc shell (Haas, 1981).

Shell is present in all conchiferan groups except in shell-less gastropods (*e.g.*, certain opisthobranchs and pulmonates) and cephalopods (*e.g.*, Octopodiformes).

Although, in the past, some researchers thought that sclerites were formed intracellularly like spicule formation in poriferans and echinoderms, aplacophoran sclerites are actually formed by a unicellular extracellular calcification process (Fig. 7A,B), and polyplacophoran sclerites as well as shell plates are formed by a multicellular extracellular calcification process (Fig. 7C). All conchiferan shells are produced by multicellular extracellular calcification processes. Based on SEM observations, sclerites of aplacophorans and polyplacophorans have an aragonitic prism structure (Salvini-Plawen, 1987; Treves et al., 2003). Apparently, mollusc calcification initially involved aragonitic prisms.

Since sclerites are comparable to shell plates/shell, it would be worthwhile to scrutinize each of the components presence of the chitin-silk fibroin gel proteins-acidic macromolecules model. Chitin is recognized as important structural component and the abundance would be one of the important biochemical characteristics in view of evolution. The presence of chitin in polyplacophoran shell plates was first reported by Poulicek and Kreusch (1986). All tested polyplacophoran shell plates contained significant amounts of chitin, which was chemically identified. Poulicek et al. found chitin in polyplacophoran sclerites as well as in some aplacophoran sclerites. On the other hand, the results of Treves et al. (2003) are controversial. They used IR to examine insoluble sclerite organics of *Acanthopleura villantii* (*A. spiniger* in the article) and concluded there was no peak indicating chitin presence. There were also no amide peaks in their IR spectrum, although they found proteins based on amino acid analysis. In our present study, we detected different IR patterns before and after chitinase treatment (Fig. 4). Furthermore, acetamide and acetamidopyrone, as chitin decomposition markers, were identified from pepsin-treated polyplacohoran (*Acanthopleura japonica*) sclerites insoluble organic matrices by pyrolysis GC/MS analysis (Fig. 5). The combined approach involving IR spectra change by chitinase and pyrolysis GC/MS clearly confirmed the presence of chitin in polyplacophoran sclerites.

The previous hypothetical concept based on anatomical data is that calcium carbonate crystals evolved as a fusion of sclerites, and that the organic layer between crystals originated from cuticle (Fig. 8). However, we still lack biochemical data to support or refute this hypothetical concept. For instance, reports on aplacophoran chitinous cuticle are not precise and lacked substantial data based on biochemistry. In an earlier study, Poulicek and Kreusch (1986) showed that shell plates of all polyplacophora species contained more chitin (159.8–411 mg/g insoluble organics) than sclerites and the cuticle. The chitin abundance in aplacophoran cuticle (Candofoveata, 15.4–32.3 mg/g insoluble organics; Solenogastres, 76.9–81.7 mg/g insoluble organics) clearly exceeded

that of spicules (0–9.8 mg/g insoluble organics). The same relationship was seen in polyplacophorans, where cuticle (15.7–156.7 mg/g insoluble organics) had more chitin than sclerites (1.8–14.9 mg/g insoluble organics). Our present pyrolysis GC/MS data revealed the same relationship, although all samples were pepsin treated in order to clearly elaborate chitin and neutral polysaccharide decomposition peaks. Indeed, the chitin:protein relative decomposition ratio was the largest in shell plates (2.407) and the smallest in polyplacophoran sclerites (0.373). The Solenogastres ratio was intermediate (0.674).

To date there is no evidence to support the presence of silk fibroin gel proteins in shell plates or sclerites or cuticle of aplacophoran/polyplacophorans. Our preliminary insoluble protein analysis of cuticle insoluble proteins by LC/MS/MS showed actin and myosin peptides in aplacophoran cuticles (data not shown). Since aplacophoran cuticle is strongly attached to muscles below the cuticle, we do not exclude the possibility that the actin and myosin peptide were from muscle structure. Future research will clarify these ambiguous parts.

With regard to acidic macromolecules, both shell plates and sclerites of polyplacophorans showed acidic proteins (around pI 4.5, 37 kDa) in 2D gel electrophoresis, and their acidity was relatively high among soluble proteins obtained from aragonite shell layers. Recent LC/MS/MS peptide analysis showed that these appear to contain collagenous peptides (unpublished data). At the same time, the ratio of sulphated groups:soluble proteins differed between sclerites and shell plates. In contrast to shell plates, polyplacophoran (*Acanthopleura villantii* and *Acanthopleura japonica*) sclerites contained more sulphated glycosaminoglycans to soluble proteins (unpublished data). Biochemical data on cuticle is quite limited, but we tested DMMB staining (with CPC fixation) on aplacophoran sections histochemically in order to determine the distribution of sulphate groups in their cuticle. Aplacophoran cuticle was positive, polyplacophoran cuticle negative (Fig. 6A–C), indicating a potential biochemical difference between aplacophoran and polyplacophoran cuticles. Although it is too early to draw definitive conclusions, the relationship between aragonitic prismatic structures in sclerites and sulphated groups might be interesting.

Colored shell organic matrices are an interesting phenomenon. All polyplacophoran soluble organics tested were deep brown in both shell plates and sclerites. In the UV/Vis spectra analysis (300–800 nm), one of ABS max of these was at 385 nm in *Acanthopleura villantii* shell plates and 370 nm in *Acanthopleura japonica* shell plates (data not shown). One previous report of carotenoids isolated from polyplacophorans was from tissues (intestine, gonad and mantle tissues) (Tsushima et al., 1989), but no reports on pigments from either polyplacophoran shell plates or sclerites are available. Since aplacophoran cuticle with sclerites has no special color, the presence of pigment in calcified tissues is a development first seen in the class Polyplacophora. Coincidentally, the evolution of midgut glands occurred in polyplacophorans (Salvini-Plawen, 2007). However, the potential role of a pigment complex and secretion in calcified tissues by the midgut gland is still speculative, and more data are required to elucidate the coloration mechanism.

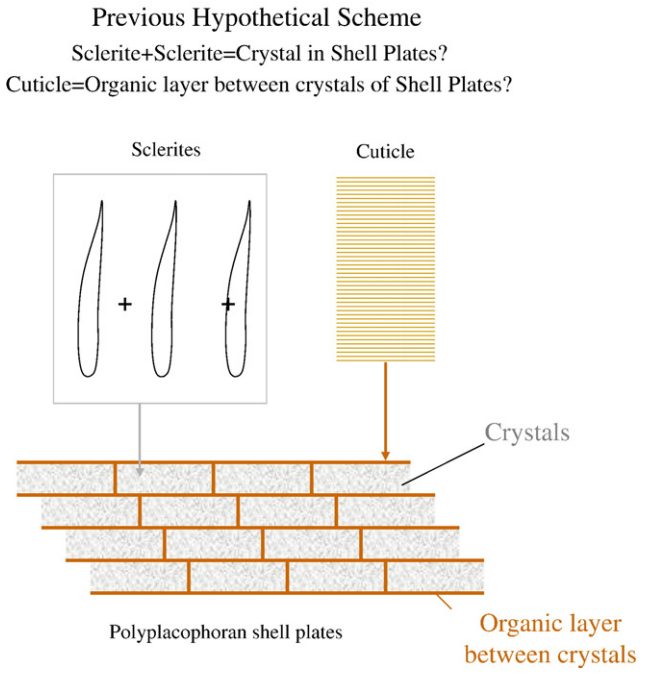


Fig. 8. Previous hypothetical scheme of shell plate evolution. Crystal and organic layer between crystals were considered to originate from sclerites and cuticle respectively.

Treves et al. (2003) stated that polyplacophoran sclerites contain 2–3% of organic matrices in calcified sclerites. Shell plates and nacre shells generally contain relatively large amounts of organic matrices as well (Addadi et al., 2006; Marin et al., 2008). Given that nacre has been recognized as a plesiomorphic shell structure (Checa et al., 2009a), the ancestral form of polyplacophorans and conchiferans might have had organic matrix-rich calcified parts. Furthermore, both shell plates and nacre shells tend to have a lower ratio of neutral polysaccharides:chitin, suggesting that the low ratio might be a plesiomorphic characteristic as well.

4.2. Conchiferan shell structure evolution

The diversification of mollusc shells occurred in the Lower Cambrian. Conchiferan diversification occurred in the deep sea. Firstly, helcionelloids and Tergomya, including Tryblidiida, were separated. Nacre has been present since the advent of monoplacophorans (Helcionelloida and Tergomya), which are a rather basal group of mollusc. Foliated structure appeared in bivalves, and a calcitic prismatic layer evolved with the appearance of pteriomorphs (Bivalvia) and haliotids (Gastropoda) The original condition in Vetigastropoda as well as Protobranchia (Bivalvia) is an outer fibrous prismatic aragonitic layer and an inner nacreous layer; Only Haliotidae have an outer blocky prismatic calcitic layer with nacre shells. Some advanced taxa in gastropods and cephalopods reduced/lost their shells, as seen in chitious shells and shell-less molluscs. The next sections introduce some biochemical relationships within shell structures, although the data are still fairly sparse.

4.2.1. Nacre/Foliated/Prismatic/Cross lamellar structures

Nacre has been observed in Tryblidiida (*e.g.*, *Veleropilina zografi*) (Checa et al., 2009a), Bivalvia (*e.g.*, *Pinctada*), Gastropoda (*e.g.*, *Haliotis*), and Cephalopoda (*e.g.*, *Nautilus*) (Bøggild, 1930; Grégoire et al., 1955; Mutvei, 1979; Chateigner et al., 2000). Some nuculoid, neotrigoniid and, to some extent, anomalodesmatan bivalves, freshwater bivalves (*e.g.*, *Unio pictorum*) and *Nautilus* possess entirely aragonitic shells, others have an outer calcitic prismatic layer. The biological significance of nacre evolution is not fully clarified. One plausible explanation is related to the hardness of nacre shell. Barthelat et al. (2006) found that it is stronger than inorganic aragonite. In fact, it is the strongest of all shell structures (cross lamellar, prismatic, and foliated structures) (Vincent, 2000; Lin et al., 2006; Esteban-Delgado et al., 2008) (Tables 2-1, 2-2 and 2-3). Accordingly, gaining nacre would provide the best mechanical protection.

The actual evolution of nacre is a matter of debate. Carter and Clark (1985) considered that it might be a transformation from the prismatic layer. Some tryblidiidans secrete nacre (*e.g.*, *Veleropilina zografi*) but it has been substituted by aragonitic foliated structure in other monoplacophorans (*e.g.*, *Rokopella euglypta* and *Micropilina arntzi*), due to absence of interlamellar membranes (Checa et al., 2009a).

Table 2-1
Mechanical property of calcified tissues.

Type	Tension (MPa)	Compression (MPa)	Bending (MPa)	*E* (Young's modulus) (GPa)	Hardness
Columnar nacre	78–116	320–401	193–267	47–64	122–211
Sheet nacre	35–86	304–419	117–211	31–58	106–221
Cross lamellar (Gastropoda)	31–60	198–336	58–165	30–58	162–270
Cross lamellar (Bivalvia)	9–43	163–336	35–106	50–82	242–298
Foliate	40–42	73–133	44–110	29–34	11–110
Cross foliate (Patella)	16–33	196–208	39–171	18–60	173
Prism (Bivalvia) calcitic	60–62	210–295	139	21–39	162
Cnidaria (Aragonitic coral skeleton)	X	22–47	25	10–62	X
Bryzoa semi nacre	X	X	24–50	42–65	X
Echinodermata (calcitic test)	X	X	95–190	31–69	X

Modified from Vincent (2000).

Two theories of nacre shell formation have been proposed and discussed (Checa et al., 2009b). The first is the heteroepitaxial theory developed by Weiner and Traub (1980). It holds that minerals grow epitaxially onto the protein chains of the organic matrices. The second is the mineral-bridge theory proposed by Schäffer et al. (1997) and the existence of mineral bridge was confirmed by Gries et al. (2008). Mineral-bridge theory can explain the difference between columnar nacre and sheet nacre based on the number and size of pores in the organic matrix membranes, which was also suggested by Cartwright and Checa (2007). To date, there is no clear evidence to rule out either theory completely. For example, the presence and the size of pore on organic layer itself was also pointed out in early study by Grégoire et al. (1955), but the pores have been sometimes doubted as artifact from preparative process. Recent study still could not preclude the possibility of artifact pores. On the other hands, the heteroepitaxial theory cannot always explain different type of nacres nor the retention of crystallographic orientation across superimposed plates. In addition, the theory was based on observation of the chitin-silk fibroin complex by X-ray diffraction, but there is still no substantial evidence for silk fibroin protein.

Our data indicated that the acidity of soluble nacre shell proteins varies, and that chitin abundance could differ (unpublished data). Indeed, chitin abundance in columnar nacre shells tends to be higher than that of sheet nacre shells in previous chitin research (Peters, 1972; Goffinet and Jeniaux, 1979; Zentz et al., 2001). In our pyrolysis GC/MS analysis, the chitin:insoluble protein decomposition ratio showed a similar tendency (*P. fucata*, 0.031; *Atrina japonica*, 0.046; *Nautilus* sp, 0.887; *Haliotis discus*, 0.231), although all samples were pepsin treated in order to clearly elaborate chitin and neutral polysaccharide decomposition peaks and the ratio was based on decomposition peaks. Moreover, this analysis showed that one of the common characteristics of nacres is a lower neutral polysaccharides:chitin decomposition peak ratio (<0.03) (Table 1). This value was higher than that of the prismatic layer in the same species as well as that of entirely aragonitic shells with cross lamellar structure. This relationship is the first common characteristic between nacres we have discovered.

Table 2-2
Mechanical property of calcified shells.

Type	Compression (kg/mm^2)	Bending (kg/mm^2)	Impact strength (number of impacts)	Density (relative)
Pinctada maxima (nacre)	38.2	36.1	69	2.74
Pinctada maxima (calcitic prismatic)	23.6	9.94	24	2.56
Pecten maximus (calcitic foliate)	10.2	ND	ND	2.67
Crassostrea gigas (calcitic foliate)	0.64	0.41	6	2.52

Modified from Esteban-Delgado et al. (2008). ND is none detected.

Columnar nacre and sheet nacre should differ biochemically. Apparently, columnar nacre shells tend to have more polysaccharides (*e.g.*, chitin) and bigger size and number of pores in the organic membrane (Cartwright and Checa, 2007), but the actual relationship between the abundance of polysaccharides and membrane pores is still speculative. The role and function of these polysaccharides are interesting and need to be clarified in future investigations.

Foliated structure is only present in few bivalves, mainly pteriomorphs. Compared with nacre, the amount of organic matrices in foliated structure tends to be quite small (Esteban-Delgado et al., 2008). It is reasonable that some shells have a foliated structure because nacre tends to be organic rich (Addadi et al., 2006; Marin et al., 2008) and this appears to be costly for molluscs.

Esteban-Delgado et al. (2008) conducted XRD texture analyses and discovered a similarity between foliated layers and the internal side of prismatic layers. Consistent with this observation, unusually acidic proteins that were cloned were from calcitic foliated/prismatic shell structures. Examples include MSP1 (Sarashina and Endo, 2001) and MPP1 (Samata et al., 2008) from pteriomorph foliated layers as well as Aspein (Tsukamoto et al., 2004) and Asprich (Gotliv et al., 2005) from pteriomorph prismatic layers. Most of these unusually acidic proteins are aspartic acid-rich form. Sharing similar, unusually acidic proteins supports the theory of a transition from a prismatic layer to a foliated layer (Esteban-Delgado et al., 2008). It is reasonable to envisage that unusually acidic proteins are especially important for calcitic shell formation.

Prismatic layers are of 2 types — calcitic prismatic layers and aragonitic prismatic layers. Most research has been done on the calcitic prismatic layer of pteriomorphs (Bivalvia), *e.g.*, *P. fucata* and *A. rigida*. Although data are still sparse, calcitic prismatic layers appear to be rich in sulphated glycosaminoglycans. In XANES analysis, sulphate groups (apparently associated with sugars) were identified in *Pinctada* and *Pinna* (Dauphin, 2003). In addition, the sulphate groups were more abundant in the calcitic prismatic layer than in the nacre of *Pinctada* (Dauphin et al., 2005, 2008). The data we obtained with colorimetric assays of soluble shell organic matrices confirmed that the calcitic prismatic layer contains more sulphated groups than the nacre layer in *Pinctada*, *Atrina*, and *Haliotis*. Data on prismatic layers composed of aragonite are not available, but polyplacophoran sclerites with prismatic aragonite structures also showed a higher abundance of sulphated groups over soluble proteins, as stated above.

Table 2-3
Mechanical property of calcified shells.

Type	Bending stress (MPa)	Compression (MPa)
Strombus gigas (cross lamellar)	29–74	166–218
Tridacna gigas (cross lamellar)	79.6 (outer layer only)	87–123
Haliotis rufescens (nacre)	177–197	233–540

Modified from Lin et al. (2006).

A higher abundance of sulphated groups appears to be common in calcitic prismatic layers, albeit *Nautilus* nacre also showed a higher abundance. These data preclude deducing a simple relationship between sulphated groups and calcitic prismatic layers. Moreover, most calcitic prismatic layers have an Mg/Ca ratio higher than other aragonitic shells; some studies show that prismatic calcite also has a higher abundance of sulphated groups (Dauphin et al., 2008).

Aspartic acid-rich proteins are present in some pteriomorph calcitic prismatic layers, for example Aspein (Tsukamoto et al., 2004) and Asprich (Gotliv et al., 2005). On the other hands, no reports are available on aspartic acid-rich proteins from gastropod calcitic prismatic layers which are present only in Haliotidae. Our current amino acid analysis of *Haliotis discus* calcitic prismatic soluble proteins showed a lower proportion of Asx than in pteriomorphs (Table 3), although nacre-soluble proteins in same species showed higher Asx.

No silk fibroin gel protein has yet been identified from either aragonitic or calcitic prismatic layers.

Cross lamellar structure is always aragonitic and is widespread in conchiferan groups, except for the calcitic cross foliated structures in patellogastropod limpets (Gastropoda). Cross lamellar structures also present in polyplacophoran shell plates. Indeed, this is one of the most ubiquitous structures in the phylum Mollusca. With regard to mechanical strength, it is quite rigid (Hou et al., 2004), although shells with this structure appear to be made from small amounts of shell organics (Dauphin and Denis, 2000). As such, it would theoretically be advantageous for many molluscs to possess relatively hard cross lamellar structures without secreting much organic matrices.

Although shells with cross lamellar structure are quite common, biomineralization research here is spotty. For instance, only relatively few shell proteins have been cloned. Dermatopontin is the only shell protein whose whole cDNA sequence has been cloned. Dermatopontin was originally found in the cross lamellar structure of *B. glabrata* (Marxen et al., 2003). Recent research by Sarashina et al. (2006) also revealed Dermatopontin in other pulmonates (*e.g.*, *Mandarina*, *Euhadra*, *Lymnaea*). Other research has characterized shell proteins by SDS-PAGE (Pokroy et al., 2006), but our knowledge on protein sequences remains very poor. Furthermore, most information about organic matrices involves amino acid analysis (Ghiselin et al., 1967), and even the acidity of soluble organics is poorly understood. Hence, little is known about the acidity of soluble proteins, and the presence of silk fibroin protein has not been documented. Several studies (Peters, 1972; Furuhashi et al., 2009) detected chitin from shells with cross lamellar structures. Although these studies did not isolate such structures, the abundance of chitin varied, and the abundance of chitin between different molluscs with cross lamellar structures also differed.

Table 3
Amino acid analysis of soluble shell organic matrices.

	Haliotis discus prismatic	*Haliotis gigantea* nacre	*Patella* sp entire shell
Asx	6.0	39.0	6.4
Glx	2.8	5.2	2.4
Ser	2.3	4.3	7.6
Gly	48.1	23.7	33.9
His	0.5	1.1	0.3
Arg	3.7	2.9	2.1
Thr	2.8	2.1	5.6
Ala	1.2	3.7	4.4
Pro	8.3	3.1	16.2
Tyr	10.3	2.8	2.6
Val	2.7	2.0	5.1
Met	0.5	0.5	0.4
Cys	3.1	0.4	2.3
Ileu	2.2	1.6	1.7
Leu	2.8	3.0	2.4
Phe	1.0	0.8	0.7
Lys	1.4	3.2	5.4

The number is molar percentage.

Together with our present data (Furuhashi et al., 2009) and this study, apparently the ratio of neutral polysaccharide:chitin in conchiferan shells with cross lamellar structure was higher (0.41–4.07) than in nacre (0–0.03), in prismatic layers (0.03–0.4) and in chitinous shells (0.01–0.02) as well as in polyplacophoran shell plates with cross lamellar structures (0.02–0.04). Despite the necessity to find new, innovative methods to examine insoluble shell organic matrices and to further improve pyrolysis GC/MS analysis, the higher ratio of neutral polysaccharide:chitin appears to be characteristic in conchiferan cross lamellar shell structures. The role of neutral polysaccharides and the correlation to cross lamellar structures are entirely unknown.

4.2.2. Shell loss

Chitinous shells and shell loss are seen only in opisthobranchs and pulmonates (Gastropoda) and in many cephalopods. In shell-less molluscs, some nudibranchs, *e.g.*, *Phyllidia* and *Phyllidiella*, have calcified spicules on the dorsal side of the mantle surface (Wägele and Willan, 2000), but no solid data are available about the crystal polymorphism and biochemical analysis of the spicules.

Chitinous shells in gastropods and cephalopods are internalized in the dorsal side of mantle tissues reduced in size. There are two ways to lose shells (Thompson, 1976; Thompson and Brown, 1984; Gibson, 2003). One is to cast off the shell at an early developmental stage as seen in many nudibranchs (Gastropoda). Another one is for the mantle to absorb calcium carbonates. Many pulmonates, *e.g.*, land slugs, and cephalopods are classified in this latter type (Boletzky, 2003). These shell reduction and loss has been recognized as being "positively" evolved and seen in advanced taxa (Mikkelsen, 1996). The evolutionary meaning of shell reduction and loss could be related to rapid movement in cephalopods (Beesley et al., 1998) and chemical defense in gastropods (Cimino and Ghiselin, 1998, 1999).

Internalized chitinous shells are clearly chitin rich and are especially typical in cephalopods, *e.g.*, *Loligo* (Beesley et al., 1998). It is still under discussion whether such chitinous shells are a single layer or composed of several layers (personal communication with Dr Y, Dauphin). Except for the fact that these shells are chitin rich, little is known about their biochemical characteristics.

Shell-less molluscs are poorly studied biochemically. Nothing is known about shell organic matrices of 'shell-less molluscs' because they only have larval shells (protoconchs) at early developmental stages. Our preliminary monosaccharide analysis on the whole body mucus of opisthobranchs and some gastropods indicated that the ratio of N-Acetyl hexosamines to mannose was higher in shell-less opisthobranchs (unpublished data). Chitin is a polymer of N-Acetyl glucosamine, and such a higher ratio would reflect a similar tendency in the evolution of chitinous shells.

Little is known about insoluble proteins in chitinous shells. Only Poulicek et al. (1991) conducted amino acid analyses on insoluble shell organic matrices of opisthobranchs; they found no clear relationship with shell reduction/loss. To date, no cloned insoluble protein sequences are available from these chitinous shells. Finally, the acidity of soluble shell organics is also poorly understood. Our recent 2D gel electrophoresis data on cephalopods with chitinous shells (*Todarodes* and *Loligo*) showed that some contain acidic proteins (unpublished data). Accordingly, it is untenable to conclude that chitinous shells are due to a lack of acidic proteins.

5. Concluding remarks

Although many researchers have made great efforts to elucidate mollusc shell biomineralization, the mechanisms and evolutional traits remain speculative. Our approach to outline shell evolution from a more integrative perspective might prompt a new way of thinking about biomineralization in mollusc shell research. We hope it helps reconsider some current shell formation models and stimulates new models.

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