Covalent modification of chitin with silk-derivatives acts as an amphiphilic self-organizing template in nacre biomineralisation

Ingrid M. Weiss, Stefan Kaufmann, Birgit Heiland, Motomu Tanaka

Abstract

Molluscs have a well-deserved reputation for being expert mineralizers of various shell types such as nacre. Nacre is defined as regularly arranged layers and stacks of ~0.5 μm thick aragonite plates that are extracellularly formed within a complex mixture of organic matrix. The control of species-specific layer thickness by the animal is still enigmatic. Despite the recent findings on the periodic layer-by-layer structures of chitin layers and silk-like protein layers in nacre-type biominerals, little is known about how the interface is defined between two different layers. In this paper, we demonstrate the presence of covalently attached, hydrophobic amino acid side chains in the chitin matrix in the bivalve mollusc Mytilus galloprovincialis by the combination of infrared spectroscopy and mass spectroscopy. The accumulation of the modified chitin matrix at the interface is quantified by the critical aggregate concentration of the purified chitin matrix, which is approximately an order of magnitude smaller than that of pure chitin. Our finding suggests an active role of such chemically modified chito-oligosaccharides in the creation of a defined interface and guidance of the periodic matrix textures, which would result in unique material properties of natural mollusc shells.

1. Introduction

Molluscs have a well-deserved reputation for being expert mineralizers based on their shell-making abilities (Lowenstam and Weiner, 1989). The unifying principle behind the various “shell-making abilities” is that mineralization occurs extracellularly in a preformed organic matrix. Chitin (Sollas, 1907), a homopolymer of N-acetyl-D-glucosamine (Clark and Smith, 1936) in its β-conformation (Gardner and Blackwell, 1975; Lotmar and Picken, 1950; Picken and Lotmar, 1950) is one of the earliest biopolymers identified as an organic part of mollusc shells (Degens et al., 1967; Jeuniaux, 1963; Weiner and Traub, 1980) and plays a major structural role in the current concepts for understanding mollusc biomineralisation (Addadi et al., 2006; Cartwright and Checa, 2007; Gilbert et al., 2005; Nudelman et al., 2007, 2008; Weiner and Dove, 2003). Chitin orientation is related to crystal orientation in molluscs (Sone et al., 2007; Weiner and Traub, 1980, 1984; Weiner et al., 1983b) and influences the activity of certain aragonite-inducing proteins (Falini et al., 1996). Recently, Weiss et al. demonstrated that mollusc chitin is fabricated by transmembrane glycosyltransferases that contain myosin motor domains suggesting that cellular mechanical forces play active roles in the control of the mineral deposition process during mollusc shell biogenesis (Weiss et al., 2006). Moreover, the fact that an inhibition of chitin synthesis disturbs the larval mollusc shell development in vivo indicates that chitin and its supramolecular architecture rigorously influences the biomineralisation of mollusc shells on various levels of hierarchy (Schönitzer and Weiss, 2007; Weiss and Schönitzer, 2006).

Another key organic component in the formation of mollusc shells are gels of glycine and alanine-rich silk-like proteins (Levi-Kalisman et al., 2001; Nudelman et al., 2008; Sudo et al., 1997; Weiner and Traub, 1984; Weiner and Dove, 2003; Weiner et al., 1983a; Yano et al., 2006). In 1947, Fraenkel and Rudall used fiber diffraction and suggested that α-chitin and β-sheet domains of tyrosine-rich protein sheets take a parallel arrangement in the arthropod integument (Fraenkel and Rudall, 1947). The presence of chitin–protein complexes was confirmed by the complementary characterization of short glycopeptides (Lipke and Geoghegan, 1971; Spira, 1966). However, despite an increasing number of reports on chitin–protein complexes in various species, the discrepancy in the amount and composition of amino acids found between even closely related species cannot be accounted for (Brine and...
Importantly, there has been no systematic study of the molecular nature of chitin–protein complex formation in mollusc shells. It is practically impossible to observe the complexity of organic matrix deposition during adult shell formation on an enzymatic level (e.g., chitin synthases, chitin deacetylases) \textit{in situ} \citep{Bevelander1969, Jackson2006, Weiss2008}. To date, most \textit{in vitro} experiments with "purified" compounds indicated an intercalation of the silk into the chitins. However, X-ray diffraction and infrared spectroscopy (FTIR) experiments of Falini and colleagues suggested for the first time that a defined interface might exist between the chitin and the gels of silk-like proteins \textit{in vivo} and that the interactions are mainly through the chitin acetyl groups \citep{Falini2003}. From the structural point of view, silk proteins possess a common feature with synthetic nylon: silk proteins are rich in alanine and glycine and form β-sheets, while nylon derivatives, such as nylon-6, form a variety of highly ordered structures via inter-molecular hydrogen bonds (e.g., crystals, clay-nylon-6) \citep{Fornes2003, Kohan1995}. Recent electron microscopy work on nacre matrices from bivalves \textit{Atrina rigida} and \textit{Pinctada margaritifera} \citep{Levi-Kalisman2001, Nudelman2008} points out that the chitin and silk-like protein are assembled into alternative layer-by-layer structures. Cryo-SEM images suggest the chitin-rich, organic ma-

\begin{figure}
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\caption{(a) Macroscopic observation of initial mollusc shell formation \textit{in vivo}. During shell growth, the shell rim of \textit{Mytilus galloprovincialis} is covered by an opaque mucous substance that disappears immediately upon disturbing the animal. This makes it practically impossible to probe the initial phase of shell and nacre formation \textit{in situ}. (b–e) Confocal laser scanning microscopy images obtained from chitin binding GFP-labeled \textit{Mytilus} shell cross-sections after gentle ascorbic acid decalcification at pH 5 in the presence of fixative. (b) The shell margin (left) shows irregular chitinous deposits, whereas the main bulk of the shells (best to avoid 'body' when referring to shell) reveals regular fluorescent lines that represent the layer-by-layer architecture that is typical of nacre. Note that the laminar arrangement of chitin is partly observed in the most recently deposited irregular zone (arrows). (c) Muscle insertion region present in the same shell piece as shown in (d) and (e). The transition zone between two different shell textures is reflected in the change of supramolecular arrangements of the chitin (arrows). (d) The shape of particular nacre tablets is revealed by the chitin staining. Chitinous material bridges two different layers (arrowheads) and thus surrounds particular nacre tables completely. As observed in other specimens (b), the most recently deposited chitinous substance (arrows) assembles within the first 2–5 μm of shell deposit into highly regular mineralized nacre composite. (e) Inner part of the same specimen that appears slightly more decalcified on the surface than the shell part in (d). The layer-by-layer texture of chitin is revealed more clearly. Additional fluorescent bridges between two layers and additional globular material exposed by the etching procedure suggest the incorporation of small fractions of chitin into the mineral tablets.
\end{figure}
trix layers with the thickness of several tens of nanometers, which are separated by species-specific crystal layers of the thicknesses in the order of several hundreds of nanometers. Depending on whether the focus is on the stage prior to or after mineralization, there might be a difference of at least one order of magnitude (swelling, dewetting). Hierarchical, species-specific textures such as nacre (Bøggild, 1930; Taylor et al., 1973) are responsible for the remarkable materials properties of mollusc shells (Fleischli et al., 2008; Gao et al., 2003; Metzler et al., 2007; Meyers et al., 2008). However, although the presence of the protein-chitin interface would call for a significant change in the existing model of the hierarchical structure formation, little is understood about the molecular building blocks that create such an interface.

One of the major problems in understanding mollusc shell formation arises from the fact that supramolecular interactions are studied using in vitro systems based on “purified” compounds (Addadi et al., 2006). Particularly, the purification of self-assembled insoluble components, such as chitin fibers, can potentially result in undesired artefacts, since their insoluble nature often originates from their supramolecular self-assembly such as inter-molecular hydrogen bonds and hydrophobic effects. Thus, the purification procedure such as reflux in alkali (Campbell, 1929; Weiss et al., 2002b) would not only make the matrix soluble, but also cause irreversible damage to their chemical structures.

In this study, we chose a relatively mild chemical extraction method to purify chitin from adult and larval shells of the marine bivalve mollusc Mytilus galloprovincialis. Mass spectra of chitin derivatives extracted from both larval and adult mollusc shells indicate the covalent attachment of hydrophobic amino acid side chains to the chitin backbone, which results in a significant change in the surface activity. This finding indicates that the mode of self-assembling of amphiphilic chitin derivatives at the interface is clearly different from that of pure chitin. This observation would refine the model for the initial mechanism of the biomineralisation of mollusc shells.

2. Experimental section

2.1. Preparation of chito-oligosaccharide standard

Chito-oligosaccharides were prepared from crab shell chitin (Mw > 400,000 Da; Catalogue No. 88451; Carl Roth, Karlsruhe, Germany) according to Foster and Hackman (1957) and Rupley (1964). Crude chitin was grind in a mortar and chitin particles <500 μm were hydrolysed by incubation in HCl for 45 min at 40 °C. After complete evaporation of HCl, the hydrolysate was extracted with deionized water and insoluble material removed by centrifugation in a table top centrifuge. The supernatant was lyophilized and resuspended in deionized water several times. Insoluble material was removed by centrifugation after each step. The final lyophilized sample was dissolved in deionized water, filtered through 0.22 μm filters, and then stored in aliquots that were shock-frozen or lyophilized at −20 °C.

2.2. Ammonia extraction and hydrolysis of mollusc shell matrix

Shells of adult Mytilus galloprovincialis were obtained freshly from animals maintained in the laboratory. After intense rinsing, the shell was incubated in 10% NaOCl for 1 h to remove contaminants and the outer organic layers (periostracum) from the surface. The remaining mineralized shell parts were washed exhaustively with deionized water and then dried and ground in a mortar. Shell particles <500 μm were decalcified and processed as for larvae.

Larvae of Mytilus galloprovincialis were rinsed twice with deionized water for 30 s and incubated 3 × with 2.5% NaOCl for 10 min. The remaining shells were washed 10 × with deionized water (Milli-Q, Millipore, Schwabach, Germany) including 20 s of sonication in each step, leading to partial disintegration of the material. Pure shell pieces were treated according to slightly modified conventional methods (Foster and Hackman, 1957) with excess of 0.5 M EDTA-NH₄⁺ for fractionated decalcification at pH 6. Insoluble material was collected by 10 min centrifugation in a table top centrifuge. After complete decalcification, the remaining insoluble organic matrix was washed until the supernatant was free of EDTA according to complexometric titration. In order to minimize contaminations with Na⁺ in commonly used hot alkali extraction procedure using NaOH, the matrix was treated with concentrated NH₄OH at 95 °C for 1–2 h or overnight in order to hydrolyze and solubilize chitin-associated proteins. The insoluble material was washed several times and subjected to hydrolysed with concentrated HCl for 25 min at 37 °C. Conditions here are slightly milder than applied for the standard chitin (crab shell) in order to prevent complete hydrolysis. After evaporation of hydrochloric acid, the chitooligosaccharide-containing samples were dissolved in deionized water and insoluble material was removed by centrifugation and 0.22 μm filtration. Lyophilized samples were stored at –20 °C.

2.3. Size analysis of oligosaccharides

Chito-oligosaccharides fractionated by gel electrophoresis according to Huang et al. (2000), based on anthranilic acid derivatisation for fluorescence detection (Sato et al., 1998). Gel filtration chromatography of 0.5 mg sample was performed in 50 mM NH₄Ac, pH 7.0, at a flow rate of 0.3 mL/min. using an Amersham-Pharmaclia FPLC system equipped with Superdex Peptide HR 10/30 column (Mₗ = 800–3000 Da) (GE Healthcare Europe GmbH, Munich, Germany), and UV detector (226/280 nm) (Kontron, Munich, Germany).

2.4. Amino acid analysis

Sample extracts were hydrolysed by treatment with HCl (gas phase) (110 °C, 24 h, N₂ atmosphere) and analysed using a Dionex HPLC system equipped with a Dionex Aminopac PA10 column and amperometric detection under standard conditions for quantitative amino acid analysis (Dionex, Idstein, Germany). A Sigma amino acid standard (10 μM; Sigma–Aldrich, Taufkirchen, Germany) was used for calibration of the system. Sample peaks were aligned manually.

2.5. Confocal laser scanning microscopy

Mytilus galloprovincialis shells were obtained alive from a commercial distributor (Deutsche See GmbH, Saarbrücken, Germany) and stored frozen at –20 °C. The mantle tissue was removed from frozen shell pieces immediately prior to sample processing according to established metallographic procedures (Petzow, 2006; Schumann, 1990). Shell pieces were mounted at room temperature in epoxy resin (EpoFix, Struers, Willich, Germany) using a vacuum impregnation device (Cast N'Vac-1000, Buehler, Düsseldorf, Germany). Flat cross-sections were obtained by SiC paper grinding at grain sizes of 30, 14 and 8 μm, subsequent polishing with “Dur” cloths and abrasives of 6 μm and 3 μm, and “Mol” cloths and abrasives of 1 and 0.25 μm (Struers, Willich, Germany), respectively. Specimens were rinsed extensively with water and ethanol prior to air drying. The quality of the cross-sections and the cleanliness of the surfaces were confirmed in reflected bright field mode using a PMG3 polarized light microscope (Olympus, Hamburg, Germany). For fluorescent staining of chitinous matrices, embedded Mytilus shell cross-sections were treated as described for larval Mytilus shells in (Weiss and Schönitzer, 2006) with minor modifi-
ations. In brief, specimens were rinsed in salt solution (150 mM NaCl, 20 mM KCl, 10 mM CaCl₂, 10 mM MgCl₂) for 5 min. prior to 45 min. etching of the mineral phase in the presence of 1% ascorbic acid and 4% glutaraldehyde at pH 5. After 5 min. rinsing in deionized water and salt solution, respectively, the blocking step was performed in salt solution containing 10 mg/mL BSA (Roth, Karlsruhe, Germany) prior to treating the etched surface with fluorescent chinin-binding protein (CBFGP) at a final concentration of 8 μg/mL for 1 h. After rinsing the surface in salt solution, confocal laser scanning microscopy was performed using a Zeiss LSM 510-Meta equipped with Plan-Neofluar 20×/0.5 and Plan-Apochromat 100×/1.4 Oil DIC objectives as previously described (Weiss and Schönitzer, 2006).

2.6. IR and mass spectroscopy

IR spectroscopy was performed using a Biorad FT-IR FTS 165 spectrophotometer (Biorad, Munich, Germany) and standard KBc processed samples. Mass spectrometric analyses of chito-oligosaccharide standard and HCl-hydrolyzed mollusc shell matrix extracts were performed on a Finnigan SQ/7000 quadrupole spectrometer (Finnigan MAT, San Jose, CA, USA). Chitin oligosaccharides were further analyzed by MALDI-TOF/TOF with respect to higher molecular weight components using the Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA).

2.7. Determination of surface activity

Critical aggregation concentrations (c.a.c.) of Mytilus shell matrix extracts (soluble chito-oligo-like fraction as characterized by mass-spectroscopy) and chito-oligo standard were calculated from the measuring the surface tension of 60 μL suspensions (concentrations: 26 μg/mL–50 mg/mL) using a Kibron Micro TroughX (Kibron Inc., Espoo, Finland). Each data point corresponds to a mean value of at least three independent measurements.

3. Results

The mollusc shell matrix is synthesized by differentiated cells that form the mantle tissue. In bivalve molluscs for example, the dynamic process of shell matrix secretion and subsequent mineralization is extremely difficult to observe in situ, since the mantle is retracted immediately from the site of shell formation whenever the animal gets disturbed and closes its valves. When the valves open again, the expanding mantle returns to its original position and covers the outer site of shell formation with an opaque substance (Fig. 1a). However, the organic matrix can be visualized at high resolution within mineralized cross-sections of shell pieces using confocal laser scanning microscopy. The highly regular arrangement of nacre platelets in some inner shell parts obtained from M. galloprovincialis is reflected in the fluorescence pattern of a GFP fusion protein that specifically stains chitin and possibly also stains closely related derivatives thereof (Fig. 1b-e). As shown in Fig. 1b and d (arrows), diffuse fluorescent signals were obtained from 2 to 5 μm thick layer next to the inner surface of the nacreous part which can be identified by aligned chitin sheets that appear as relatively straight lines with the thickness close to the optical resolution (~0.2 μm) that are separated by a typical distance of 0.5–1 μm in the cross-sections. The apparent thickness of the chitin layer observed here seems to be smaller than the corresponding values for matured bivalves calculated from Cryo-SEM images (20–160 nm) (Nudelman et al., 2008). The discrepancy between our optical microscopy results and electron microscopy experiments can be attributed either to the difference in biological stages (species, developmental stages, tissues, maturity of mineral phases, etc.) or to the conditions of the measurements. The lines appear bridged by fluorescent material, indicating that particular nacre platelets are fully surrounded by chitin (Fig. 1d, arrowheads) or eventually infiltrated with chitinous substance (Fig. 1e, arrows). The high concentrations of globular fluorescent material on top of the cross-sections can hardly be interpreted with respect to structural information given the harsh surface treatments applied in order to obtain sufficiently flat surfaces as well as subsequent etching and fixation procedures. Since chitinous matrices are challenging to characterize in the native state due to their insolubility, the alternative strategy was to extract soluble chito-oligosaccharides from decalcified mollusc shell matrix for further molecular and functional characterization.

First indications for a substantial chemical and physico-chemical difference between standard chito-oligos and mollusc chito-oligo-like extracts was revealed by PAGE analysis of anthranilic acid modified oligosaccharides (ESM 1). Only the two smallest oligosaccharide fragments extracted from mollusc samples were detected by this method, whereas the expected series of chito-monomer up to chito-hexamer (or even higher) were detected in the standard. Size exclusion chromatography revealed that the mollusc shell chito-oligo-like extract contained molecules in the size range of ~800–3000 Da (ESM 2). Conceivably, the larger fragments were just not detected by PAGE and anthranilic acid staining procedure.

FT-IR spectroscopy revealed that ammonia extraction of mollusc shell matrices was suitable to replace the alkaline extraction under harsh conditions by NaOH. In general, the IR spectra of mollusc (M. galloprovincialis) shell alkaline extracts (Fig. 2a) are comparable to spectra of silk or nylon-like polyamides (see (Kohan, 1995)) for reference spectra). Fig. 2b shows IR data of this extracted M. galloprovincialis shell matrix components after acid hydrolysis (in the following referred to as mollusc chito-oligo-like components), which differed significantly from spectra obtained from standard chito-oligosaccharides (Fig. 2c). At a first glance, these data suggest silk-like peptides as one part of the mollusc chito-oligo-like fraction. The presence of silk-like peptides was also verified.

Fig. 2. Structural evaluation of the alkaline purification methods and the partial acid hydrolysis of shell matrix extracts. Infrared spectra of chitin-like shell components extracted from: (a) mollusc (Mytilus galloprovincialis) shell extract after treatment with hot NH₄OH. There was no significant difference compared with spectra obtained with standard NaOH extraction. (b) Mollusc (Mytilus galloprovincialis) shell alkaline (NH₄OH) extract after partial HCl hydrolysis. Note that only water soluble compound was processed further. (c) Standard chitin processed identically as the mollusc shell matrix extract (b). The compound obtained was identified as chito-oligosaccharides by PAGE and MALDI (Supplementary materials).

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by standard hydrolysis and subsequent amino acid composition analysis (28% Gly, 27% Ala, 11% Ser, 11% Leu, 6% Asx, 4% Arg). Thus, Mytilus chitin-associated peptides differed from soluble glycerine-rich acidic shell protein fractions (Meenakshi et al., 1971; Weiner, 1979, 1983; Weiner and Hood, 1975) but are in good agreement, taking into account differences in methodology and specimen taxonomy, with previous reports on insoluble matrices (Keith et al., 1993; Weiner and Traub, 1984; Weiner et al., 1983a). Despite the lack of quantification due to minor response factors for amino-sugars, the presence of N-acetyl-α-glucosamine (GlcNAC) and α-glucosamine (GlcN) was confirmed in our experiments by anion-exchange coupled pulsed-amperometric detection.

Silk like peptides detected by FT-IR (Fig. 2a and b) and amino acid analyses may or may not be covalently bound to the chitin/chito-oligo-backbone. In order to determine which is the case, mass spectra were measured, taking advantage of the fact that, non-covalently associated silk-like peptides can be identified via primary structure and trace contaminations from processing reagents can be tracked by using this method. As presented in Fig. 3a, the mass spectrum of standard chito-oligosaccharides revealed mass fragments as expected (see also ESM 1). However, in case of mollusc chito-oligo-like fractions obtained from both adult (Fig. 3b) and larval (Fig. 3c) M. galloprovincialis shell matrices, some unusual mass fragments were detected.

The unexpected mass fragments (Fig. 3b and c) differing in size by m/z differences of 19 can only be explained by covalent side-chain modifications of chitin with silk- or nylon-like peptide moieties, such as an additional glycine (Gly, m/z = 57) or alanine (Ala) residues in every third or sixth sugar residue, respectively (see Table 1 for assignment of mass fragments with multiply charged multimeric oligosaccharides). Additional peaks may refer to various numbers of attached acetic acid molecule (m/z = 60), which is present in the solvent. Fragment spectra of particular mass peaks could not be obtained, as the mollusc extracts cannot be ionized by ESI. Moreover, MALDI was not technically possible, either possibly due to the fact that the matrix cannot be crystallized or the matrix desorption by the laser does not occur. Amino acids other than glycine or alanine may well be associated with side-chain modifications but would escape our analysis in case of insolubility of the compound.

Chitin from mollusc shell matrices has been characterized based on common extraction methods such as boiling in hot alkali under reflux. However, this procedure is powerful enough to destroy even covalent bonding between amino acids and the chitin backbone (Campbell, 1929; Rudall et al., 1963; Toleimate et al., 2000; Weiss et al., 2002b). Here, a milder extraction technique has enabled the demonstration of covalent bonding between the amino acids and chitin backbone. Scheme 1 represents the refined structural model deduced from our experimental results, including hydrophobic amino acid side chains. The amino-acyl group of one GlN subunit is modified by a second amino-acyl group (R1). In the case of the pure chitin polymer, a methyl group (R2) is present in every amino-acyl residue. The observed fragment sizes suggest that the molecular ratio between the two types of substituents within one polymer chain is R1:R2 = 2:1. Such a model is supported further by the fact that one and the same m/z peak results from multiply charged ionic species, as indicated by splitted peaks within one mass unit.

An alternative way of chitin modification would occur at the position of 3'- or 6'-OH group, like the case of peptidoglycan of bacterial cell walls connected via pyruvic acids (MW ~ 88 Da) (Naumann et al., 1982). In our case, it has been demonstrated that the side chain modification should occur after the chitin synthesis, since the active site of mollusc chitin synthase is highly conserved. In fact, as presented in Fig. 3 and Table 1, the mass spectra of adult and larval mollusc matrix fractions clearly exhibit the fingerprint of the substrate monomers UDP-GlcNAC (MW ~ 222 Da). On the other hand, the side chain modification at 3'- and 6'-position requires cross-linkers like pyruvic acids. In our mass spectra, however, we found no such fragments, which seems to exclude the

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**Table 1** Assignment of mass fragments as shown in Fig. 3.

<table>
<thead>
<tr>
<th>Mass fragment</th>
<th>Assignmenta</th>
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<th>Larva</th>
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* a N-acetyl-α-hexosamine, i.e., glucosamine: X = unknown 57 [m/z] mass fragment; HAc = acetic acid. Note possible mass differences of 18 [m/z] due to H2O in glycopolymers.

b Standard Chito-oligosaccharides.

c Mollusc shell insoluble matrix extract (NH4OH/95°C) after HCl hydrolysis (40 min/40°C).

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**Fig. 3.** MS of chito-oligo standard (a) and Mytilus shell extracts prepared from adult (b) and larval (c) shell insoluble matrices. See Table 1 for assignment of mass fragments.
possibility of enzymatic modification of chitin with such moieties. Note that the other modes of mass spectroscopy, such as electrospray ionization mass spectroscopy (ESI-MS) which would reveal the small fragments like pyruvic acid, were not possible for our samples.

Interestingly, we observed the formation of foams when the extracted Mytilus shell components were suspended in water at 0.2 g/L (Fig. 4, inset left). This finding suggests that Mytilus shell components may be accumulated to the interface (like other surfactant molecules) and create a defined interface, which would guide the formation of layer-by-layer structures of chitin and silk-like protein (Levi-Kalisman et al., 2001; Nudelman et al., 2008). The accumulation of Mytilus shell components at the interface seems plausible, if one considers the structural model in Scheme 1 that includes hydrophobic amino acid side chains. To verify this hypothesis, the surface tension of Mytilus shell components was measured at various concentrations (Fig. 4) (Adamson and Gast, 1997). At very low concentrations (c < 0.3 mg/L), the surface tension is almost identical to that of water subphase at T = 298 K, γ ≈ 72.0 mN/m. Then, the surface tension linearly decreases with the logarithm of concentration of the shell matrix, γ = γ₀ + B log c, which generally corresponds to the accumulation of the surface active molecules at the air/water interface. Finally, the surface tension reaches to the saturation level at 43 mN/m, and further increase in the concentration of shell matrix does not cause any change in γ. At this concentration (c ≈ 0.8 g/L), called critical aggregate concentration (c.a.c.), Mytilus shell components saturate the air/water interface and start forming aggregates, such as micelles. According to size exclusion chromatography (ESM 2), the molecular weight of shell components lies between 800 and 3000 Da, corresponding to the monomer number of 9 ± 6. This agrees well with those molecular weights estimated from mass fragments (Table 1): 3100 Da coincides with 12 unmodified monomers, and 800 Da corresponds to a double-charged trimer with two modified side-chains. Taking the possible range of the molecular weight, the c.a.c. of Mytilus shell component can be calculated to be 0.3–1 mM. This value is comparable to those of conventional surfactants such as Triton X-100 (0.3 mM) and N-decyl-β-D-maltopyranoside (1.6 mM), and is about an order of magnitude smaller than that of chito-oligo standard (8 mM) measured in the same manner (data not shown). This finding gives additional supporting evidence that mollusc shell chitin possesses an amphiphilic nature by hydrophobic side chains.

4. Discussion

This study presents clear structural evidence that chitin matrices purified from both larval and adult mollusc shells consist not only of β-chitin but also of chitin with covalently coupled, hydrophobic amino acid side chains. Mass spectroscopy analyses are superior to infrared spectroscopy that largely depends on the proportion of chitin to silk-like proteins. Furthermore, the mass spectrometry excludes the possibility that there are contaminants from EDTA that have previously been reported to cause difficulties in IR spectroscopy of mollusc shell matrices.

This demonstration of the covalent modification of mollusc chitin with silk-like side chains explains, for the first time the nature of the “intimate link” of the two separate phases: β-sheet proteins with a species-specific angle (i.e., perpendicular) to the chitin backbone (Weiner and Traub, 1980) that was suggested to form unusual plywood-like constructions for increased mechanical strength while serving as an organic template for epitaxial mineralization according to the extended definition of Weiner and Traub (1984). However, the concept of epitaxy continues to be an issue of debate, since covalent modifications cannot provide the ultimate proof.

The amphiphilic functions of silk-modified chitins as suggested by c.a.c. data presented here would explain the formation of matrix structures such as the current hierarchical model, which merely considers alternative layer-by-layer structure of β-chitin and silk-like protein gels into account (Levi-Kalisman et al., 2001). In fact, an amphiphilic modified chitin fraction can provide an energetically favorable interface towards the more homogeneous fractions such as the hydrophilic silk-like gel phase observed by Nudelman and colleagues (2008) prior to mineralization.

A significantly smaller critical aggregate concentration of the chitin matrix from mollusc shells than that of pure chitin would sharply confine the interface between chitin-rich and protein-rich layers, and inter-molecular hydrogen bonds between amide groups would facilitate the self-assembly of chitin backbones into highly ordered textures. The almost identical silk-like modification patterns for the chitinous biopolymers obtained, from both adult and larval developmental stages, suggest that modified chitin plays a fundamental role in the biomineralisation of mollusc shells. Natural chito-oligosaccharides modified with silk-like residues in the same manner as presented here for M. galloprovincialis have been

Scheme 1. Structural model of the Mytilus shell matrix components deduced from MS data. Two sugar subunits are shown (blue). For clarity, the sugar subunit shown on the left contains the silk-like modification (R²) (pink). Note that the statistical molar ratio between R¹ and R² is calculated from MS data to be 2:1.

Fig. 4. Main panel: surface tensions of mollusc (Mytilus galloprovincialis) shell matrix extract measured at different concentrations. Error bars represent deviation from the mean values out of three independent measurements. Critical aggregation concentration (c.a.c.) of 0.8 g/L can be calculated. Inset: formation of foams in case of Mytilus shell components (right) at 0.2 g/L, in contrast to a transparent solution of standard chito-oligosaccharides (left).
isolated from the crayfish Astacus fluviatilis (Hertzog et al., 1975). This, together with our M. galloprovincialis results indicate that such chitin modification seems to be a clever strategy in nature to create nacre-type integument textures, such as lenticular nacre found for example in Mytilus and the cephalopod Neutilus, and bivalve sheet nacre (Taylor et al., 1973). Finding similar, but not completely identical modification patterns in matrix extracts from larval shells suggests that alternative textures such as prisms (Weiss et al., 2002a) may be encoded or at least fine-tuned by using an enzymatic chitin modification strategy. This would help to rationalize the genetic encoding and thus the species-specificity of various shell types. Further study on the interfacial contact between two organic layers would unravel the impact of side chains modification on the surface activity and self-assembly of the shell matrix, which guides the crystallization and pattern formation in mollusc shell composites.

5. Conclusions

In this study, we present experimental evidence that chitinous biopolymers isolated from mollusc shells are covalently modified by silk-like peptide side chains. Mass spectra analyses reveal almost identical modification patterns for the chitinous biopolymers obtained from both, adult and larval developmental stages. This finding gives a clear indication that modified chitin plays a fundamental role in the biomineralisation of mollusc shells. Moreover, quantitative surface tension measurements revealed for the first time that mollusc chito-oligosaccharides possess a strong surface activity (even comparable to conventional detergents) in comparison to unmodified chito-oligosaccharides. Indirect evidence for the accumulation of shell matrix at the interface is obtained by a clear sonication and the resulting composite textures, ranging from nano- to micron-scales.

Acknowledgments

We thank Prof. S. Weiner for his valuable critical comments, and Prof. M. Sumper for helpful suggestions. We thank Dr. R.G. Oliveira, R. Hett, W. Bieffeldt, and S. Schmidt for experimental support, and Prof. R. Deutzmann and E. Hochmuth for assistance with mass spectroscopy. This work has been financially supported by the German Science Foundation (DFG Ta259/3, Ta 259/6) and the Fonds der Chemischen Industrie. I.M.W. acknowledges financial support from the University of Regensburg and the INM–Leibniz Institute for New Materials.

Appendix A. Supplementary data


References


