CHITINOPROTEIC COMPLEXES AND MINERALIZATION IN MOLLUSK SKELETAL STRUCTURES

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STRUCTURE AND CHEMICAL COMPOSITION OF CHITINOPROTEIC MATRICES OF MOLLUSK SHELLS

The deposition of shell material by Mollusks is one of the more fully studied processes of extracellular calcification, and a large amount of data has been collected on shell structure, formation and regeneration, mineralogy, trace elements and organic compounds biochemistry. Like in many other invertebrates, the nucleation and growth of mineral crystallites occur on a well defined structure of organic components, these components being more or less "frozen" within developing crystals : this constitutes the "organic matrix template theory".

Both vertebrate and invertebrate organic matrices seem to be built on the same general scheme: a core of structural macromolecule (polysaccharide or protein) sheathed by one or several envelopes of acidic proteins, glycoproteins and mucopolysaccharides. All these components may be stabilized by structural bonding, mostly quinone-tanning and sulfur bonds.

The organic matrix isolated through decalcification of calcified layers of Mollusk shells (the so-called "conchyolins") is very complex in composition and structure. Proteins are the main component and account for 50 to 80% of matrix dry weight (1). Chitin was demonstrated in the shells of several species (2,3) and recent investigations made obvious its presence in every calcified layer of Mollusk shell so far examined (ca. 150 species). Its amount, estimated by the enzymatic method of Jeuniaux (2,4), is quite variable : from 0.01 to 40 % of the matrix dry weight (1 - 3).

The conformation and orientation of the matrix macromolecules were relatively poorly understood until the studies of Weiner and co-workers (5,6, 7,8). As stressed by Weiner (8), the main difficulty was the fact that X-ray and electron diffraction studies need to remove the minerals and, during this operation, some hydrophilic components are lost. The use of non-calcified material (for example, *Loligo* pen) may lead to misinterpretation (9). X-ray and electron diffraction studies (5) of the insoluble components of the matrix reveal that there is an association of chitin in its β-form (parallel chains crystalline form) with proteins that, similarly, adopt an antiparallel β-sheet conformation (8). The chitin polymer would be oriented approximately perpendicular to the protein-polypeptide chains (5,6,7), so that this crossed construction presumably contributes to the mechanical strength of the matrix (6).

This theory is consistent with transmission electron microscopy results

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(shadowcast preparations, ultrathin sections) (10 - 12). The representative ultrastructure of the matrix is a micro-meshwork made of dense grains (2.3 - 4.0 nm average diameter) united by short, straight and thin organic connections (10). The sheets of the matrix are composed of several (up to five) different layers. The two surface layers are composed mainly of more soluble, acidic constituents. The core comprises a thin layer of chitin sandwiched between two thicker layers of proteins (12,13).

Bearing in mind those structural results, the calcophillic matrix of Mollusk shells appears, from a chemical point of view, as composed of two structural units :

1. An acidic polypeptide fraction with strong affinity to Ca^{++} ions, mostly soluble in decalcifying reagents (1, 14, 15, 16, 17). Its most probable arrangement involves a spiraled peptide chain (18). It is generally called "Mineralization Matrix"(MM).

2. A high molecular weight chitinoproteic complex with no affinity to calcium, arranged in the form of sheets and layers (1, 18), called "Carrier Protein" (CP).

The attachment of the soluble "mineralization matrix" to the "carrier protein" complex will activate the mineralizing substrate, leading to epitaxial $CaCO_3$ deposition (18). A "sandwich" structure thus appears with the chitinoproteic CP embedded between two sheets of the mineralization matrix, as presented on Figure 1. Subsequently, quinone-tanning can stabilize this whole molecular framework (1).

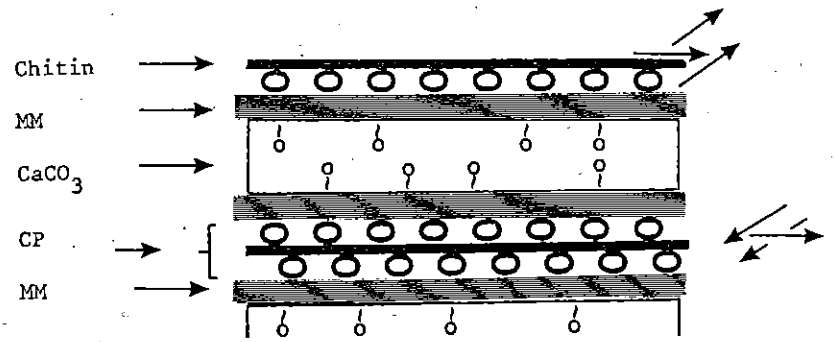

Fig. 1 : Schematic interpretation of the structure of the organic matrix in Mollusk shells (not drawn to scale).

CHITIN-PROTEIN BOND IN MOLLUSK SHELLS

The nature of the chitin-protein association in Mollusk calcified shells is still poorly understood, particularly as far as linkage is concerned. As chitin always appears accompanied by proteins, it is obvious that there is/are site/s in the polysaccharide polymer through which covalent binding to protein occurs. This proteoglycan may be accompanied by some other proteins or glycoproteins, specifically or not specifically associated with it by weak forces (9, 19).

According to the generalized high aspartic acid content of the insoluble protein complex (1), Hackman datas on chitin-protein relations may tentatively be extended to calcified Mollusk shells.

N-acetylglucosamine and chitin can react with α-aminoacids and peptides to give stable complexes and it seems that chitin can form more or less stable glycoproteins, probably through aspartyl and histidyl residues(20,21). Similarly, Brine and Austin (22) showed that predominant aminoacids in the residual chitin after vigorous alkaline hydrolysis were aspartic acid,

serine and glycine, which may be involved in the chitin-protein linkage. It should be remembered that those three aminoacids represent 35 to 50 % of aminoacid residues in the soluble matrix of Mollusk shells (1). In a similar perspective, Gottschalk proposed a linkage in the form of glycosidic ester between acetylglucosamine residues and β-carboxyl groups of aspartyl residues (23). According to these ideas, Stegeman reported that, in the non-calcified pen of Cephalopods, the proteins most firmly bound to chitin contain large amounts of aspartic acid (24).

In contrast, Atwood and Zola (25) found that, after prolonged alkali treatment, there is no predominance of aspartic acid and histidine, nor indeed, of any other aminoacid in the chitin isolated from *Loligo* pen. According to these authors, these results do not indicate the lack of protein-chitin covalent linkages, but only that the use of this method is inadequate (25). The results of hydroxylamine and lithium borohydride treatments on the *Loligo* pen chitinoproteic complex show that ester linkages are probably absent. If present, the protein/s joined to chitin by these links is/are also joined to chitin through another type of bond (25).

Hydroxylamine cleaves ester and amide linkages and has been used extensively in studies of protein-polysaccharide linkages (26). Lithium borohydride cleaves esters but has less action on amides (25).

These controversial results clearly show that existing evidences for covalent binding between chitin and proteins must be reexamined, particularly in the case of calcified matrix.

CHITIN AND CALCIFICATION IN MOLLUSK SKELETAL STRUCTURES

It seems well established that $CaCO_3$ nucleation occurs through a Ca^{++} high affinity soluble proteic complex, associated with an insoluble chitinoproteic complex. Chitin does not appear to be directly related to calcification process, but physiological and evolutive evidence indicates indirect relationship of chitin with $CaCO_3$ metabolism. Three kinds of indirect arguments were recently proposed.

A) Correlative chitin and $CaCO_3$ content in slug shells during annual cycles

The internal shells of the slugs *Agriolimax reticulatus*, *Milax rusticus*, *Limax maximus*, *Limax cinereoniger* and *Lehmania marginata* do contain chitin, and an important part of this chitin (60 %) is not firmly bound to the proteins of the insoluble complex (27).

$CaCO_3$ and chitin estimations in shells taken at different periods of the year (under natural conditions) show important cyclic fluctuations (fig.2). An obvious decrease of the amount of chitin in the organic matrix of the shell is observed during hibernation of the slugs (October to March) and this is parallel with the decrease of the amount of $CaCO_3$ during the same period. During the period of activity (March to October) the internal shell simultaneously restore the $CaCO_3$ content and the amount of chitin of the organic matrix (maximum just before hibernation). During shell resorption, histoenzymological methods make obvious a chitinolytic activity from the whole palleal epithelium (28).

Correlatively, a high amount of hydrophillic acidsoluble proteins (20% of total shell proteins, with an aminoacid pattern similar to other mineralization matrices (27)) should be referred to an increased calcium metabolism of the structure. The simultaneous metabolic variations of chitin and $CaCO_3$ can be simulated, under laboratory conditions, by starvation of the slugs or sexual maturity induction (28).

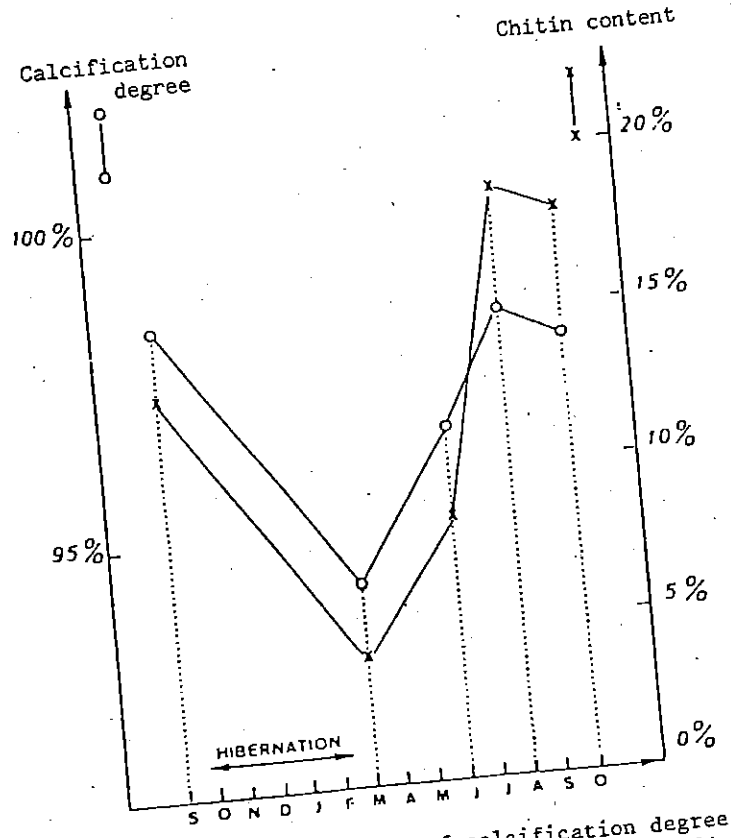

Fig. 2 : Cyclic fluctuations of calcification degree and chitin content in the shell of *Agriolimax* (from (27), slightly modified).

B) Chitin content of Gastropod operculi

The operculi of 114 species of Gastropods representing 38 families with wide ranging morphological, ecological and geographical differences have been examined for the presence of chitin (29,30). In all the species so far analysed, calcified operculi were found relatively rich in chitin ; the amount of chitin was similar in operculi of neighbouring species. No chitin was found in the non-calcified, corneous operculi. The level of calcification was the unique character found to be correlated to the presence of chitin and no other parameter showed any reciprocity.

The organic matrix of calcified operculi is built of two layers, a quinone tanned proteic sheet, and a calcified glycoproteic matrix. The tanned sheet of all operculi tested is totally devoid of chitin. Mainly proteic, its aminoacid composition is similar with the composition of the whole typical corneous operculum. The calcophillic glycoproteic matrix (insoluble complex) does contain chitin, the amount of which depends directly on the calcification degree of the operculum (31). Aminoacid composition of this matrix appears very close to that of insoluble shell proteins.

C) Evolution of the chitin content of Mollusk shells

From an evolutive point of view, chitin content and calcification degree of Mollusk shells appear to vary concommitantly, but in opposite directions. During the evolution of the external shells of Conchifera (Gastropods,

Cephalopods, Bivalvia, Scaphopods and Monoplacophora), the less calcified chitin rich microstructural layers (mother-of-pearl) are replaced by highly calcified crossed-lamellar layers, much poorer in chitin (1).

On the contrary, in the evolutive lines where the shells become more and more embedded within dorsal palleal expansions (Cephalopods, Opisthobranchs, Pulmonates, Placophora,...), the increase of the chitin content is connected with a decrease of the calcification degree (1).

In both instances, the mineralization matrix remains remarkably stable in its aminoacid composition pattern. The proteins associated with chitin to form the insoluble "carrier protein" complex, show only slight modifications, with no general obvious tendency.

CONCLUSIONS

The main results on chitin-protein complexes in Mollusk shells were here summarized and focused on the problems linked with calcification, one of the most tremendous features of Mollusk shells. It is suggested that chitin may behave as a molecular skeleton onto which structural proteins and mineralization proteins are bound. This is consistent with electron microscopy results (10,11,12,13) and X-ray diffraction data (5,6,7,8). It is likely that the binding of proteins to chitin reveals some active site used for nucleation and growth of $CaCO_3$ crystals.

According to this preliminary hypothesis of indirect influence of chitin on mineralization of Mollusk shells, the nature of chitin-protein bond and fine molecular organization of chitin-protein complexes in calcified shells will certainly need further research.

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