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Protein mapping of calcium carbonate biominerals by immunogold

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Abstract

The construction of metazoan calcium carbonate skeletons is finely regulated by a proteinaceous extracellular matrix, which remains embedded within the exoskeleton. In spite of numerous biochemical studies, the precise localization of skeletal proteins has remained for a long time as an elusive goal. In this paper, we describe a technique for visualizing shell matrix proteins on the surface of calcium carbonate crystals or within the biominerals. The technique is as follows: freshly broken pieces of biominerals or NaOCl then EDTA-etched polished surfaces are incubated with an antibody elicited against one matrix protein, then with a secondary gold-coupled antibody. After silver enhancement, the samples are subsequently observed with scanning electron microscopy by using back-scattered electron mode. In the present case, the technique is applied to a particular example, the calcitic prisms that compose the outer shell layer of the mediterranean fan mussel *Pinna nobilis*. One major soluble protein, caspartin, which was identified recently, was partly *de novo* sequenced after enzymatic digestions. A polyclonal antibody raised against caspartin was used for its localization within and on the prisms. The immunogold localization indicated that caspartin surrounds the calcitic prisms, but is also dispersed within the biominerals. This example illustrates the deep impact of the technique on the definition of intracrystalline versus intercrystalline matrix proteins. Furthermore, it is an important tool for assigning a putative function to a matrix protein of interest.

Keywords: Calcium carbonate; Immunogold; SEM; Caspartin; Back-scattered electrons; Surface treatment

1. Introduction

In the metazoan world, calcium carbonate skeletons are the most commonly encountered biomineralizations, from the most "simple" diploblastic animals, sponges and corals, to deuterostomes, echinoderms and vertebrates. Their study offers promising perspectives in biotechnology: the synthesis at room temperature of materials that mimic the molluscan nacre, both in its structure and in its superior mechanical properties [1,2]; the use of bioactive coral or mollusk implants in bone substitution medicine [3,4].

All metazoan calcium carbonate biomineralizations share a remarkable property. They are organo-mineral assemblages, where the dominant mineral—calcite, aragonite, or less frequently, other polymorphs of $CaCO_3$—is closely associated with a minor organic matrix [5]. This latter (0.1–5 wt% of the skeleton) represents a mixture of proteins, glycoproteins and polysaccharides, which are secreted by the calcifying tissues during skeletogenesis, and sealed within the skeleton during its growth. The matrix displays essential functions in biomineralization: beside physico-chemical interactions, i.e., nucleation, polymorph selection, crystal growth and inhibition [6] the organic matrix is suspected to display enzymatic functions [7] and to be involved in cell signaling [8].

One fundamental aspect in biomineralization research is the comprehension of the topographic relations between the organic and the mineral phases [6]. Localizing the matrix components around and within biominerals helps to understand how they interact together, giving thus information on putative functions of matrix components during crystal synthesis. The precise localization of matrix components is critical, since it is usually the basis from

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which new models of biomineralization are proposed. The best example is that of molluscan nacre, for which the "classical" model published in the early 1980s [9] evolved drastically until recently [10–13].

For localizing the organic matrix in calcium carbonate biominerals, several techniques are available from the most easy-to-handle to the most sophisticated ones: at low magnification, the distribution of organic matrix within biominerals can be visualized by coupling light microscopy with cathodoluminescence [14], with epifluorescence or by using different fluorochrome staining [15]. In the magnification range [1000–50,000], scanning electron microscopy (SEM) gives interesting results when samples surfaces are pre-treated for deciphering the fine topography of mineral-matrix assemblages [16]. At higher magnification, transmission electron microscopy (TEM) and cryo-TEM reveal nanostructural details [11,17], but their implementation requires a special expertize and skill. Finally, at the molecular scale, atomic force microscopy gives spectacular results, which however, may be difficult to interpret [18]. Other physical techniques, like high-resolution energy/wavelength dispersive X-ray analysis (EDX/WDX), Raman microspectroscopy, X-ray diffraction, X-ray absorption near edge structure spectroscopy (XANES), nanoscale secondary-ion mass spectrometry (NanoSIMS) may be useful. However, most of them do not localize a particular matrix component. At the best, XANES [19] and NanoSIMS [20] can only localize some chemical groups.

To localize a single component within a calcified skeleton, immunological techniques represent a valuable approach. The basic principle of these techniques is the use of antibody molecules, which specifically bind to their target antigens. The recognition domain of a given antibody is usually a short portion of the antigen, for polypeptides, 5–8 amino acids. The technique applied to skeletal matrices has not received a great deal of attention, in spite of having been successfully used with calcium carbonate biominerals: mollusc shells [21,22] and coral skeletons [23]. Two drawbacks of these earlier experiments were that, in two cases [21,23], the antibody preparations were made from crude mixtures of different matrix components and, if not, that these preparations were observed by optical microscopy, which implies a limited magnification [22].

In this paper, we describe an immunological staining, which overcomes these technical obstacles. The studied biominerals are the shell calcitic prisms of the pteriomorphid bivalve *Pinna nobilis*, from which caspartin, a shell soluble protein, was previously characterized [24]. In this paper, the characterization was pursued and caspartin was partly *de novo* sequenced after enzymatic digestions. Polyclonal antibodies were obtained against caspartin and used for immunogold localization with SEM, under back-scattered electron mode, after different surface treatments. We assess that this technique represents a substantial improvement for the localization of matrix components. Furthermore, it allows redefining the concepts of intracrystalline versus intercrystalline matrix.

2. Materials and methods

2.1. Materials

The shells of the bivalve *P. nobilis* were kindly provided by CERAM (Centre d'Etudes et de Recherches Animales Marines, Prof. Nardo Vicente). Like several Pteriomorphid bivalves, *P. nobilis* exhibits a nacro-prismatic shell microstructure. The surface of the shells was carefully cleaned by abrasion with a dental drill, and the two shell layers were separated mechanically. In this study, only the outer prismatic calcitic layer was used for subsequent observations. On the one hand, small shell fragments were used for immunogold assay. On the other hand, several fragments of the prismatic layer were treated for protein purification. In this second case, they were soaked in dilute sodium hypochlorite (0.2 wt% active chlorine), for 4 days, under constant stirring. This operation resulted in the isolation of the calcitic prismatic biocrystals by degrading their periprismatic organic sheath [24]. Prisms were collected on a membrane, extensively rinsed with Milli-Q water, dried and crushed under liquid nitrogen. The soluble matrix (intra-prismatic) was extracted from this powder preparation.

2.2. Shell protein purification and polyclonal antibodies

The intra-prismatic matrix was obtained by overnight dissolution of the prism powder (20 g) with cold dilute acetic acid (5% v/v, 4 °C). The clear solution (about 1 l) was centrifuged (4500 rpm, 10 min), ultra-filtered (10 kDa cut-off) and extensively dialyzed [24]. Caspartin, a 17 kDa-protein and one of the two main soluble macromolecules of the prisms, was obtained by a blind fractionation of the matrix on preparative gel electrophoresis, followed by a dot-blot detection as previously described [24]. The quality of the preparation was checked on mini denaturing electrophoresis gels (Bio Rad Protean III), which were subsequently stained with silver [24].

Sera containing polyclonal antibodies raised against caspartin were obtained from the purified caspartin, in a white rabbit, according to a standard protocol (Eurogentec, Seraing, Belgium). The immunization procedure was performed with injections at 0, 14, 28 and 56 days, and bleedings at 0, 38, 66 and 80 days. The sera (1st, 2nd and 3rd bleeding) were tested on ELISA for the determination of their respective titer. The specificity of each serum for caspartin was subsequently checked by running the prism soluble matrix on mini-gel, and by transferring it on PVDF membrane. The membrane was then incubated with the anti-caspartin serum, extensively rinsed, incubated with the second antibody (peroxidase conjugate Goat Anti-Rabbit, Sigma A6154), rinsed and revealed by luminol chemoluminescent staining [24].

2.3. Protein sequencing

A caspartin extract was *de novo* sequenced [25] at the Biology Department of the Technion (Smoler Proteomics Center), Haifa, with an electrospray-quadrupole-TOF mass spectrometer (Q-TOF Ultima, Micromass, UK). To this end, the caspartin extract was digested either by trypsin, either by pepsin or by aspN. This yielded peptides of different lengths, the sequences of which were determined. Sequences were analyzed for homology search in SwissProt database. A complementary analysis was performed with SIM computer program (http://www.expasy.ch/tools/sim-prot.html), by aligning two per two each obtained sequence with each of the 43 known full-length shell proteins, characterized by their SwissProt accession number.

2.4. Immunogold localization of caspartin

Following titer determination, the 2nd antiserum preparation was used for localizing caspartin in the prismatic layer. All the incubation steps were performed in Falcon multiwell tissue culture flat bottom plates (12 or 24 wells). NaOCl-isolated prisms or shell fragments were used for the