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► To cite this version:

Frédéric Marin, Virginie Morin, Frédéric Knap, Nathalie Guichard, Benjamin Marie, et al.. Caspartin: thermal stability and occurrence in mollusk calcified tissues. 9th International Symposium on Biomineralization, Dec 2005, Pucón, Chile. pp.281-288. hal-00197158

HAL Id: hal-00197158

<https://hal.science/hal-00197158>

Submitted on 11 Nov 2020

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Caspartin: Thermal Stability and Occurrence in Mollusk Calcified Tissues

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ABSTRACT

*The fan mussel *Pinna nobilis* secretes a two-layered shell, the external layer of which is composed of long calcitic prisms. The dissolution of these prisms releases an assemblage of acidic intracrystalline proteins, among which caspartin, an Asp-rich protein (Marin et al., 2005). A polyclonal antibody raised against purified caspartin was used for estimating its thermal stability, and for investigating the presence of cross-reacting proteins in the shell matrices of several mollusks.*

*In the first case, prisms of *Pinna nobilis* were heated at 100°C, for one to eleven days. The degradation of caspartin was followed on SDS-PAGE and on Western-blot. The experiment shows that caspartin is rather stable, and suggests that this protein might be preserved in recent fossil specimen.*

In the second case, shell matrices were extracted from numerous mollusks including bivalves, gastropods and cephalopods. They were subsequently tested on ELISA and on dot-blot, to check the presence of caspartin or immunologically related proteins. The pattern obtained is not superimposed with the mollusk phylogeny nor it is strictly correlated with the pattern of shell microstructures. The implications of our findings are briefly discussed.

INTRODUCTION

Among mollusks, the biomineralization of the shell is regulated by a mixture of glycoproteins and polysaccharides, collectively named the shell matrix. This amalgamate is secreted by the calcifying mantle epithelium. It interacts with the mineral ions, to form calcium carbonate biominerals, calcite or aragonite. The matrix remains closely associated with the formed minerals. It can be retrieved by dissolving the mineral phase with a weak acid or with a calcium-chelating agent like EDTA. It is then usually analyzed by classical fractionation techniques, mostly electrophoresis or chromatography. One drawback of these approaches is that they are tedious and time-consuming, and that they do not allow the comparison of numerous matrices at the same time.

One way to overcome this difficulty is to use immunological techniques. Antibodies constitute indeed powerful tools in biomineralization research (Muyzer *et al.*, 1984). They have been successfully employed for serotaxonomical studies (Muyzer, 1988; Curry *et al.*, 1991) and for detecting macromolecules from fossil shells (Muyzer, 1988; Collins *et al.*, 1991). Typically, an antibody recognizes specifically a short portion of the target-antigen, for proteins, 4 to 10 amino acids. An antibody allows large-scale comparisons against several matrices, and, because the immunological recognition is sensitive, the technique does not require large quantities of antigens.

In the present case, a polyclonal antibody was obtained from caspartin, a shell protein extracted from the outer prismatic layer of the bivalve *Pinna nobilis* (Marin *et al.*, 2005). Caspartin is an Asp-rich protein, migrating at 17 kDa of apparent molecular weight. It is not glycosylated, weakly binds calcium, and is able to polymerize. It is located around and within the prisms. Its complete primary structure has not been elucidated yet. In the present study, the anti-caspartin antibody is used for two purposes:

- To check the thermal stability of caspartin.
- To investigate the presence of caspartin, or immunologically related proteins, in the calcified tissues of several mollusks.

MATERIALS AND METHODS

Shell material preparation

Shells of juvenile specimens of the mediterranean fan mussel *Pinna nobilis* were provided by CERAM (Centre d'Etude des Ressources Animales Marines, Prof. N. Vicente, Marseille, France). Only the prismatic calcitic shell layer was used for the study. Prismatic shell fragments were scrupulously cleaned. Isolated calcitic prisms were obtained after a long treatment with dilute sodium hypochlorite, as described in a previous study (Marin *et al.*, 2001).

Protein purification and polyclonal antibody production

The sieved cleaned powder of calcitic prisms was decalcified with dilute acetic acid, and the acetic acid-soluble matrix was treated as previously described (Marin, 2003). Caspartin, an Asp-rich 17-kDa protein, was obtained by preparative SDS-PAGE (Marin *et al.*, 2001). Polyclonal antibodies against caspartin were raised in a white rabbit, according to a standard immunization procedure (Marin, 2003). The specificity of the antiserum was checked on Western-blots (Towbin *et al.*, 1979).

Thermal stability of caspartin

The thermal stability of caspartin was measured as follows: prism preparation (1 g) were heated at 100°C in a drying oven, for different durations, from 1 to 11 days. The preparations were crushed, dissolved in cold dilute acetic acid (10% vol/vol) overnight then centrifuged (3900 g) for separating the insoluble matrix from the soluble fraction containing caspartin. The volumes of the soluble matrices were reduced by ultrafiltration (10 kDa cutoff membrane), then dialyzed against water. All the matrices were freeze-dried, before being analyzed on SDS-PAGE (15% acrylamide). The gels were stained with silver or 'Western-blotted' on PVFD membranes (Immobilon), which were subsequently incubated with the anti-caspartin antiserum (for technical details, see Marin *et al.*, 2005).

Occurrence of caspartin or caspartin-related peptides in several shell matrices

The presence of peptides, which are immuno-reactive with the anti-caspartin antibody, was checked as follows: NaOCl-cleaned shells of several mollusks including bivalves, and outgroups like gastropods and cephalopods, were crushed, and 300 mg of sieved powder of each species were dissolved overnight with EDTA (6 ml, 20% vol/vol). The solutions were centrifuged (3900 rpm, 15 min.), and the supernatants were tested against the anti-caspartin antibody, by conventional ELISA and by dot-blot. For each antigen, serial dilutions were performed. Each sample was tested twice with each technique. We took care to exclude from the analysis samples which cross-reacted with the preserum, or with the secondary antibody.

RESULTS

Production of a polyclonal antibody against caspartin

Large-scale extraction of caspartin from the prism soluble matrix yielded few mg of this protein. One mg of the freeze-dried protein was used for the production of polyclonal antibodies. The working dilutions of the first, second and third bleeding antisera are respectively 1:2000, 1:3000 and 1/10000. The specificity of the different antisera is tested by Western blots (Fig. 1, right) on the whole acetic acid-soluble matrix. We observe that the antiserum (2nd bleeding) reacts against caspartin but does not cross-react with any other component of the prism matrix. Note that this antiserum cross-reacts with a 17 kDa band of the acetic acid-soluble matrix of the nacre of *Pinna nobilis*. This suggests that caspartin is also present in the nacre matrix, but in much lesser amount (data not shown). This antiserum may be used for subsequent studies.

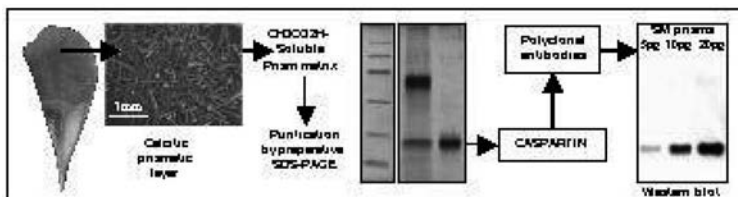


Figure 1. Elaboration of a polyclonal antibody elicited against caspartin, a soluble shell protein purified from the calcitic prismatic layer of the bivalve *Pinna nobilis*. For further details, see Marin et al. (2005).

Thermal stability of caspartin

The results of the stability experiments at 100°C are shown on Fig. 2. Both the silver stained gel and the Western blot suggest that caspartin is stable at 100°C. We do not record a significant decrease of the reactivity, which would indicate a degradation of caspartin. Nor we observe that caspartin smears towards low molecular weight compounds. On the contrary, the patterns observed after 192 h (8 days) or after 264 h (11 days) are not significantly different from those obtained at T0 or after 12 hours heating.

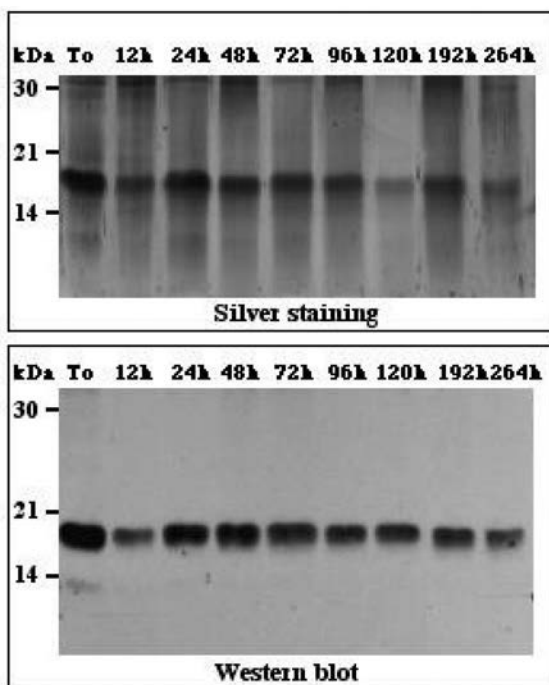


Figure 2. Thermal stability of caspartin, checked on silver stained gel (top) and on Western blot (bottom). As both gels suggest, caspartin is rather stable when heated at 100°C for several days. Each lane = 30 to 40 µg acetic acid-soluble matrix.

Occurrence of caspartin –or immunologically related proteins– in calcified tissues

The results of the dot blot experiments and of the ELISA are shown in Table 1. In addition, for the bivalve class, we also indicate for each species the mineralogy (calcite or aragonite or both) and the shell microstructures, as defined by Taylor and coworkers (1969; 1973). The immunological signals obtained with the different species are not correlated to the mollusk phylogeny. Some genera which are phylogenetically distant from *Pinna nobilis*, like the outgroup *Haliotis*, give a strong cross-reactivity with the anti-caspartin antibody. On the other hand, some closely related genera, like *Pteria*, give a moderate cross-reactivity. Within the bivalve class, the immunological pattern is not correlated to the shell microstructures. For example, the two representatives of the paleoheterodont bivalves, which exhibit a nacro prismatic shell, do not cross-react at all, while the three representatives of the pectinoids, characterized by shell foliated microstructures, give a strong cross-reactivity.

In few cases (*Pinctada radiata*, *Mercenaria mercenaria*, *Trochus maculatus*, *Crepidula fornicata*), discrepancies are observed between the two techniques. These discrepancies are not explained yet, but may relate to technical limitations: for ELISA, the lack of binding of immunoreactive peptides on the microplate polymer; for dot blot, the removal of peptides, which pass through the nitrocellulose membrane during the vacuum transfer. We are also fully aware that the analysis may be biased. In particular, only the EDTA-soluble fraction is taken into account. Furthermore, the two tests are only qualitative, because the amount of dissolved matrix may vary in a broad range from sample to sample. At last, the target epitopes of our antibody are not identified yet.

DISCUSSION

The stability of shell proteins is still a debated question since the late fifties. Precursor works of Abelson (1954), Vallentyne (1969), Hare & Mitterer (1969), Wyckoff (1972), Akiyama (1980) showed that the amino acids that constitute the shell matrices exhibit a different diagenetic stability. One drawback of these fundamental works was to consider diagenesis at the amino acid level, on bulk matrix, and not at the macromolecular level. Because no shell proteins were identified at that time, it was not possible to check their respective stability, for detecting putative differential degradation patterns. In the present case, we are able to follow a single protein, caspartin. Our experiment at 100°C shows that caspartin is rather stable at this temperature. Other experiments performed at higher temperatures (120°C, 200°C, not shown) indicate that the rate of degradation is dramatically increased. This suggests that there may be a threshold in the thermal stability of caspartin. These results have to be compared with those of Muyzer *et al.* (1984) on the soluble matrix of *Mercenaria mercenaria*.

Table 1. Presence of caspartin - or immunological related proteins - in the EDTA-soluble extracts of several shells. The reactivities are measured by ELISA and by dot blot. Significance of the reactivity signs. For ELISA: 0: OD < 0.15; +: 0.15 < OD < 0.25; ++: 0.25 < OD < 0.5; +++: OD > 0.5; OD = optical density at 405 nm. For dot-blot: 0: no cross-reactivity; +: weak cross-reactivity; ++: cross-reactivity; +++: strong cross-reactivity. For the bivalves, the mineralogy and microstructures are indicated, according to Taylor *et al.* (1969; 1973). All the one-letter abbreviations are explained above.

Phyl.	Class	Sub-class	Order	Genus and species	ELISA	Dot-Blot	Miner.	µstructure
		PALAEOTAXODONTA	Nuculoidea	<i>Nucula sulcata</i>	+/+	++	A	CP+N CL+CCL
			Arcoidea	<i>Arca noae</i>	0/0	+	A	CL+CCL
			Mytiloidea	<i>Glycymeris glycymeris</i>	0/0	++/0	A	CL+CCL
				<i>Mytilus edulis</i>	+/+	+/+	C+A	FP+N
				<i>Mytilus galloprovincialis</i>	+++/>++	+++/>++	C+A	FP+N
	B	PTERIOMORPHIA		<i>Pinna pectinata</i>	+++/>+++	+/+	C+A	SP+N
	I		Pterioidea	<i>Pteria sp</i>	+/+	+/0	C+A	SP+N
				<i>Pinctada margaritifera</i>	0/>++	+/+	C+A	SP+N
	V			<i>Pinctada radiata</i>	+++/>++	0	C+A	SP+N
M				<i>Chlamys varia</i>	+++/>++	++	C+A	F+CL
			Pectinoidea	<i>Spondylus gaederopus</i>	+++/>++	+	C+A	F+CL
O				<i>Anomia ephippium</i>	+++/>++	++	C+A	F+CCL
L	L		Ostreoidea	<i>Ostrea edulis</i>	+++/>++	++	C(+A)	P+F
			Unionoidea	<i>Unio pictorum</i>	0/0	0/0	A	P+N
L	V			<i>Anodonta cygnea</i>	0/0	+/+	A	P+N
				<i>Laevicardium oblongum</i>	0/+	+/0	A	CL+CCL
U	I			<i>Cerastoderma edule</i>	0/0	+++/>0	A	CL+CCL
				<i>Callista erycina</i>	+/>0	0	A	CL+CCL
S	A	HETERODONTA	Veneroidea	<i>Maetra subtruncata</i>	0/>++	+	A	CL+CCL
				<i>Tellina tenuis</i>	+/>0	+/+	A	CP+CL+H
C				<i>Macoma balthica</i>	+/>0	0	A	CL+CCL
				<i>Venus verrucosa</i>	0/0	0/0	A	CP+CL+H
A				<i>Mercenaria mercenaria</i>	0/0	+++/>++	A	CP+CL+H
				<i>Ruditapes philippinarum</i>	+++/>++	++	A	CP+H
		ANOMALODESMATA	Pholadomyoidea	<i>Pandora inaequalis</i>	+/>+	0	A	SP+N
G				<i>Haliotis lamellosa</i>	+++/>+++	++	A	aragonite
A			Archaegastropoda	<i>Haliotis laevigata</i>	+++/>++	+++/>++	A	calcite
S				<i>Trochus maculatus</i>	+++/>+	0		CP: composite prismatic
T				<i>Turbo coronatus</i>	+/>++	+/+		FP: finely prismatic
R				<i>Crepidula fornicata</i>	0/0	+/+++		SP: simple prismatic
O		PROSOBRANCHIA	Mesogastropoda	<i>Strombus decorus persicus</i>	+++/>+	0/+		N: naere
P				<i>Cerithium vulgatum</i>	+/>+	0		F: foliated
			Neogastropoda	<i>Pisania maculosa</i>	+/>++	++		H: homogeneous
								CL: crossed-lamellar
								CCL: complex crossed-lamellar
CEPH.		NAUTILOIDEA	Nautilida	<i>Nautilus pompilius</i>	0/0	0/0		

The apparent stability of caspartin at 100°C is intriguing. Caspartin is an intracrystalline Asp-rich protein (Marin *et al.*, 2005). In thermal degradation experiments performed in aqueous solutions, aspartic acid is rather unstable (Vallentyne, 1964), and peptidic bonds involving aspartic acid residues are the most easily broken (Partridge and David, 1950). The discrepancies between these theoretical data and our experiment suggest that caspartin is stabilized by the mineral phase or by other macromolecules of the matrix. Clearly, this idea needs to be tested further.

The second aspect of our study concerns the occurrence of caspartin-like proteins in the shell of several mollusks. The pattern obtained is neither correlated with the mollusk phylogeny, nor with the shell microstructures. In a paper published some years ago (Marin *et al.*, 1999), we obtained similar conclusions with an antiserum raised against a whole soluble matrix. The present data suggest that caspartin cannot be used for serotaxonomy, and that it is not a marker of shell microstructure.

This work will be refined in two manners: first, for the samples, which give the highest cross-reactivity with the anti-caspartin antibody, we will identify on Western blots, the cross-reacting bands, in order to obtain N-terminal sequences. In parallel, when the full primary structure of caspartin is known, we will develop monoclonal antibodies against selected domains of caspartin. These antibodies will be screened again against several molluscan matrices. Since most of the known shell proteins are composed of functional modules arranged in tandem, the microstructures may be determined by a number of key-modules. Another alternative possibility is that shell microstructures emerge at supra-molecular level, from complex spatial rearrangements of the matrix proteins. If true, this hypothesis has major implications on our ability to produce *in vitro* biomimetic materials with complex microstructures.

ACKNOWLEDGMENTS

This paper is a contribution to an ACI (“Aide Concertée Incitative” JC3049) awarded to F. MARIN by the French “Ministère Délégué à la Recherches et aux Nouvelles Technologies”. An additional support is provided by EGIDE via a “Programme d’Action Intégrée” with Croatia (PAI Cogito 09084XG).

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