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Comparison of aragonitic molluscan shell proteins

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ABSTRACT

Acidic macromolecules, as a nucleation factor for mollusc shell formation, are a major focus of research. It remains unclear, however, whether acidic macromolecules are present only in calcified shell organic matrices, and which acidic macromolecules are crucial for the nucleation process by binding to chitin as structural components. To clarify these questions, we applied 2D gel electrophoresis and amino acid analysis to soluble shell organic matrices from nacre shell, non-nacre aragonitic shell and non-calcified squid shells. The 2D gel electrophoresis results showed that the acidity of soluble proteins differs even between nacre shells, and some nacre (*Haliotis gigantea*) showed a basic protein migration pattern. Non-calcified shells also contained some moderately acidic proteins. The results did not support the correlation between the acidity of soluble shell proteins and shell structure.

1. Introduction

Mollusc shells are made of calcium carbonates. Several different shell structures (*e.g.*, nacre and cross lamellar structure) are composed of aragonite, which is the orthorhombic calcium carbonate mineral phase (Mann, 2001). Aragonite shell layers are typically found in the shell interior, but may also be external (*e.g.*, monoplacophorans, vetigastropods, nautiloids, nucloids, scaphopods) (de Paula and Silveira, 2009). It is therefore important to investigate aragonite shells as the most dominant type of molluscan shell material.

Past research was mostly based on data from nacre shells, such as *Pinctada fucata* (Samata, 2004) and *Atrina rigida* (Nudelman et al., 2006, 2007), although shell calcification models were sometimes intended to be generally applicable to molluscs (Addadi et al., 2006). Within the widely accepted mollusc shell formation concept, chitin/silk fibroin gel/acidic macromolecules have been recognized as the main components (Levi-Kalisman et al., 2001; Addadi et al., 2006).

Accordingly, acidic macromolecules are expected to be a key factor for calcium carbonate nucleation based on their negatively charged bonds. Two different types of acidic macromolecules have been widely recognized. One is acidic proteins (Weiner and Hoods, 1975) and the other is sulphated sugars (Wada, 1961).

In the case of acidic proteins, some early studies on conchiferan shell proteins would have overestimated the acidity of these proteins by focusing only on Asx (aspartic acid and asparagine) and Glx (glutamic acid and glutamine) (Marin and Luquet, 2004). Such amino acid analyses would not reveal the actual proportion of aspartic and glutamic acid, and the estimated acidity has sometimes been doubted.

Relatively recent research has overcome this problem and identified the shell protein sequence. This made it possible to calculate the theoretical pI value for each protein sequence. As reviewed by Marin et al. (2008), extremely acidic proteins (pI<4.5) are mostly localized in calcitic prismatic layers. One outstanding example is aspartic acid-rich proteins—*e.g.*, MSP1 (pI 3.2) from *Patinopecten yessoensis* calcitic layer (Sarashina and Endo, 2001), Aspein (pI 1.67) from *P. fucata* prismatic layer (Tsukamoto et al., 2004), and Asprich 1–10 (pI 2.53–3.34) from *A. rigida* prismatic layer (Gotliv et al., 2005). To date, the reported pI values of aragonitic shell proteins were above 4.7.

These results were based on pI values calculated from amino acid acidity, but acidity may have shifted due to protein modification (*e.g.*, glycosylation of sialic acid and phosphorylation). Therefore, the application of 2 D gel electrophoresis to scrutinize actual acidity is important, and the results should be compared with calculated pI values to check for the presence of modifications in soluble proteins.

Previous studies have yielded some 2 D gel electrophoresis data on soluble mollusc shell proteins. The calcitic prismatic layer of *Pinctada margaritifera* and *Pinna nobillis* (Dauphin, 2003), the entirely aragonitic shells of *Nautilus marcomphalus*, *Spirula spirula*, and *Sepia* sp. (Dauphin, 2004), and the nacre layer of *Unio pictorum* (Marie et al.,

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2007) were shown to contain acidic proteins. Except for the data from *U. pictorum*, which clearly showed main protein spot migration around pI 5–6, most of the data do not allow clear conclusions to be drawn about pI and the molecular size of proteins in gels. This calls for continued application of 2D gel electrophoresis to soluble shell proteins in various samples.

The necessity of acidic proteins for calcification in aragonite shells is still uncertain. Gastropods and cephalopods contain shell-less species such as sea slugs (Opisthobranchia) and octopus (Octopodiformes), with the variety ranging from reduced to no shell. Many squids have a relatively large non-calcified shell inside their body, and these are considered to be derived from entire aragonitic shells. Although such internal shells have been thoroughly studied and the presence of chitin well established (Falini et al., 2001; Dauphin and Marin, 1995), very little information is available about non-calcified shell soluble organic matrices. Only Dauphin (1996) analyzed shell organic matrices (solely in *Loligo* sp.) by using gel isoelectric focusing (IEF), concluding that the main shell organic matrix was basic. This calls for reconsidering the acidity of soluble proteins and the negative charge effect for the nucleation process by analyzing non-calcified shell soluble protein.

Another negatively charged factor, sulphate groups, was recognized as being an important type of acidic macromolecule for shell formation in some previous prismatic layer studies on *Pinna noblis* and *P. margaritifera* (Dauphin, 2003; Dauphin et al., 2008), and *N. marcomphalus* (Dauphin, 2002). Nevertheless, data on sulphated groups remain scarce, and comprehensive investigations with a broad taxonomic perspective are completely missing.

Overall, previous studies on acidic macromolecules have narrowly focused on a few species of bivalves and gastropods with nacre. For instance, cross lamellar structure is a more dominant shell structure than nacre, and the respective data might not reflect other general aragonitic shell characteristics. Information on the acidic proteins and sulphated glycosaminoglycans in polyplacophoran sclerites/shell plates or in scaphopod shells is completely missing, although all conchiferan classes are expected to have acidic macromolecules both in calcitic and aragonitic layers.

In this study, we applied 2D gel electrophoresis and amino acid analysis to examine the acidity of soluble proteins in nacre shells, non-nacre aragonitic shells and the non-calcified shells of so-called shell-less cephalopods (cephalopods with internal shells are termed endocochleate, as opposed to those with an external shell, ectocochleate). This approach was designed to encompass a broad taxonomic range of molluscs.

2. Materials and methods

2.1. Sample preparation

Calcified conchiferan shells and shell plates or sclerites of two polyplacophorans (*Acanthopleura japonica*) 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 16,200 *g* for 3 min at room temperature and the supernatant and insoluble organics were separated.

Non-calcified shells of *Todarodes pacificus* (Cephalopoda) were washed with 3% NaOCl for 3 min to remove organic contaminants. After rinsing with distilled water and drying in air, shells were ground with a machine grinder and soluble organic matrices were subsequently extracted with deionized water. The solution was centrifuged at 20,000 *g* at 10 °C for 3 min and the supernatant—representing soluble shell organic matrices—was lyophilized.

The shell samples of each species (1–10 g dry weight) were pooled and analyzed.

2.2. 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) following the manufacturer's instructions. Protein hydrolysis was done in HCl vapor (6 M HCl with 2% phenol) for 20 h at 110 °C in a vial with an inert atmosphere of nitrogen under vacuum. Each sample was independently replicated 3 times. The relative standard deviation (RSD) of individual amino acids was always less than 10%.

2.3. 2D gel electrophoresis

Each lyophilized sample (100–300 μg protein) was dissolved in 350 μL of rehydration buffer (8 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 0.5% DTT and 0.5% ampholytes (IPG buffer 3–11 NL, Amersham-Pharmacia, Freiburg, Germany)). Each sample was then applied on commercially available IPG gel strips (pH 3–11 NL, 18 cm, Amersham-Pharmacia) by in-gel rehydration (Rabilloud et al., 1994). Focusing was done on an IPGphor unit (Amersham-Pharmacia) to a total of 83 kVh. Transfer to the second dimension, where we used laboratory-made gels (12.5% Acrylamide, 20 × 20 cm), was done after equilibration for 2 × 15 min in 2 × 5 mL equilibration solution (50 mM Tris, pH 8.8, containing 6 M urea, 30% v/v glycerol, 2% w/v SDS). DTT (1% w/v) was added to the first equilibration solution, and iodoacetamide (2.5% w/v) to the second. The gels were silver stained according to the following sequence (Blum et al., 1987); at least 1 h fixation in 40% v/v methanol, 10% v/v acetic acid; 2 × 20 min washing in 30% v/v ethanol; 20 min washing with MilliQ water; 1 min sensitizing in 0.02% sodium thiosulphate; 3 × 20 s washing with MilliQ water; 20 min staining in 0.2% silver nitrate with 0.2 mL/L 37% w/v formaldehyde, which has to be added to the solution just before use; 3 × 20 s washing with MilliQ water; 3–4 min developing in 3% w/v sodium carbonate, 5 mg/L sodium thiosulphate, 0.5 mL/L 37% w/v formaldehyde; 20 s washing with MilliQ water; 5 min stop solution with 0.5% glycine; finally, at least 3 × 10 min washing with MilliQ water. All solutions were prepared with MilliQ water and at least reagent-grade chemicals; the whole procedure was performed under gentle shaking (Schlags et al., 2005). 2D gel electrophoresis of *Haliotis gigantea*, *T. pacificus*, *Loligo* sp., and *A. japonica* shell plates were triplicated. Other samples, except polyplacophoran sclerites, were replicated twice. Similar spot migration patterns were obtained.

2.4. Conditions for HPLC-MS/MS

HPLC-MS/MS analysis was made according to a previously published method (Pataridis et al., 2008). Chromatographic separation was carried out in a Jupiter Proteo 90 A column, 250 × 2 mm (Phenomenex, Torrance, CA, USA). The HPLC apparatus used was a HP 1100 LC system (Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostated column compartment and a diode array detector. It was coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra).

The separation was achieved via a linear gradient between mobile phase A (water-formic acid, 100:0.03, v/v) and B (acetonitrile-formic acid, 100:0.025 v/v). Separation was started by running the system isocratically for 2 min with 2% mobile phase B, followed by a gradient elution to 35% B at 40 min. Finally, the column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by washing with 2% mobile phase B for 10 min. The flow-rate was 0.25 mL/min, the injection volume was 40 μL, and the column temperature was held at 25 °C.

Atmospheric pressure ionization-electrospray ionization (API-ESI) positive mode ion-trap mass spectrometry was used. Operating conditions: drying gas (N_2), 10 L/min; drying gas temperature, 350 °C; and nebulizer pressure, 172.37 kPa. Ions were observed over the mass range m/z 100–2200 (MS—standard mode, MS/MS—enhanced mode). Analysis was done in auto MS/MS mode (10 precursor ions, excluded after 2 spectra for 0.5 min). The fragmentation amplitude was set to 1.14 V.

MS/MS data were analyzed using SpectrumMill software (v.3.02, Agilent). Searches were performed in the full protein databases SwissProt and NCBInr and then on the data extracted from these databases.

3. Results

3.1. Amino acid analysis

Almost all samples tested contained a high proportion of glycine (12.7–34.56%) (Table 1). Asx was generally higher than Glx, except in two bivalve species and *Loligo* sp. Although the actual proportion of Asp and Glu was not determined, the potential acidic amino acid proportion of polyplacophoran shell plates (*A. japonica* and *Haliotis discus*) was high (Asx + Glx, 36.82% and 31.1%, respectively). Entirely aragonitic conchiferan shells showed moderate amounts of Asx + Glx (18.7–21.87%). In contrast, the values of the non-calcified shells of the two squid species were the lowest (10.8 and 12.81%). With regard to basic amino acids (His + Arg + Lys), the non-calcified squid shells contained the highest proportion (10.82 and 13.62%). Moreover, higher proportions of Ala, Pro, and Val are a common characteristic in these non-calcified shells (versus calcified shells). Another factor, polar amino acid, differed between samples, and no clear tendency was associated with taxa, calcification, or nacre.

3.2. 2D gel electrophoresis

The tested taxa differed in the acidity and molecular size of nacre soluble proteins. *Atrina japonica* nacre soluble proteins showed smear spots (Fig. 1a). These spots represented only the acidic part (pI<4) and the lower molecular mass region (<37 kDa). The acidity of *P. fucata* nacre soluble proteins, in contrast, ranged mostly from pI 4 to 8 (Fig. 1b). Protein size also varied from 75 kDa to 20 kDa. In gastropod nacre, an interesting difference was observed between two Haliotidae (*H. gigantea* and *H. discus*). The main spots in *H. gigantea* nacre soluble proteins migrated pI 6–7, MW 37 kDa, as well as pI 11, around MW 15 kDa (Fig. 1c). The corresponding values in *H. discus* were pI 4, MW around 40 kDa and 15 kDa (Fig. 1d). One of the spots in the lower region is considered to be Perlucin (pI 5.7, MW 15 kDa) (Marin et al., 2008). *Dentalium elephantinum* (Scaphopoda), whose shell has 3 calcified aragonite layers, showed only acidic spots (Fig. 1e). There are some spots at pI 5.5, MW 75 kDa, pI 5.2, 50 kDa and 30 kDa. *T. pacificus* (Fig. 1f) shell organic matrices contained moderately acidic and neutral spots (around pI 6) as major components, and *Loligo* sp. (Fig. 1g) shell organic matrices were slightly more acidic (main spots around pI 5.5). In both shell organic matrices, the cluster of spots was from 10 kDa to 50 kDa. One spot around pI 5, MW 15 kDa in *Loligo* sp. appears to be different from that in *T. pacificus*. 2D gel electrophoresis data revealed that *T. pacificus* and *Loligo* sp. lack basic proteins or any basic spots. In polyplacophoran soluble proteins, the 2D gel electrophoresis data showed spots in the acidic region. Electrophoresis of polyplacophoran shell plates yielded only one distinct spot around pI 4.5, 37 kDa (Fig. 1h). Sclerites of both species showed spots at a similar position (Fig. 1i). In all cases, the spots migrated to the acidic area. There were no neutral or basic spots. LC/MS/MS data indicated that the main spot of the polyplacophoran shell plate would contain some collagenous peptides, although the entire molecular mass of collagen does not match this spot (Supplementary Table 1).

4. Discussion

2D gel data revealed that the acidity of soluble shell organic matrices varied even between nacre-shelled mollusc species. The mass of insoluble shell organic matrices also varied between species. In general, nacre shells contained more organics than other shells.

2D gel electrophoresis of soluble proteins showed a broad range of acidities. Those of *A. japonica* (nacre) are mainly acidic, while those of *P. fucata* (nacre) showed variation in acidity. Such a wide range of soluble protein migration patterns in *P. fucata* nacre was previously reported by Takakura et al. (2008). This indicates that acidity could differ even between pteriomorphs.

There was a huge difference in the distribution of nacre soluble protein in Haliotidae: *H. discus* mostly contained acidic proteins, *H. gigantea* basic and neutral proteins. The presence of very basic shell proteins in *H. gigantea* nacre is unique. The currently reported most basic shell proteins are schematrin7 in the prismatic layer of *P. fucata* (theoretically calculated pI: 10.3) and N66 (pI 8.66) in the nacre of *Pinctada maxima* (Marin et al., 2008). Data on *H. gigantea* suggested that the nacre shell could have more basic proteins, although it is highly unlikely that nacre is formed by basic proteins. Surprisingly, our 2D gel

Table 1
Amino acid analysis of soluble shell proteins. Unit indicates mol%. Asx means Asp + Asn, and Glx means Glu + Gln. In this hydrolysis condition, Trp is not detectable. The relative standard deviation (RSD) of individual amino acids was always less than 10%.

	Acanthopleura japonica	*Dentalum elephantinum*	*Solecurtus strigillatus*	*Mya arenaria*	*Nautilus* sp.	*Todarodes pacificus*	*Loligo* sp.	*Haliotis discus* (nacre)	*Helix pomatia*
Asx	25.7	14.94	8.9	9.0	16.27	5.22	4.53	26.8	10.5
Glx	11.1	6.85	10.0	9.7	5.60	4.86	8.28	4.3	8.7
Ser	4.8	7.80	7.7	6.4	8.38	3.59	3.62	4.7	7.3
Gly	16.2	30.04	12.9	12.7	34.56	17.12	17.29	27.5	18.0
His	0.8	0.67	1.1	0.5	0.75	11.20	3.27	0.9	0.5
Arg	4.8	2.71	5.8	4.9	2.94	0.72	3.83	3.7	3.0
Thr	3.1	2.87	6.5	8.5	2.47	4.54	4.95	2.8	7.1
Ala	4.1	9.67	3.3	3.7	8.87	11.75	11.59	6.8	2.6
Pro	5.1	6.09	13.3	16.7	6.08	12.97	11.49	4.7	11.2
Tyr	3.1	2.17	3.5	1.8	2.31	6.96	3.69	2.1	3.3
Val	5.3	2.74	6.2	8.9	1.99	7.27	7.43	3.1	6.0
Met	1.3	0.19	2.4	2.0	0.14	0.17	1.23	0.8	3.2
Cys	1.2	0.04	2.3	2.1	0.03	0.04	0.30	0.4	1.5
Ileu	4.1	2.28	4.3	5.5	1.67	1.63	4.50	1.9	4.3
Leu	6.7	3.29	5.9	5.0	2.28	6.83	6.06	5.2	7.7
Phe	0.7	6.43	4.1	1.0	4.96	3.43	4.21	1.7	2.8
Lys	1.8	1.21	1.6	1.3	0.73	1.70	3.71	2.5	1.5

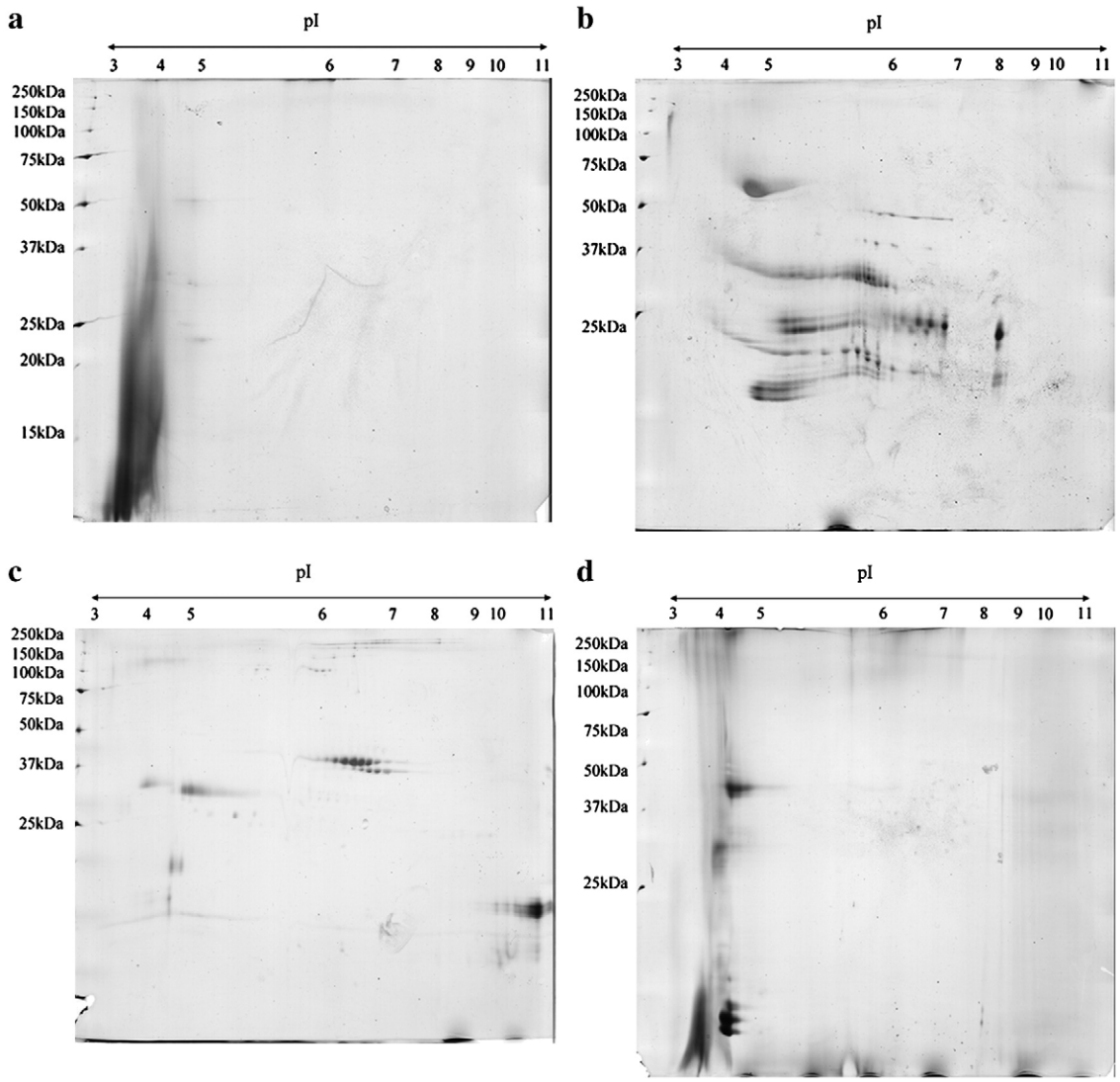

Fig. 1. 2D gel electrophoresis gel of soluble shell organic matrices. a; *Atrina japonica* (nacre), b; *Pinctada fucata* (nacre), c; *Haliotis gigantea* (nacre), d; *Haliotis discus* (nacre), e; *Dentalium elephantinum*, f; *Todarodes pacificus*, g; *Acanthopleura japonica* shell plate, h; *Acanthopleura japonica* sclerites. Approximate pI value (3–11 NL) is indicated above gel photos. Molecular mass is shown on left side of gel.

electrophoresis data on non-calcified squid shells (*T. pacificus*) showed a "more acidic" migration pattern of spots than *H. gigantea* nacre.

Our initial data on scaphopods confirm the presence of acidic proteins in soluble shell organics in this class. The main spots were at a higher molecular mass than other shell proteins, but more information must be collected on scaphopod shells before drawing definitive conclusions.

Our 2D gel electrophoresis data for non-calcified squid shell contradict previous studies (Dauphin, 1996), which concluded that the main soluble organics in the non-calcified squid shell (*Loligo* sp.) were basic. The electrophoresis sample preparation and operation did not differ significantly from Dauphin's experiment. As such, the difference between *Todarodes* (Oegopsida) and *Loligo* sp. (Myopsida) could be attributed to different taxonomic groups within the cephalopods. Another possible explanation for the observed difference is that the recently commercially available IEF strips for 2D gel electrophoresis could be better than gel IEF electrophoresis. Making gel for IEF is more difficult and the gel quality is less uniform. The soluble proteins detected by 2D gel electrophoresis in both polyplacophoran sclerites and shell plates were acidic. Compared with previously reported shell proteins for aragonite formation, the protein sizes were relatively close to nacrein (*P. fucata* nacre, pI 6.85, 50.1 kDa; *Turbo marmoratus*, pI 5.76, 57.6 kDa) (Marin et al., 2008). The soluble proteins in polyplacophoran shell plates and sclerites would be classified as moderately acidic, and their acidity is relatively high among reported soluble proteins obtained from aragonite shell layers. Proteins with pI 5.5–6 are classified as moderately acidic (Marin et al., 2008), and recent 2D gel electrophoresis data of *U. pictorum* soluble proteins also showed that the main spots migrated around pI 5–6 (Marie et al., 2007). Except for smear spots in *A. japonica*, there was no distinctive spot at pI < 3. Asprich proteins whose pI is less than 3 are apparently absent in other nacre shell layers.

2D gel electrophoresis data suggest that non-calcified shell proteins (*T. pacificus*) are consistently present on the more neutral side than other calcified shells analyzed in this study, except *H. gigantea*. Amino acid analysis showed that the soluble proteins of *T. pacificus* contained more His as a basic amino acid, which would contribute to the pI shift to the neutral side. The Asx + Glx proportion in non-calcified soluble shell proteins was relatively small.

Polyplacophoran shell plates and *H. discus* nacre soluble proteins contained a higher proportion of Asx than squid. At a glance, such a bigger proportion fits the Weiner and Hoods (1975) template model, *i.e.* that the Asp-Gly/Ser repeat is the template for the nucleation process. A high proportion of Asx, however, might not fit to the protein migration pattern in 2D gel electrophoresis. For instance,

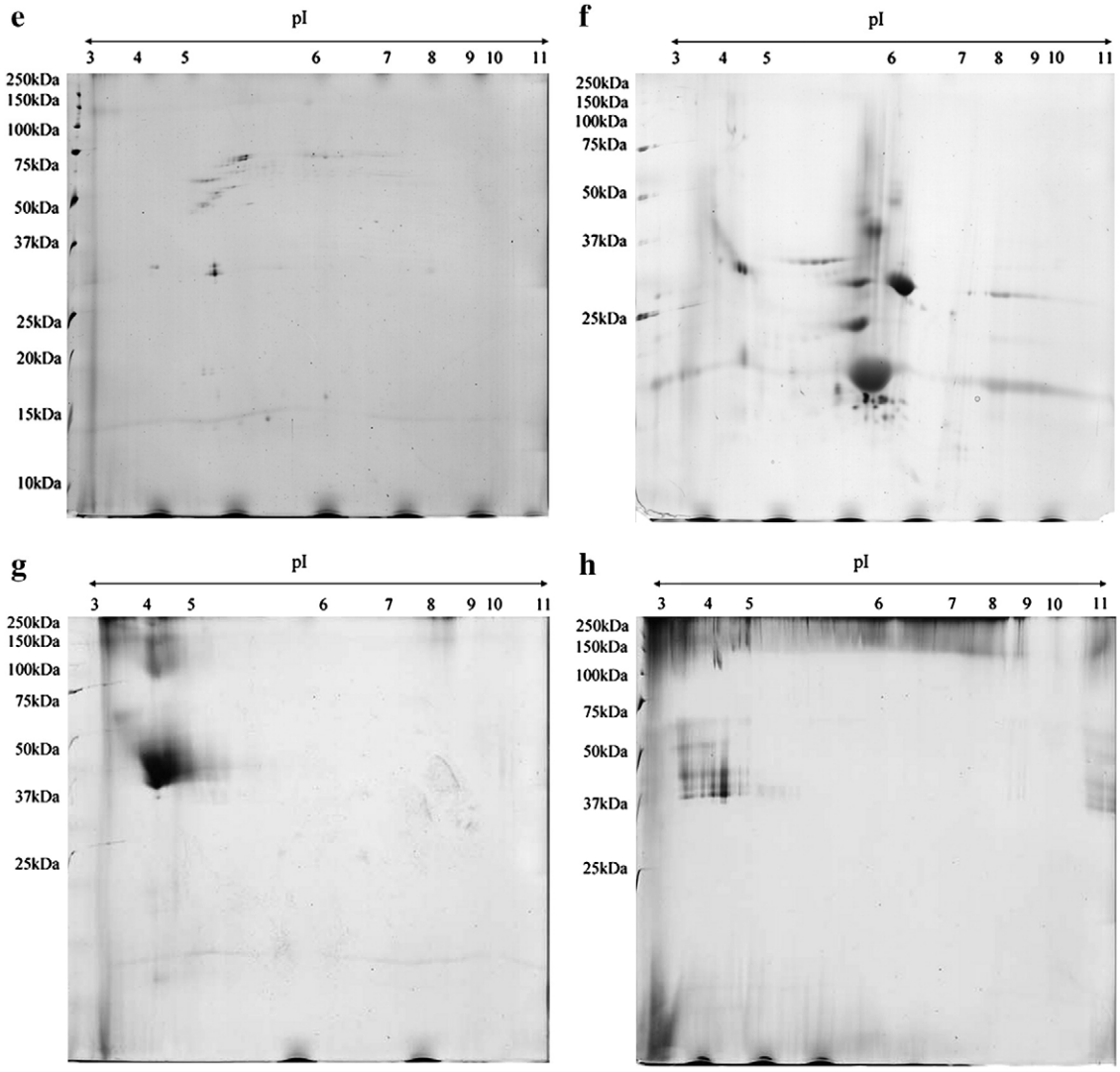

Fig. 1 (*continued*).

H. gigantea has many basic soluble proteins in 2D gel electrophoresis. There are some plausible explanations for these complicated data. The first possibility is that Asx was mostly Asn, and that the proportion of Asp was small. In this case, some of the proteins should contain more Asn, but previously reported *Haliotis* shell proteins have neither much Asp nor Asn (Marin et al., 2008; Wang et al., 2008). The second possibility is the presence of some other unknown factors that contributed to Asx peak height in the amino acid analysis. This explanation might be feasible if some other amino acids were present that do not contribute to pI but to Asx peak height. The third possibility is that proteins with high molecular mass were stacked between the stacking gel and running gel and were not visible in the 2D gel. Proteoglycans would be a candidate.

Given that many entirely aragonitic shells and sea urchin tests (single calcitic layer) contain fewer insoluble organics than nacre shells, the calcification process for both calcite and aragonite crystals itself does not require a bigger mass of insoluble organics. Some previous studies stated that the proportion of insoluble organic matrix varies widely and that non-nacre shells have less insoluble organic matrices than nacre-shelled molluscs (Esteban-Delgado et al., 2008; Marin et al., 2008). Our data were consistent with this statement. In particular, Esteban-Delgado claimed that nacre shells are stronger mechanically, and that this strength would allow the respective molluscs to secrete more organic matrices for the shell. We cannot find a plausible explanation for the smaller amount of insoluble organics in *H. gigantea*. This species also showed a unique migration pattern in 2D gel electrophoresis, and its nacre formation might be different. Accordingly, different calcification mechanisms might exist even within the genus *Haliotis*. In fact, previously reported shell proteins from *Haliotis* showed a variety of protein acidities and molecular sizes (Marin et al., 2008). Another interesting comparison is with freshwater nacreous bivalves: *U. pictorum* shells contained 0.5% insoluble organic matrices, less than in other nacre shell organic matrices (Marie et al., 2007). This different insoluble organic mass between nacre shells supports the idea of several different nacre formation mechanisms.

In summary, there were clear differences between shell organic matrices with respect to the acidity of soluble shell proteins and the mass of insoluble organics. These factors varied considerably even between aragonitic shells, making it difficult to explain nacre formation by one simplified hypothetical concept.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.cbpb.2009.11.007.

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