Preservation of the shell matrix protein Dermatopontin in 1500-year-old land snail fossils from the Bonin islands

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Abstract

Organic molecules such as proteins are preserved in certain fossils. The bulk properties of fossil proteins of both vertebrates and invertebrates have been studied for over a half-century. Named proteins have so far been identified, however, only from vertebrate fossils, such as Collagen from mammoth bones. We examined the presence of Dermatopontin, a molluscan shell matrix protein, in 1500-year-old land snail fossils from the Bonin islands using immunological assays. Firstly, we examined shell microstructure and mineralogy of the fossil shells of *Mandarina luhuana*, an extinct land snail, by SEM and powder XRD to estimate the extent of diagenetic alterations. The results suggest that the original microstructure and mineralogy of the fossil shells are preserved. Antiserum raised against Type-1 Dermatopontin fragment of the living land snail *Euhadra brandtii* showed significant immunological reactivity with the extracts from the fossil shells of *M. luhuana*. Immunological binding curves drawn for the shell extracts of extant *Mandarina aureola* and extinct *Mandarina luhuana* confirmed the presence of Dermatopontin in fossil shells, and further gave an estimate that about 75 to 98% of original Dermatopontin was lost in the *M. luhuana* fossils. This is the first report on a named protein identified from invertebrate fossils.

Key words: antiserum; Dermatopontin; invertebrate fossils; immunological assays; immunological binding curves; *Mandarina luhuana*; molecular paleontology; mollusks; shell matrix proteins.

1. Introduction

The fossil record is a key to go into ancient world, and has provided invaluable information particularly for geology and evolutionary biology. Some fossils contain organic molecules, sometimes in the form of macromolecules such as proteins. The bulk properties of the mixtures of protein fragments in fossils have been studied for over a half-century (cf. Abelson, 1954). To utilize those pieces of information in phylogenetic and other evolutionary studies, however, it is desirable to identify and characterize proteins. Over the last few decades, some named proteins have been identified from vertebrate fossils using immunological assays, such as Osteocalcin from bovid bones (12 thousand years (Ka) old to 13 million years (Ma) old), rodent teeth (30 Ma) (Ulrich et al., 1987), deer antlers (1 Ma), moa bones (3.6 Ka) (Huq et al. 1985), bison bones (0.8-450 Ka), an elk (5.8 Ka), a mastodon (12 Ka), a walrus bone (53 Ka) (Ostrom et al., 2000), and dinosaur bones (75 Ma) (Muyzer et al., 1992), Albumin from fossil urines of rodents and hyraxes (1-20 Ka) (Lowenstein, 1991), rodent bones (1.7 Ka) (Montgelard, 1992) and a frozen mammoth (44 Ka) (Prager et al., 1980), and Collagen from bones of emus (0.15-26 Ka), kangaroos (26 Ka), wallabies (0.15 Ka) (Rowley et al., 1986), Cro-Magnons, Neanderthals, *Homo electus* (0.5 Ma) and *Australopithecus robustus*

(Lowenstein, 1980, 1981). More recently, partial amino acid sequences of some fossil proteins have been determined, such as those of Osteocalcin from moa bones (3.6 Ka) determined by N-terminal degradation (Huq et al., 1990), bones of camels (21 Ka) (Humpula et al., 2007), Neanderthals (75 Ka) (Nielsen-Marsh et al., 2005) by mass spectrometry, and Collagen from bones of mammoth (0.1-0.3 Ma) (Schweitzer et al., 2002), mastodon (160-600 Ka) and *Tyrannosaurus* (68 Ma) (Asara et al. 2007) by mass spectrometry. On the other hand, no named protein has been identified so far from invertebrate fossils, although some bulk properties of the invertebrate fossil proteins have been reported (Jope, 1967; Matter et al., 1969; Akiyama and Wyckoff, 1970; de Jong et al., 1974; Westbroek et al., 1979; Collins et al. 1991; Endo et al., 1995). In the past six years, the complete sequences of Osteocalsin from bones of bison (55 Ka) (Nielsen-Marsh et al., 2002) and a horse (42 Ka) (Ostrom et al., 2006).

Almost 100 species of proteins have been identified from the skeletons of living invertebrates to date (Sarashina and Endo, 2006), and about one-fourth of them are molluscan shell matrix proteins. Molluscan shells are one of the most abundant sources of fossils. Molecular information from shell fossils is, therefore, expected to be useful for evolutionary studies. Dermatopontin is one such molluscan shell matrix protein, found in shells of pulmonate snails (Marxen et al., 2003; Sarashina et al., 2006),

that can provide historical information from fossils. In order to determine if Dermatopontin is preserved in fossil shells of pulmonate snails, we performed immunological assays, and we demonstrate its presence in 1500-year-old shells. This is the first report on a named protein identified from invertebrate fossils.

2. Materials and methods

2.1. Samples

Japanese land snails, *Euhadra brandtii* and *Mandarina aureola* were collected in Tsukuba city, Ibaraki Prefecture and Hahajima, Bonin (Ogasawara) Islands, respectively. Freshwater snails, *Lymnaea stagnalis* and *Biomphalaria glabrata*, originally collected in Neustadt, Donau, Germany and Costa Rica, respectively, were maintained in deionized and calcium carbonate saturated tap water on lettuces at about 23ºC. Fossil shells of *Mandarina luhuana* were collected in Chichijima, Bonin (Ogasawara) Islands, Japan. *M. luhuana* fossils previously collected from the same bed in Chichijima had been dated at 1500 ± 60 yrBP using carbon 14 (Chiba, 1989).

2.2. Scanning electron microscopy

Fractured shells were sputter-coated with gold using JFC-1200 (JOEL, Tokyo). Specimens were examined with JSM-5500V scanning electron microscope (JOEL).

2.3. Powder X-ray diffraction (XRD)

A 50 μg of shell was ground to a powder with an agate mortar and a pestle. The powdered shell was put on a glass plate and flattened. XRD measurements were carried out between 20º to 40º 2θ using a RAD-A system (Rigaku, Tolyo).

2.4. Extraction of soluble organic materials from shells

Both Recent and fossil shells were thoroughly cleaned mechanically and incubated in a 5 % (v/v) aqueous solution of bleach with gently shaking at room temperature for 48 h in order to destroy surface contaminants. After thorough washing with ultrapure water, shells were crashed to fine fragments. Organic material was extracted by dissolution of 0.2 g of the shell fragments in 10 ml of 0.5 M

ethylenediaminetetraacetate (EDTA), pH 8.0, with agitating by a magnetic stirrer at room temperature for 24 h. To remove insoluble materials, the sample solution was centrifuged at 3000 g at 4 ºC for 10 min, and the supernatant was used for immunological assays. The sample solution was appropriately concentrated using a centrifugal filter device, Amicon Ultra-15 (Millipore; Billerica, MA, USA) by successive centrifugations at 5000 g at 4 ºC for 1 h.

2.5. Antiserum preparation

Rabbit antiserum was prepared against a synthetic peptide corresponding to the position 26 to the position 4 upstream of the C-terminus of Type-1 Dermatopontin of *E. brandtii* (*Eb*Derm1) (Sarashina et al., 2006), comprising 23 amino acid residues (Fig. 3). The purified synthetic peptide was conjugated at the C-terminus to Keyhole Limpet Hemocyanin (KLH) as a carrier protein. One rabbit was injected with 1 mg of the synthetic peptide five times at interval of two weeks with Freund's complete adjuvant (FCA) and incomplete Freund's Adjuvant (IFCA) as an immunoactivator. Serum was collected and titers were determined one week after the last injection.

2.6. Enzyme linked immunosorbent assay (ELISA)

Five microliters (μl) of the sample solution described in the section 2.2. in 95 μl of Tris-buffered saline (TBS; 0.8% (w/v) NaCl, 0.2% (w/v) KCl, 25 mM Tris, pH7.4) were incubated at 37ºC for 90 min in each well on a multiwell plate. After emptying the wells, they were washed with 0.2% (v/v) Tween 20 in TBS (TBS/Tween) three times. The wells were blocked by incubation with 100 μl of 2% (w/v) gelatin in TBS at 37ºC for 30 min. After emptying the wells, 100 μl of rabbit antisera diluted appropriately (1/50-1/25600) by 0.2% (w/v) gelatin in TBS/Tween (gelatin/TBS/Tween) were added to each well and incubated at 37ºC for 90 min, followed by the TBS/Tween wash as described above to remove unbound antibodies. Then 100 μl of 0.05% (v/v) Anti-rabbit IgG alkaline phosphatase conjugate (A8025, Sigma; St Louis, MO, USA) in gelatin/TBS/Tween was added to each well and incubated at 37ºC for 90 min. After the TBS/Tween wash to remove unbounded second antibodies, 100 μl of the 1% (w/v) 4-nitrophenyl phosphate (pNPP) disodium salt hexahydrate (S0942, Sigma) in 1 M diethanolamine, pH 9.8, with 0.5 M $MgCl_2$, was added to each well and incubated in dark at 37ºC for 30 min, followed by adding 100 μl of 1N NaOH to each well to stop the staining reaction. The color intensity was measured spectrophotometrically at 405

nm using a micro plate reader (MPR-A4i II; TOSOH, Tokyo). All assays were carried out in triplicate.

3. Results and discussion

2.1. Estimating the preservation of microstructure and mineralogy of fossil shells

In order to estimate the extent of diagenetic alterations of the fossils of *M. luhuana*, we examined their shell microstructure and mineralogy by SEM and powder XRD, respectively. SEM images of the cross-lamellar layers of shells of extant *E. brandtii* and *M. aureola*, and a fossil shell of *M. luhuana* are shown in Fig.1a-c, respectively. The microstructure of the fossil shell showed no sign of dissolution or overgrowth (Fig. 1c), when compared with that of the shells of extant species (Fig. 1a, b). It appeared that the original structure of the fossil shell was preserved.

X-ray diffraction patterns for shells of extant *E. brandtii* and *M. aureola*, and a fossil shell of *M. luhuana* are shown in Fig. 2a-c, respectively. All these three showed a typical pattern for aragonite (Fig. 2). At the temperatures and pressures found near the Earth's surface, aragonite is known to be metastable and recrystalize spontaneously

during diagenesis to calcite. We could not, however, even detect the peak of 29.5 degrees corresponding to the (104) planes of calcite, which is the most intense calcite peak, in XRD patterns of the fossil shell (Fig. 2c) as well as the shells of extant snails (Fig. 2a, b). The results suggest that the diagenetic transition of aragonite to calcite have not occurred and the original mineralogy was preserved in the fossil shell.

2.2. Immunodetection of Type-1 Dermatopontin in shell extracts

The antiserum was raised against the synthetic peptide corresponding to a partial amino acid sequence near the C-terminus of Type-1 Dermatopontin of *E. brandtii* (*Eb*Derm1). Pulmonate snails have two or three types of Dermatopontins, and only Type-1 Dermatopontin (Derm1) is considered to be a shell matrix protein (Sarashina et al. 2006). The antiserum reacted positively with the extracts from the shells of the extant land snail *E. brandtii* served as the positive control (Fig. 4a). The antiserum also reacted positively with shell extracts of the other living land snail *M. aureola* and the two living freshwater snails, *B. glabrata* and *L. stagnalis* (Fig. 4a). The intensities of the reaction with the two freshwater snails were lower than those with the land snail *M. aureola* and *E. brandtii*. The reaction intensities correlate with the similarities of the amino acid

sequences of the antigen Derm1 (Fig. 3), which probably reflect the evolutionary relationships. The two freshwater snails are evolutionarily more distantly related to *E. brandtii* than to the land snail *M. aureola*. Although the antiserum was expected to react most strongly with the shell extracts of *E. brandtii* served as the positive control, it reacted slightly higher with *M. aureola*, a species closely related to *E. brandtii* (Fig. 4a). The antiserum might detect relative quantities of Derm1 in shell extracts in these two closely related species.

We also performed immunochemical analysis on the extracts from the 1500-year-old fossils of *M. luhuana*. The antiserum reacted with the extracts from the *M. luhuana* fossils much weaker than those from the shells of *M. aureola* or *E. brandtii* (Fig. 4a). Because the extinct species *M. luhuana* belongs to the same genus as *M. aureola*, the low reaction intensity is probably due to small quantity and degradation rather than due to amino acid sequence differences of Derm1. The reactions with the extracts from the *M. luhuana* fossils were slightly but significantly higher than those with EDTA served as the negative control, suggesting that Derm1 or fragments of Derm1 are preserved. To reconfirm the presence of fossil Derm1 fragments and to quantify them, we performed serial dilution assays (Endo et al., 1995) and drew immunological binding curves for the reactions with the shell extracts from living *M.*

aureola, extinct *M. luhuana* and EDTA served as a negative control. To obtain the same level of immunological reaction as extant *M. aureola*, about 4- to 40-fold concentrated antiserum was required for the extinct *M. luhuana* (Fig. 4b). This means that about one-fourth to one-fortyth of the original Derm1 is preserved in the fossil shells of *M. luhuana*, that is, about 75 to 97.5% of original epitopes of Derm1 was lost.

Land snail fossils, especially in tectonically uplifted islands, are interesting in evolutionary studies, because their limited active dispersal promotes speciation. But it is often difficult to estimate phylogenetic relationships and divergence times of extinct land snails only by using morphological data. This in fact is the case for *Mandarina luhuana*. It is classified as belonging to the genus *Mandarina*, which is endemic to the Bonin Islands, by virtue of possession of certain morphological traits. It appears possible, however, that the diagnostic shell features of *Mandarina* evolved convergently through adaptation to insular environments. Our immunological analysis determined that at least some peptide fragments of Drem1 are preserved in the fossil shells of *M. luhuana*. When we determine their amino acid sequences to compare with known sequences of living species (Fig. 3), we should be able to make clear whether *M. luhuana* is phylogenetically closer to living *Mandarina* species than to the mainland *Euhadra* species, or vice versa. Moreover, these analyses will certainly be useful for

palaeobiological and palaeoecological reconstructions in future. In this paper, we identified a named protein from invertebrate fossils for the first time, and this would open a new field of invertebrate molecular paleontology.

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Fig. 1. Cross section SEM images of fractured shells of (a) extant *E. brandtii*, (b) extant *M. aureola* and (c) a fossil of *M. luhuana*. Bottom to top in each image corresponds to the direction toward the outer shell surface.

Fig. 2. X-ray diffraction patterns for shells of (a) extant *E. brandtii*, (b) extant *M. aureola* and (c) a fossil of *M. luhuana*. Intensity means “diffraction intensity in counts per secomd”.

Fig. 3. Alignment of the part of the Type-1 Dermatopontin sequences for which the peptide for immunization was synthesized. *Eb*Derm1, *Ma*derm1, *Bg*Derm1 and *Ls*Derm1 stand for Type-1 Dermatopontin of *Euhadra brandtii*, *Mandarina aureola*, *Bionphalaria glabrata* and *Lymnaea stagnalis*, respectively. Identical amino acids are indicated by asterisks. The peptide was synthesized for the sequence of *Eb*Derm1, which is located at the position 26 to position 4 upstream from the C-terminus of *Eb*Derm1.

Fig. 4. Results of ELISA. (a) Immunological reactivity for soluble organic materials from shells of four living pulmonates (*E. brandtii* (*Eb*), *M. aureola* (*Ma*), *B. glabrata*

(*Bg*) and *L. stagnalis* (*Ls*)) and fossil shells of *M. luhuana* (*Ml*). EDTA (E) solution served as a negative control. Error bars represent standard deviations. Antiserum used was at a dilution of 1:1000. (b) Immunological binding curves for the shells of extant *M. aureola* (A), the fossil shells of *M. luhuana*(B) and EDTA served as a negative control (C).