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Molecular characterization of the major yolk protein of the Japanese common sea cucumber (*Apostichopus japonicus*) and its expression profile during ovarian development

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ABSTRACT

The most abundant protein in the coelomic fluid of the Japanese common sea cucumber (*Apostichopus japonicus*) was purified through two steps of liquid chromatography. Subsequent peptide sequencing and cDNA cloning demonstrated that the purified fraction contained two similar but distinct proteins. Deduced amino acid sequences of these proteins revealed about 30% identity with those of sea urchin major yolk protein (MYP) and therefore they were designated *Aj*MYP1 and *Aj*MYP2. The full-length cDNAs for *Aj*MYP1 and *Aj*MYP2 consisted of 4600 and 4420 bp, with predicted protein lengths of 1365 and 1345 amino acid residues, respectively. RT-PCR detected transcripts for both types of *Aj*MYPs in all the tissues and organs examined. The transcript levels of both *Aj*MYPs in the ovary were apparently elevated at late stages of ovarian development whereas the MYP content of the ovary examined by SDS-PAGE remained stable throughout ovarian development.

1. Introduction

During oogenesis of oviparous animals, the growing oocytes accumulate large quantities of yolk materials, including proteins, lipids, carbohydrates, vitamins, and carotenoids, which supply essential nutrients until feeding begins. The most abundant yolk protein in many oviparous animals is the vitellin, which is an enzymatic cleavage product of a precursor protein, vitellogenin. Initially, the term, vitellogenin, was not applied to a specific molecular structure but to the biological features of a female-specific blood protein sequestered by vitellogenic oocytes (Pan et al., 1969). However, more recent biochemical and molecular analyses have demonstrated that vitellogenins of diverse animals, including vertebrates, insects, crustaceans, nematodes, mollusks and corals, are structurally similar to varying degrees. The genes encoding vitellogenins of those animals are therefore considered to have diverged from a common ancestral gene (Smolenaars et al., 2007).

The yolk protein precursor of sea urchins has also been called vitellogenin (Shyu et al., 1986). At present, however, it is generally termed major yolk protein, MYP, since the sea urchin yolk protein is structurally distinct from vitellin/vitellogenin. Sequencing of complementary DNA (cDNA) for MYPs demonstrated that the protein was not a homologue to a vitellogenin found in other animals but was a transferrin-like protein (Unuma et al., 2001; Brooks and Wessel 2002; Yokota et al., 2003). To date, no vitellogenin or vitellin protein has been identified in sea urchins although three genes that encode vitellogenin-like proteins were discovered in the genome of the American purple sea urchin (*Strongylocentrotus purpuratus*) by a whole genome sequencing (Song et al., 2006). Thus, a protein structurally homologous to vitellins appears to be absent from sea urchin eggs or, if it is present, it is not a major constituent in developed oocytes.

Beside its molecular structure, the MYP of sea urchins has unique features that distinguish it from vitellins. MYP is a substantial constituent of the coelomic fluid irrespective of sex and reproductive season (Harrington and Easton, 1982; Giga and Ikai, 1985a). It is also abundant in the immature gonads of both sexes (Unuma et al., 1998). Nutritive phagocytes, which are somatic cells in the ovary and testis, store MYP before the onset of gametogenesis. During oogenesis some of the MYP in the nutritive phagocytes is transferred to the oocytes as a yolk component and the remaining MYP is metabolized to produce materials necessary for egg development (Unuma et al., 2003). During spermatogenesis, on the other hand, most of the MYP is utilized as a source of materials for sperm production, resulting in the disappearance of MYP from the mature testis (Unuma et al., 2003). Additionally, it has been proposed that MYP functions as a transporter of metals, such as zinc (Unuma et al., 2007) and iron (Brooks and Wessel, 2002), from the coelomic fluid to oocytes during oogenesis, and as a cell adhesion molecule during embryogenesis (Noll et al., 2007; Hayley et al., 2008).

Yolk proteins are excellent biomarkers for understanding the process of ovarian development and its regulatory system. As described

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above, the involvement of MYP in sea urchin oogenesis has been well documented but, except for a starfish yolk protein (Reimer and Crawford, 1995), yolk proteins of other echinoderms have not yet been reported. Thus, it remained unknown if sea cucumbers possess MYP and/or vitellogenin as yolk proteins. In this study we clarified the molecular characteristics of sea cucumber MYP and the expression pattern of MYP in relation to ovarian development.

2. Materials and methods

2.1. Sea cucumbers

Japanese common cucumbers (*Apostichopus japonicus*) were purchased from a local fish market in Mie, Japan, transferred to the National Research Institute of Aquaculture, and stocked in a 1000 L tank. The tank also contained sea urchins, *Pseudocentrotus depressus*, which were fed with the kelp species, *Eisenia bicyclis* and *Laminaria* sp. Thus, the sea cucumbers were reared mainly on the feces of the sea urchins for four months until maturation. Coelomic fluid was collected using a syringe from an opening made in the dorsal body wall and was centrifuged at 3000×*g* for 10 min at 4 °C. The supernatant was filtered through a 0.2 μm membrane to obtain cell-free coelomic fluid and stored at −80 °C before analysis. Mature ovaries were excised, rinsed in filtered seawater, and stored at −80 °C until use.

To analyze the changes in MYP expression throughout ovarian development, sea cucumbers were purchased from the local fish market at intervals of a few weeks from December until April. A portion of the ovary was fixed in Davidson's fixative for the histological examination of reproductive stages and the reminder was quickly frozen in liquid nitrogen and stored at −80 °C before analysis.

2.2. Reproductive stages of the ovary

Ovarian stages were classified as follows, based on their histological features and the gonad-somatic index (ovary weight/body weight %, GSI):

UG (undeveloped gonad). Germinal cells exist along with the gonad wall but sexes are indistinct. GSI<0.01.

Stage 1 (immature). Small oocytes (about 30 μm in a diameter in histological sections) become distinct. Yolk accumulation in oocytes has not started. GSI<0.1.

Stage 2 (early mature). Oocytes (30–60 μm) with basophilic ooplasm are located in the lumen of the ovary as well as at the ovarian wall. GSI<1.

Stage 3 (mid mature). Oocytes (60–100 μm) become slightly eosinophilic because of the initiation of yolk accumulation in ooplasm. GSI<5.

Stage 4 (fully mature). Ooplasm is extremely basophilic. Oocytes reach their maximum size (100–125 μm) and fill the ovary entirely. GSI<10.

Stage 5 (fully mature). Histological features of the ovary are the same as those at the stage 4. The GSI is greater than in Stage 4. GSI≥10.

2.3. Liquid chromatography

Cell-free coelomic fluid was subjected to gel filtration chromatography using Superose 6 10/30 (GE Healthcare Biosciences) equilibrated with 20 mM Tris/HCl buffer (pH 8.0) containing 100 mM NaCl (NaCl/Tris). Proteins were eluted with NaCl/Tris at a flow rate of 0.8 mL/min using an FPLC system (GE Healthcare Biosciences). Aliquots of eluate (2 mL) were collected. Fractions rich in the targeted protein were pooled and subjected to ion exchange chromatography using Mono Q 5/50 GL (GE Healthcare Biosciences) equilibrated with 20 mM Tris/HCl buffer (pH 8.0). The retained proteins were eluted with an NaCl linear gradient from 0 to 800 mM (10 or 20 mL in total) at a flow rate of 0.5 mL/min using the FPLC system. Aliquots of eluate (0.5 mL) were collected. Proteins contained in each fraction were analyzed by SDS-PAGE.

2.4. Protein electrophoresis and peptide sequencing

SDS-PAGE was performed using a 5–20% gradient polyacrylamide gel under reducing or nonreducing conditions and the separated proteins were stained with Coomassie brilliant blue R-250 (CBB). For the determination of N-terminal amino acid (aa) sequences, proteins separated by SDS-PAGE were transferred onto a PVDF membrane without staining. The targeted bands were then cut out and subjected to N-terminal aa sequencing by Edman degradation using an HP G1005A sequencer (Hewlett Packard). To determine the internal aa sequences, the proteins separated by SDS-PAGE were excised with gel, fragmented in the gel with lysyl endopeptidase and applied to reverse phase HPLC. Five peak fractions were subjected to aa sequencing by Edman degradation on a Procise 494 HT protein sequencing system (PE Applied Biosystems).

2.5. Construction of a normalized EST library and cDNA cloning

Total RNA extracted from a single ovary at stage 3 using Trizol reagent (Invitrogen) was reverse transcribed to cDNA using Powerscript reverse transcriptase (Clontech Laboratories, Inc.), followed by the synthesis of the double-stranded cDNA. The redundancy of cDNA formed by abundant RNA was reduced by duplex-specific nuclease, according to the instructions of a Trimmer-Direct cDNA Normalization kit (Evrogen JCS). Subsequently, cDNAs were amplified by PCR using SMART techniques (Clontech Laboratories, Inc.). The quality of the cDNA products during normalization and SMART PCR processes was confirmed by gel electrophoresis. A directional cDNA library was constructed by inserting cDNAs into pDNR-LIB vector (Clontech Laboratories, Inc.). The vector was transformed into *Escherichia coli*-competent cells (DH10B) and about 15,000 randomly-selected colonies were subjected to one-pass sequencing.

A full-length cDNA sequence for MYP was determined from transcripts of a single ovary by the RACE (5′- and 3′-rapid amplification of cDNA ends) method using a SMART RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) according to the manufacturer's protocol. Specific primers used for RACE and subsequent PCR amplification of internal regions were designed from EST sequences encoding the internal peptide sequences of purified MYPs (Table 1). A partial cDNA sequence for actin was also obtained from our EST data (accession no., **AB510191**).

A homology search and a sequence analysis were carried out using the Blast program (Altschul et al., 1997) and Genetyx-Mac version 14 (Genetyx Co. Ltd., Tokyo, Japan). The predicted cleavage site position of the signal peptide was inferred using the program SignalP 3.0 (Bendtsen et al., 2004).

2.6. RT-PCR

Total RNA was extracted from the water lung, anterior intestine, posterior intestine, body wall, rete mirabile, and ovary of a female at the stage 3, from a developing testis (GSI=0.4%) and from coelomocytes of an immature individual (sex was unidentified). These total RNA were treated with DNase (Ambion) and reverse transcribed to cDNA with oligo (dT) primer using the Super Script III First-Strand synthesis system for RT-PCR kit (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed with the following specific primer sets: *Aj*MYP1F1 and *Aj*MYP1R2 for *Aj*MYP1 (product size, 596 bp), *Aj*MYP2F1 and *Aj*MYP2R2 for *Aj*MYP2 (450 bp), *Aj*ActinF1 and *Aj*ActinR2 for actin (384 bp) (Table 1). The