Localization of major yolk protein in the digestive tract of the sea urchin, *Strongylocentrotus intermedius*

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Abstract

In the present study, we examined the localization of the major yolk protein (MYP) in the intestine of the sea urchin (*Strongylocentrotus intermedius*). First, a partial MYP cDNA was isolated from the sea urchin intestine. The expression level of MYP mRNA is the highest in the intestine of sea urchin, therefore, we performed *in situ* hybridization and immunohistochemical analysis using the intestine. No MYP mRNA was detected in the luminal epithelium, connective tissue, muscle tissue, or coelomic epithelium by *in situ* hybridization analysis. Positive immunohistochemical staining was observed in the luminal epithelium, inner epithelium and connective tissue, signal being strongest in the latter. We conclude that MYP synthesized in the inner epithelial cells is moved to and stored in connective tissue and the luminal epithelium, before being secreted into the body cavity and the inner digestive cavity of the sea urchin.

Keywords:
sea urchin; major yolk protein; immunohistochemistry; *in situ* hybridization; intestine

Introduction

The most abundant protein in sea urchin eggs is called the major yolk protein or major yolk glycoprotein (MYP) [1-3]. The molecular mass of MYP in eggs is 600–700 kDa, as determined by gel filtration chromatography [4-6]. Whereas the major yolk protein in vertebrates, vitellogenin, is a female-specific protein, sea urchin MYP is synthesized and stored in the gonads of both females and males [7,8]. Moreover, MYP is detectable in coelomic fluid and is considered to be a precursor of MYP in the gonad of sea urchins [1]. The molecular mass of the MYP contained in coelomic fluid is also 600–700 kDa [4,5].

Results of sodium dodecyl sulfate- polyacrylamide gel electrophoresis analysis under reducing conditions revealed egg MYP to yield a major protein band with a molecular mass of 170 kDa, whereas the major protein band in the coelomic fluid has a molecular mass of 180 kDa. To avoid confusion, we refer to the MYP in egg as EG-MYP and to the MYP in coelomic fluid as CF-MYP, in keeping with the terminology by Unuma et al. [9].

To date, the full length of MYP cDNA has been isolated from *Pseudocentrotus depressus* (AF318300), *Hemicentrotus pulcherrimus* (AB097218), *Strongylocentrotus purpuratus* (AY090112), *Strongylocentrotus intermedius* (AB192414), *Mesocentrotus nudus* (DQ102372) and *Tripneustes gratillia* (AY026514), and the encoded, deduced protein is transferrin-like [10-12]. MYP can bind iron, zinc, calcium, magnesium, barium, cadmium, and manganese [9,11,13]. MYP mRNA expression has been confirmed in the esophagus, stomach, intestine, rectum, coelomocytes, testis, and ovary of sea urchins [7,10]. Unuma et al. [14] reported that MYPs are synthesized mainly in the inner epithelium of digestive tract and gonadal nutritive phagocytes of sea urchins. Moreover, the CF-MYP is synthesized in the digestive tract and secreted into the coelomic fluid. CF-MYP is taken up by the gonadal nutritive phagocytes in the ovary and testis. Finally, the CF-MYP is transported to eggs in female sea urchins [9]. However, the localization of CF-MYP after synthesis in the inner epithelium of digestive tract remains unclear. In the present study, we therefore investigated the localization of CF-MYP in the digestive tract using *in situ* hybridization and immunohistochemical analyses in adult *Strongylocentrotus intermedius*.

Materials and methods

Animals

Six adult sea urchins (*Strongylocentrotus intermedius*) were collected by diving in Usujiri Bay, Hakodate, Japan in June 2014. They were transferred to a 1000-l tank supplied with sand-filtered seawater at 20 l/min, and reared for one month at the Usujiri Fisheries Station, Field Science Center for Northern Biosphere, Hokkaido University, for use in this study. Urchins (n = 6; weight = 139.3 ± 36.7 g) were sampled one by one in July 2014; the pharynx, esophagus, stomach, intestine and rectum were excised, snap-frozen in liquid nitrogen and stored frozen at -80°C until use or tissue was preserved in fixative as detailed below.

RNA isolation

Total RNA was prepared from a intestine with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Poly(A)$^+$RNA was isolated from total RNA using oligo(dT)-Latex beads (Oligotex™-*dT30* <*Super*> mRNA Purification Kit, Takara Bio, Shiga, Japan) as instructed by the manufacturer. The concentration of the extracted RNA was determined by spectrophotometry (Nano Drop ND-1000 Spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA).

Partial cDNA cloning of MYP from the intestine

The poly(A)$^+$RNA (500 ng) from a intestine was reverse transcribed using an oligo(dT) primer and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. PCR primers were designed within the cDNA sequences of *S. intermedius* MYP (AB192414) (Table 1). Thirty-five amplification cycles were carried out with EX *Taq* HS DNA polymerase (Takara Bio) under the following conditions: 95°C for 15 s, 58°C for 15 s, and 72°C for 120 s. The amplicons were T-A ligated to the pGEM-T-Easy vector (Promega, Madison, WI, USA) and Sanger-sequenced.

Real-time quantitative PCR

Abundance of MYP transcript was determined as a measure of gene expression in the digestive organs by real-time quantitative PCR analysis. Samples were reverse transcribed from 500 ng of total RNA using the Prime Script™ RT reagent kit with gDNA Eraser (Perfect Real time) (Takara Bio), according to the manufacturer's protocol. Aliquots (1 μl) of the first-strand cDNA solutions were diluted and subjected to PCR with 0.5 μM of each forward and reverse primers for MYP or β-actin shown (Table 1) in a 15 μl reaction volume (Fast Start Universal SYBR Green Master; Roche, Basel, Switzerland). The samples were amplified in duplicate. Forty amplification cycles were carried out under the following conditions: 95°C for 10 min,

95°C for 10 s, 58 (β-actin) or 60 (MYP) °C for 10 s, and 72°C for 20 s. The quantities of the PCR products were monitored using the Light Cycler Nano (Roche). The gene transcript levels in the tissues were normalized to those of the housekeeping gene β-actin.

In situ hybridization

In situ hybridization of the intestine was carried out according to the procedure of Iimura et al. [15]. Digoxigenin (DIG)-labeled antisense and sense strand riboprobes were prepared using a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). The intestines from three individuals were excised and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 0.1 M NaCl, pH 7.4). Tissues were dehydrated in ethanol, embedded in paraffin (Tissue Prep; Fisher Scientific, Fair Lawn, NJ, USA), and cut into 6-μm-thick sections. After removing the paraffin, the sections were treated with 20 mM Proteinase K (Nippon Gene) in PBS (pH 7.4) for 10 min at 37°C. The antisense or sense probes (100 ng/ ml) were mixed with hybridization buffer (50% formamide, 4 × SSC, 1 ng/ ml calf thymus DNA). After 1 h of prehybridization at 45°C, hybridization was performed for 16 h at 45°C. The sections were washed twice with 2× SSC and twice with 0.1× SSC for 30 min each at 58°C. The signals were visualized with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) using a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Roche Diagnostics) as the substrate.

Immunohistochemistry

Immunohistochemistory of the intestine was carried out according to the procedure of Takagi [16] The intestines were excised from six adult sea urchins and fixed for 24 h in a mixture 4% paraformaldehyde in 0.1M phosphate buffer (PB: pH 7.3) at 4°C. Tissue samples were dehydrated through a graded ethanol series at room temparature, and embedded in LR White Resin (London Resin Company Ltd, Cambridge, UK). The serial semithin section (1-μm) were cut transversally with a glass knife, mounted on a glass slide and subjected to immune histochemical staining for MYP based on the silver enhancement method. The tissue sections were treated with 1 mg/ ml trypsin for 20 min at 37°C and washed in PBS. The sections were treated with blocking buffer (3% normal goat serum, 0.1% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide) for 30 min at room temperature to reduce non-specific binding. Primary antiserum (anti-MYP) [6] or pre-immune normal rabbit serum (NRS) at 1:1,000 dilution was applied to the sections overnight at 4°C. After washing with PB, the tissues were incubated with a gold-labeled, goat anti-rabbit IgG solution at a 1:50 dilution for 1 h at room temperature, and then washed with PB. The final reaction product was visualized using a Silver Enhancing Kit (BBI Solutions, Cambridge, UK). The immunostained and adjacent sections stained with methylene blue-Azure II were observed with differential interference contrast microscopy (Eclipse E800, Nikon) connected to an LV-TV camera (Nikon).

Statistical analysis

Data are presented as the mean and standard deviation. The level of MYP mRNA in the digestive organs were analyzed by one-way analysis of variance (ANOVA) and post-hoc Tukey-Kramer's test.

Results

Isolation of the partial MYP cDNA from the intestine

The deduced amino acid sequence of *S. intermedius* MYP from the intestine is shown in Fig. 1. The cDNA encoded 395 amino acids. The amino acid sequence was compared with that of other sea urchin MYPs and had high homology (88–94%) to MYPs of other sea urchins. The cDNA sequences have been deposited in the DNA Data Bank of Japan under Accession Number LC170478.

Messenger RNA levels of MYP in the digestive organs

Transcripts of MYP were detected by qPCR in the pharynx, esophagus, stomach, intestine and rectum of *S. intermedius* (Fig. 2). In the digestive organs, the highest MYP transcript abundance was observed in the intestine.

Localization of MYP mRNA in the intestine

In situ hybridization analysis was performed on *S. intermedius* intestine to identify where MYP is synthesized (Fig. 3). Hybridization with antisense probe in the intestine revealed the MYP mRNA signal in the inner epithelium under the luminal epithelium, but not in the luminal epithelium, connective tissue, muscle tissue, or coelomic epithelium (Fig. 3A). Hybridization with a sense control probe produced no significant signal (Fig. 3B).

Immunohistochemical analysis

Immunohistochemical staining of intestinal sections from *S. intermedius* is presented in Figs. 4–7. The antibody stained the luminal epithelium, inner epithelium, and connective tissue in the intestine. And, strong positive staining was observed in connective tissue compared to that luminal epithelium and inner epithelium (Fig. 4B). At high magnification, positive immunostaining for MYP was evident in connective tissue underneath the coelomic epithelium, but the coelomic epithelium did not itself immunoreact (Fig. 5B). Similarly, inner epithelial cells (Fig. 6B) and epithelial cells that line the intestine (Fig. 7B) also clearly immunoreacted with the MYP antibody at high magnification. Negative control sections using NRS produced no significant signals (Fig. 4C, 5C, 6C, 7C).

Discussion

In the present study, we show that the sea urchin MYP mRNA were detected mainly in the inner epithelium by *in situ* hybridization analysis. We further demonstrate that MYP were localized in the luminal epithelium, inner epithelium, connective tissue, and MYP is mainly localized in the connective tissue in the intestine of the sea urchin.

The most abundant protein in sea urchin eggs is called the major yolk glycoprotein or MYP [1-3,17]. Unlike the female-specificity of vertebrate MYP, i.e., vitellogenin, sea urchin MYP is synthesized in the gonads of both sexes [7,10]. MYP is stored in the nutritive phagocytes before gametogenesis [8,18] and is then transferred to the developing gametes to form the yolk granules [18]. MYP disappears from the testis in male sea urchins because it is used as nutritive energy for spermatogenesis [8].

To date, MYP cDNA has been isolated from several sea urchin species. The MYP amino acid sequence is not homologous to that of vertebrate vitellogenins but rather, to transferrins [10-12,19]. MYP mRNA is expressed in the esophagus, stomach, intestine, rectum, coelomocytes, testis, and ovary [7,10]. MYP is also secreted into the coelomic fluid in the body cavity after synthesis in the digestive organ [11,20]. Moreover, the most abundant protein in coelomic fluid is MYP (CF-MYP) [1,4,5,20]. Unuma et al. [9] reported that MYP in coelomic fluid makes its way to the testis and ovary and is finally incorporated into growing oocytes. Unuma et al. [14,20] also reported that MYP mRNA is expressed in the inner epithelium of the red sea urchin digestive organ. However, localization of MYP after synthesis in the inner epithelium remains unclear.

In the present study, we isolated the partial MYP cDNA from the intestine, quantified MYP mRNA abundance and localized MYP, both at the protein and mRNA level, in a number of tissues from *S. intermedius*. The expression of MYP mRNA was highest in the intestine of the digestive organs (Fig. 2), indicative of synthesis of CF-MYP principally in the intestine of *S. intermedius*.

Histological analysis revealed that the intestine consisted of a luminal epithelium, inner epithelium, connective tissue, muscle tissue, and coelomic epithelium. *In situ* hybridization analysis showed that the MYP mRNA were detected in the inner epithelium (Fig. 3), which agrees with the report of Unuma et al. [14,20]. We performed immunohistochemical analyses using an anti-MYP antibody to determine the location of CF-MYP after synthesis in the inner epithelium. Positive staining was observed in the luminal epithelium, inner epithelium, and connective tissue (Figs. 4–7). Strong positive staining was observed in connective tissue compared to that in the inner and luminal epithelia (Fig. 5), suggesting that CF-MYP is synthesized in the inner epithelium, moves through the connective tissue and then makes its way into the coelomic fluid via unknown mechanisms (paracellular / transcellular). Unuma et al. [9,21] reported that CF-MYP binds zinc; therefore, zinc from intestinal connective tissue may

be bound to CF-MYP in coelomic fluid and then accumulate into nutritive phagocytes in the gonads during gametogenesis.

Interestingly, positive MYP staining was also observed in the lumen epithelium (Fig. 7), suggesting that CF-MYP could be secreted into the inned digestive cavity in sea urchins. Indeed, we found that MYP inhibited bacterial growth *in vitro* (unpublished observations). If in fact this proves to be the case, then MYP may well have antibacterial properties in sea urchins. Indeed, MYP is a transferrin-like protein and it can bind many divalent cations [9,11,13]; one of these, iron, is important for the growth of many microbes [22] and limitation of iron availability may be an effective way to restrict their growth – transferrins have been credited with restricting iron availability, thus providing innate immunity by inhibiting bacterial growth [23], as shown, for example, in insects [24]. Moreover, the protective effect of transferrin can be modulated by its genotype, as has been shown, among others, in coho salmon (*Oncorhynchus kisutch*) [25].

In conclusion, MYP synthesized in the inner epithelial cells is moved to and stored in connective tissue and the luminal epithelium, before being secreted into tha body cavity and inner digestive cavity of the sea urchin. However, further studies are needed to examine the CF-MYP secretory pathway in digestive organs by electron microscopical analysis.

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Figure legends

Fig. 1 Alignment of the major yolk protein (MYP) amino acid sequences. ClustalW multiple sequence alignment was used to compare *Strongylocentrotus intermedius* (Si) with MYP forms derived from *Hemicentrotus pulcherrimus* (Hp), *Pseudocentrotus depressus* (Pd), *Mesocentrotus nudus* (Mn), and *Strongylocentrotus purpuratus* (Sp). The percentages beside the sequence are the percent identities with Si MYP. This partial MYP cDNA is used for in situ hybridization analysis as probe in this study. Amino acids identical to those in other species are indicated by asteristcs (*). Colon (:) indicated conserved substitution and dot (.) indicates semi-conserved substitution. .

Fig. 2 Messenger RNA expression levels of the major yolk protein (MYP) in the digestive organs. MYP mRNA level were quantified by real time quantitative PCR in the pharynx, esophagus, stomach, intestine and rectum. All data were normalized to those of β-actin mRNA and are presented as the relative expression levels. The results are presented as the means±S.D. (n=3). Significant ($p < 0.05$) differences among digestive organs are denoted by different letters.

Fig. 3 Localization of the major yolk protein (MYP) mRNA by in situ hybridization in the intestine. Hybridization with antisense probes in the intestine revealed the MYP mRNA signal (A), hybridization with a sense control probe produced no significant signal (B). ie, inner epithelium; le, luminal epithelium; cc, coelomic cavity; dl, digestive lumen; co, connective tissue; mu, muscle layer; ce, coelomic epithelium. Bar, 50 μm.

Fig. 4 Adjacent intestinal sections of *S. intermedius*, stained with methylene blue-Azure II (A), a specific antibody against the major yolk protein (MYP) (B), or with normal rabbit serum (C). The immuno-positive staining indicated by asterisks. ie, inner epithelium; le, luminal epithelium; cc, coelomic cavity; dl, digestive lumen; co, connective tissue; mu, muscle layer; ce, coelomic epithelium. Bar, 50 μm.

Fig. 5 Immunohistochemical staining inside the intestinal coelomic epithelium of *S. intermedius*, stained with methylene blue-Azure II (A), a specific antibody against the major yolk protein (MYP) (B), or with normal rabbit serum (C). The immuno-positive staining indicated by asterisks. ie, inner epithelium; cc, coelomic cavity; co, connective tissue; mu, muscle layer; ce, coelomic epithelium. Bar, 20 μm.

Fig. 6 Immunohistochemical staining of the intestinal inner epithelium of *S. intermedius*, stained with methylene blue-Azure II (A), a specific antibody against the major yolk protein (MYP) (B), or with normal rabbit serum (C). The immuno-positive staining indicated by

asterisks. ie, inner epithelium. Bar, 20 μm.

Fig. 7 Immunohistochemical staining inside the digestive gland lumen in the intestine of *S. intermedius* stained with methylene blue-Azure II (A), a specific antibody against the major yolk protein (MYP) (B), or with normal rabbit serum (C). The immuno-positive staining indicated by asterisks. le, luminal epithelium; dl, digestive lumen. Bar, 20 μm.

Table 1. Primer nucleotide sequences used

Gene	Primer sequences	Accession No.
For RT-PCR (cloning)		
MYP	F: 5′-TCCAAGGGCTATGAGGGC-3′	LC170478
	R: 5′-TTCTTGCTTGCGTTGCAT-3′	
For Real-time PCR		
MYP	F: 5’-AGATCAACGTCGGTCGCTTT-3’	LC170478
	R: 5’-GTAGCCGAAACCATTGTCGC-3’	
β-actin	F: 5’-CATCTACGAGGGTTACGCCC-3’	LC176103
	R: 5’-TGAAAGAGTAGCCACGCTCG-3’	

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Si (probe)      FKGYEGALRCLKSGVADMAFFDEQTLRDTDLLDRVGFT----YNDLRLLCPNGQVVEIDVNMDIAKVCNF
Hp              FKGYEGSLRCLKSGVADLAFFDEQTLRDTDLLSRVGFT----YNDLRLLCPNGQVVEIDVNMDIAKVCNF
Pd              FKGYEGSLRCLKSGVADMAFFDEQTLRDQDLLSRVGFT----YNDLRLLCPNGQVVEIDVNLDITKVCNF
Mn              FKGYEGSLRCLKSGVADMAFFDEQTLRDEDLLSRVGFT----YNDLRLLCPNGQVVEIDVNLDIAKVCNF
Sp              FKGYEGALRCLKSGVADLASSTSRPSVTRTLRDTDLSTGWVHLQRPPPSLPQRQVVEIDVNMDIAKVCNF
                ******:**********:*   .:.     * .    *     :      *: ********:**:*****

Si              GEVMNPVLVTAYNTSGSWRWNITKALMIAHQSVALPALFGEGTVMGKDYDMLLPIAPLNQSYQPFLGPKP
Hp              GEVMNPVLVTAYNTSGSWRWNITKALMIAHQSVALPALFGEGTVMGKDYDMLLPIAPLNQSYQPFLGPKP
Pd              GEVMNPVLVTAYNTSGSWRWNITKALMIAHQSVALPALFGEGTVLGKDYDMLLPIAPLNQSYQPFLGPKP
Mn              GEVMNPVLVTAYNTSGSWRWNITKALMIAHQSVALPALFGEGTVMGKDYDMLLPIAPLNQSYQPFLGPKP
Sp              GEVMNPVLVTAYNTSGSWRWNITKALMIAHQSVALPALFGEGTVMGKDYDMLLPIAPLNQSYQPFLGSKP
                ********************************************:**********************.**

Si              LRSMEAIVKASSYDWFKDQTGICYGETYTNIVKQRNETCQAIVKDVTCVGTPRVKKINVGRFGAKQYKMI
Hp              LRSMEAIVKASSYDWFKDQTGICYGETYTNIVKQRNGTCQAIVKDVTCVGTPRVKKISVGRFGAKQFKMI
Pd              LRSMEAIVKASSYDWFKDQTGICYGETYTNIVKQRNGTCQAIVKDVTCVGTPRVKKISVGRLGAKQYKII
Mn              LRSMEAVVKASSYDWFKDQTGICYGETYTNIVKQRNETCQAIVKDVTCVGTPRMKKISVGRFGAKQYKMI
Sp              LRSMEAIVKASSYDWFKDQTGICYGETYTNIVKQRNETCQAIVKDVTCVGTPRMKKISVGRFGAKQFKMI
                ******:***************************** ****************:***.***:****:*:*

Si              KMCSRPSKFVRKMADFQCDNGFGYLKPVITAVACECMPCEEMIEYNTSFTEDHMWSDVSNKYMLTGEQDI
Hp              KMCSRPSKFVRKMADFQCDNGFGYLKPVITAVACECMPCEEMIEYNTSFTQDHMWSDVSNKYRLTGEQDI
Pd              KMCSRPSKFVRKMADFQCDNGFGYLKPVITAVACECMPCEEMIEYNTSFTEDHMWSDVSNKYRLTGEQDI
Mn              KMCSRPSKFVRKMADFQCDNGFGYLKPVITAVACECMPCVEMIEYNTSFTEDHMWSDRSNKYSLRGEQDI
Sp              KMCSRPSKFVRKMADFQCDNGFGYLKPVITAVACECMLCEEMIEYNTSFTEDNMWSDVSNKYVLTGEQDI
                ************************************ * **********:*:**** **** * *****

Si              YKQIPIWGNNSYFYDHTLNKNFELGNRSIIVEHVRTVVVEGQIPGIMSQVNSEVEPEVQVQMDSASITKT
Hp              YRQIPIWGNNSYFYDHTLNKNFELGNHSIIVENVQTVVVDRPIPGISSQVNLEVDPEVQVQMDSASITKT
Pd              YSQIPIWGNNSYFYDHTLNKNFELGNHSVIVEHVRTVVVERPIPGIVSQVNPEVDPEVQVQMDTVNISKT
Mn              YNQIPIWGNNSYFYDHTLSKNFELGNHSIIVEHVQTVVGERPISGVMSQRIPEVDSEVQVQMDTVSITKT
Sp              YRQIPIWGNNSYFYDHTLNKNFELGNHSIIVEHVQTVVVERPSPGILSQVNSEVDPEVQVQMDSASLTKT
                * ****************.*******:*:***:*:*** :   .*: **   **:.*******:..::**

Si              CETVWNGQSWLPERFQGYKTSGSCVIPETAANLKSRVDRFRQIMQRKQQ
Hp              CETVWNGQSWLPERFQGYKTSGSCVIPEYGANAKSRVDRFRQIMQRKHQ (94%)
Pd              CESVWNGQSWLPERFPDSKISGSCVAPEYGVNAKSRVDRFREMMRRKQQ (91%)
Mn              CESVWNGQSWLPERFPGYKTSGSCAVPEYGVNAKSRVERFRQIMQRKQQ (91%)
Sp              CETVWNGQSWLPERFQGYKTSGSCVVPETGANAKSRVDRFRQIMQRKQQ (88%)
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```

Fig. 1

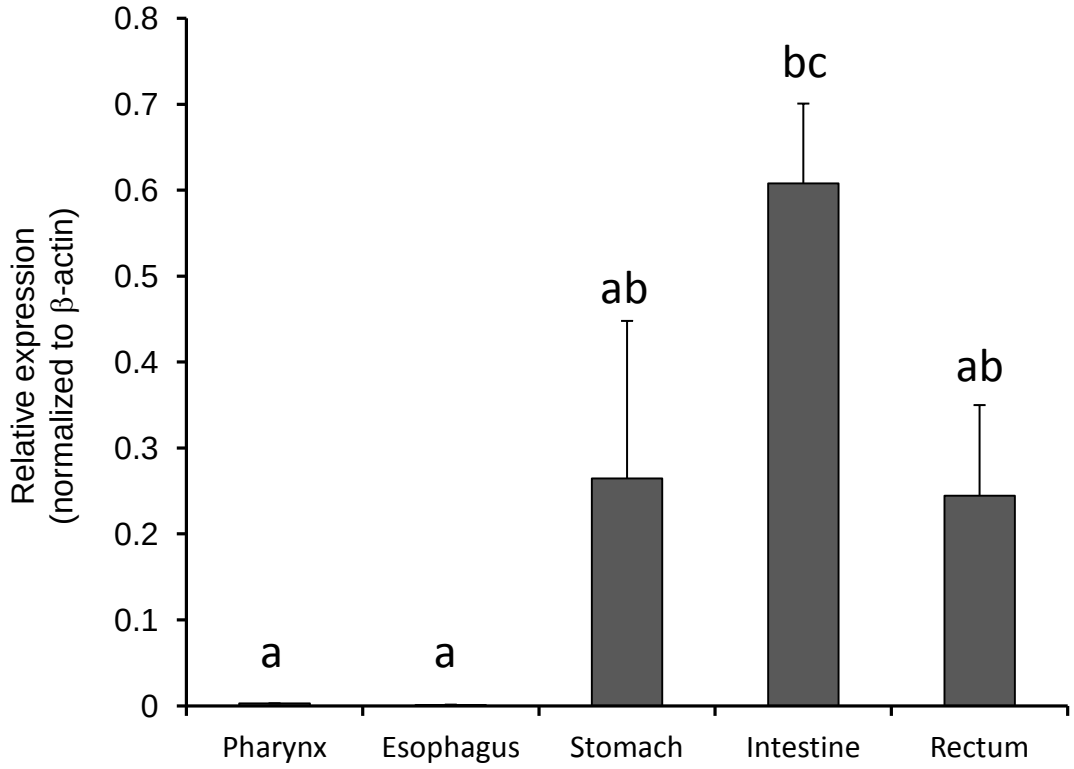
Relative expression
(normalized to β-actin)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
ab
bc
ab
Pharynx
Esophagus
Stomach
Intestine
Rectum

Fig. 2

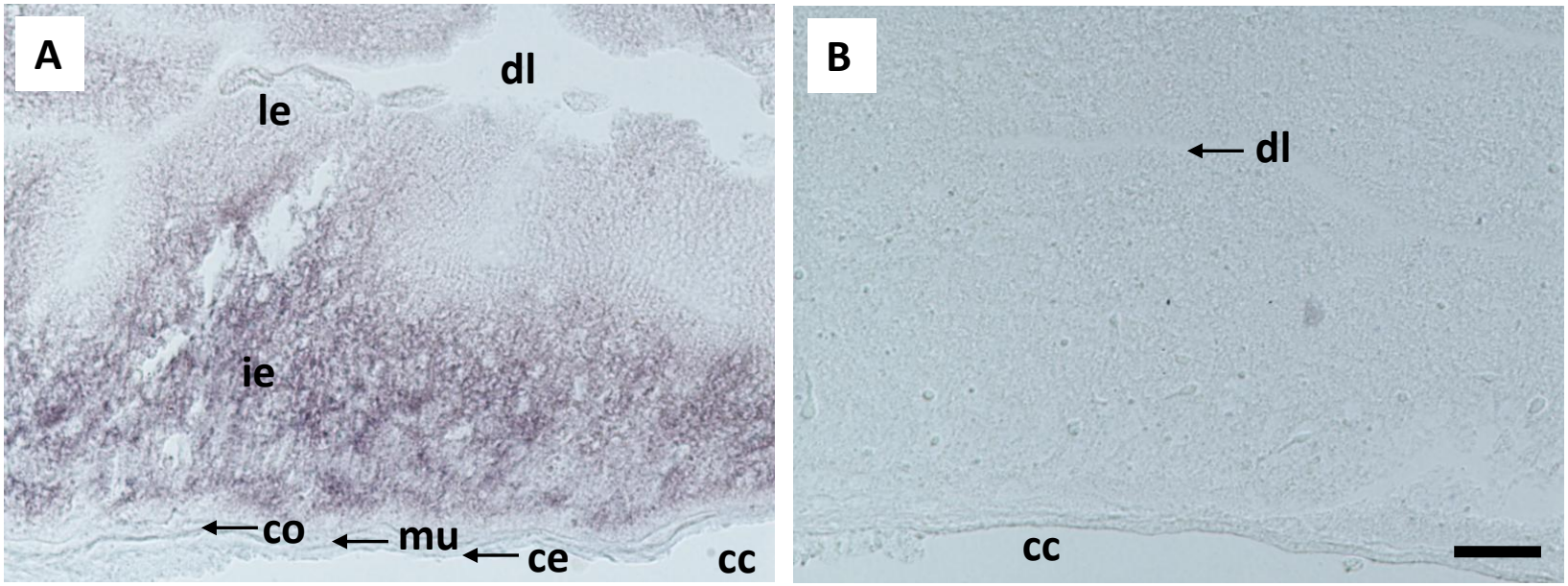

Fig. 3

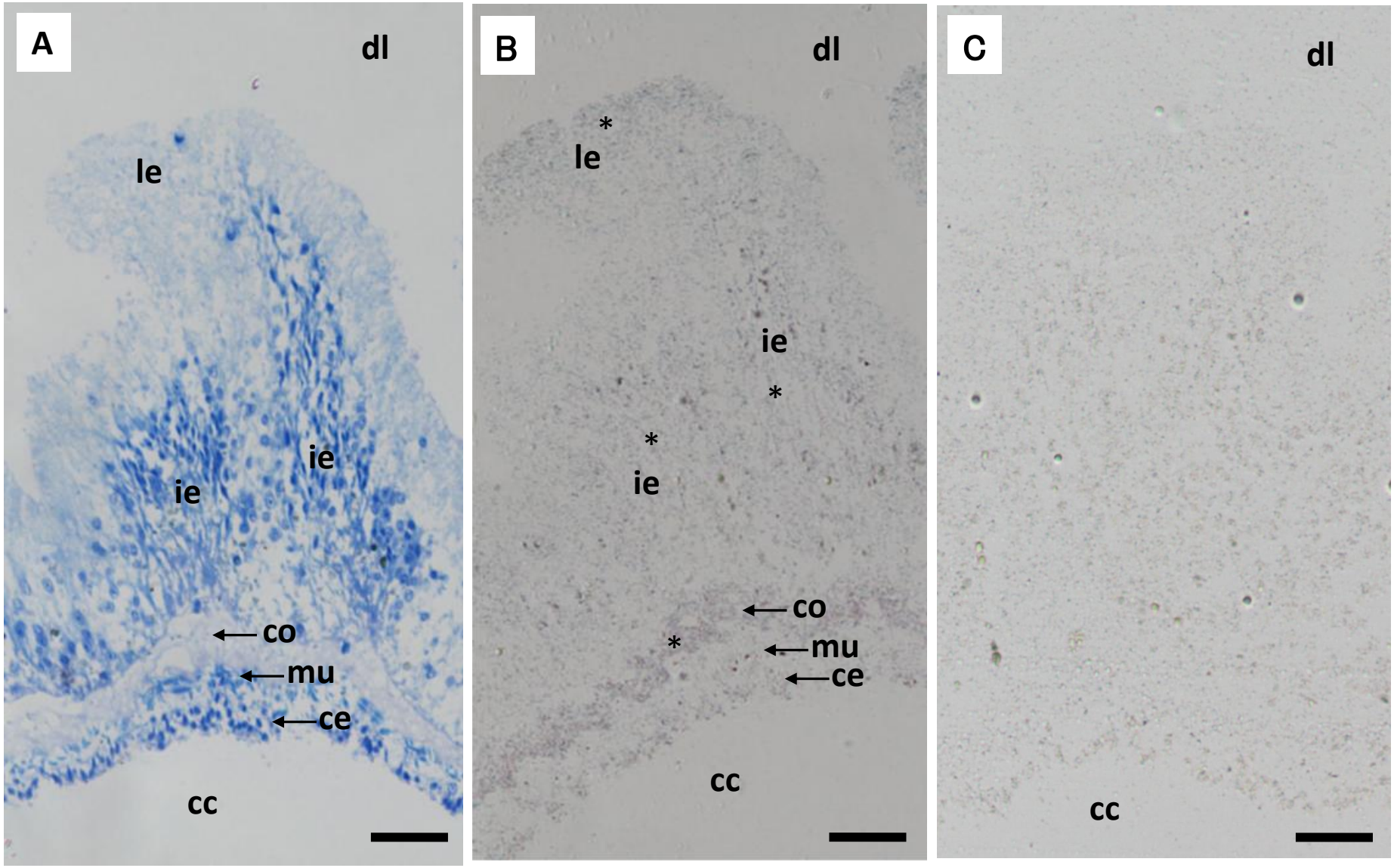

Fig. 4

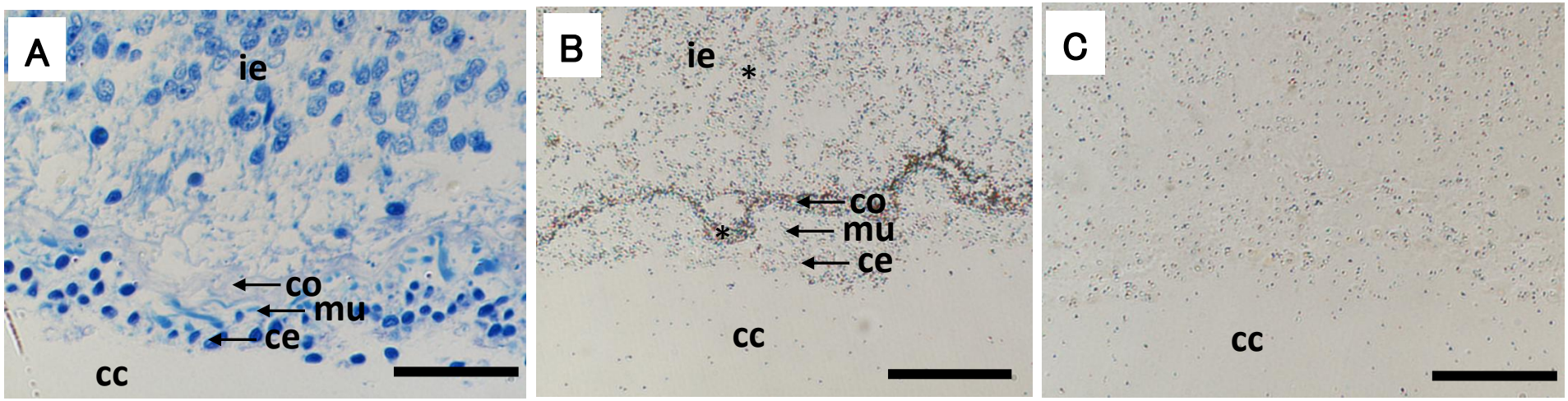

Fig. 5

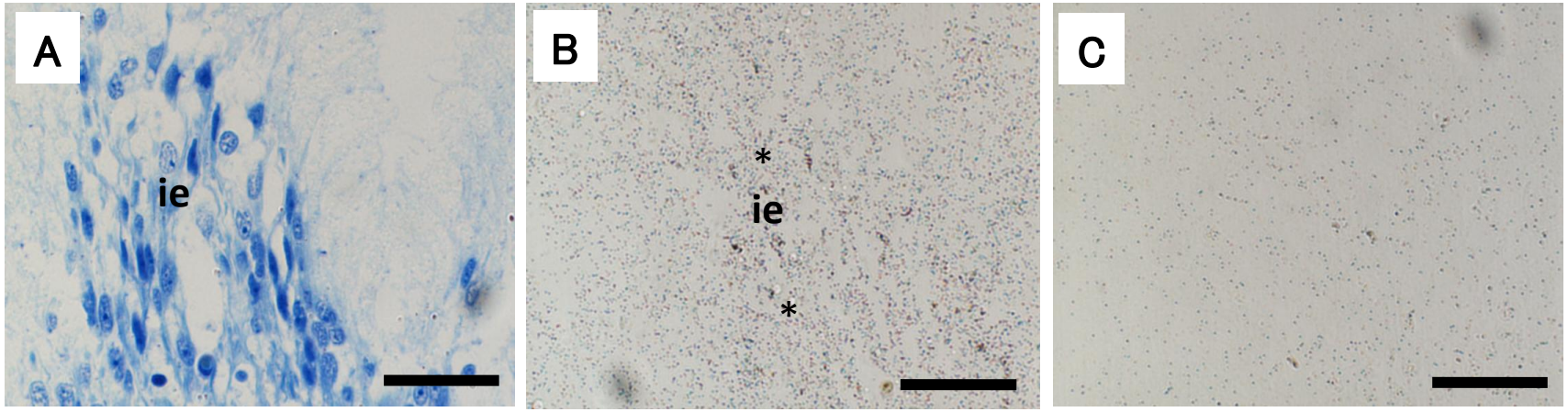

Fig. 6

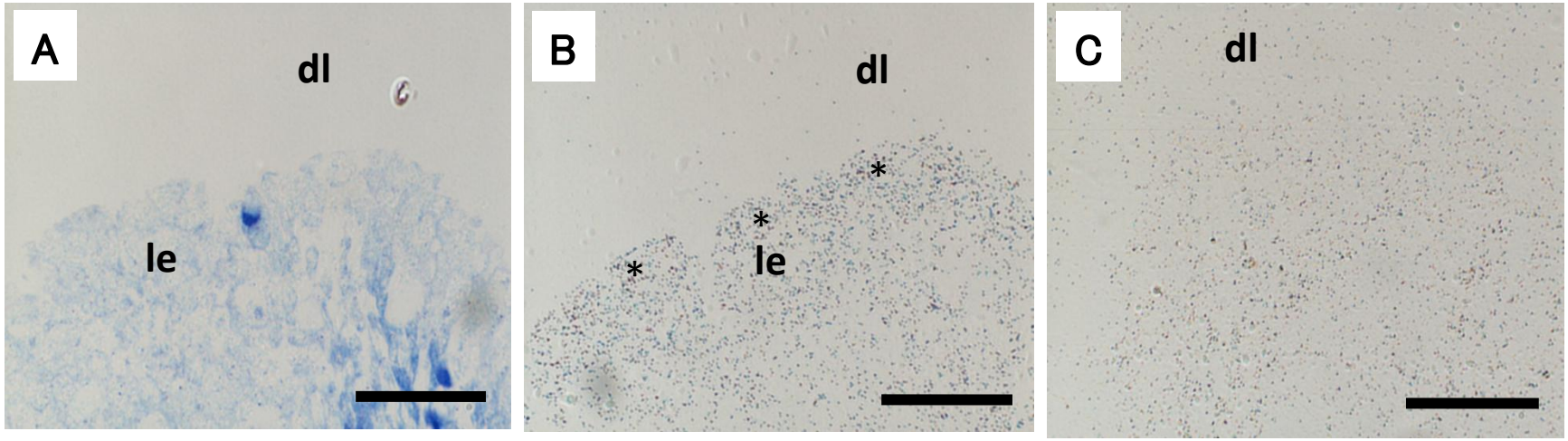

Fig. 7