学位論文内容の要旨

博士の専攻分野の名称：博士（水産科学）　　氏名：王　姮

学 位 論 文 題 目

Biochemical and molecular biological study of major yolk proteins (MYPs) in sea urchin

(ウニの主要卵黄タンパク質 MYPs に関する生化学および分子生物学的研究)

Sea urchin gonad of both sexes, commonly termed “roe”, is one of the expensive seafood delicacies. Japan, as the largest importer and consumer of sea urchins, consumes more than 80% of the world’s production, and Hokkaido accounted for 56% of the Japanese total catch of sea urchin in 2014. To promote stock enhancement, a significant number of artificially-produced juveniles of sea urchin have been released every year since the 1980s. However, the harvest of sea urchin has not increased and has instead declined around Japan. Although a number of factors may be involved in the failure of the stock enhancement, reproductive abnormalities of the supplemented stock seems to be the major reason. Thus, it is important to have a reliable biomarker to identify normal or abnormal reproduction of sea urchin, and then to solve the problem of the reproductive abnormalities in order to recover the wild stocks. In teleost fish, the yolk precursor protein, vitellogenin (Vtg) is established as a reliable biomarker of the onset of puberty and ovarian development in females. In other echinoderm, i.e., in sea cucumber and sea star, the yolk protein is thought to be the biomarker to study the regulatory mechanism of the ovarian development. Similarly, in sea urchin, the major yolk protein (MYP) served as the most abundant yolk protein, is a candidate biomarker to identify normal or abnormal reproduction and gametogenesis.

MYP was originally identified as the predominant component of yolk granules of sea urchin eggs. Unlike other oviparous animals in which the major yolk protein in the egg is usually female-specific, MYP is produced in both male and female sea urchins. The major sites of MYP synthesis have been reported to be the inner epithelium of the digestive tract and the nutritive phagocytes (NPs) within the ovary and testis. The sequence of MYP cDNA reveals that MYP is not grouped with Vtg, but is a member of the transferrin superfamily of iron-binding proteins. However, at present, no studies have intensively investigated and compared (1) the protein structure of MYPs in various tissues of different sea urchin species employing the same purification method, and (2) the transcripts of *myp* genes in each species. If the structure of MYP and the *myp* genes differ in each sea urchin species, then there will be a need to characterize the MYP in each

species to develop reliable biomarkers for application in aquaculture. For these objectives, in this thesis, two sea urchin species, *Strongylocentrotus intermedius* and *Mesocentrotus nudus*, which are commonly harvested and economically important species in Hokkaido, Japan, were chosen. Unlike the other sea urchins, MYP of these two species have not yet been intensively studied. Thus, the present study was designed for characterization of MYPs in these two important sea urchins at the protein and genetic level.

Firstly, MYP (EGMYP, egg-type major yolk protein) was purified from the unfertilized eggs of *S. intermedius* and *M. nudus* and biochemically characterized. Additionally, the yolk granules were isolated from mature unfertilized eggs of both species. The structure of EGMYP purified from the whole eggs was compared to that of EGMYP in the yolk granules to determine the differences and similarities arising from these two sources in each species. The molecular weight of native EGMYP purified from the whole eggs was 595 kDa in *S. intermedius* and 625 kDa in *M. nudus*. The purified EGMYPs of both species were positive to PAS staining and suggesting that EGMYPs could bind carbohydrates. The use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis under reducing conditions revealed that the EGMYP of *S. intermedius* separated into four bands (approximately 172, 116, 74 and 68 kDa), while that of *M. nudus* resolved as a set of bands ranging from 175 to 58 kDa (approximately 175, 165, 153, 115, 102, 90, 78, 65 and 58 kDa). Similar SDS-PAGE patterns were observed in the yolk granules isolated using sucrose density ultracentrifugation in both species. Therefore, the multiple bands of SDS-PAGE in purified EGMYPs were likely not the products of proteolysis but were instead the constituents of EGMYP. Taken together, the EGMYPs of both sea urchins were a glycoprotein complex consisting of polypeptide components with different molecular weight, and the structures of these proteins varied between the two species.

It is possible that other types of MYPs which are stored in different tissues could be also a complex, and the structure of the complex varies among species. Thus, three types of MYP [EGMYP, coelomic fluid-type MYP (CFMYP) and nutritive phagocytes-localized-type MYP (NPMYP)] were purified form the eggs, coelomic fluid (CF), and nutritive phagocytes (NPs) of *S. intermedius* and *M. nudus* with the same purification process, respectively. Then, their protein structures were compared to determine the differences and similarities in MYPs contained in different tissues in each species. The molecular weight of three MYPs (EGMYP, NPMYP, CFMYP) were about 560 kDa, 620 kDa and 700 kDa in *S. intermedius*, respectively, while that of *M. nudus* MYPs were about 580 kDa, 640 kDa, and 670 kDa, respectively. In *S. intermedius*, three types of MYPs revealed distinctly different structures under reducing conditions. The EGMYP produced the same bands as described above. The CFMYP produced a set of bands (about 180, 135, 127, 95 and 64 kDa). The NPMYP exhibited a different set of bands (about 175, 103, 100, 95, 78, 70 and 65 kDa). In *M. nudus*, the EGMYP resolved the same bands as described above, while CFMYP and NPMYP produced a same band pattern (about 180, 140,

120 and 70 kDa) different from that of EGMYP. In line with the results of SDS-PAGE, analyses of N-terminal amino acid sequences and peptide mapping also suggested that there may be three types of MYPs (EGMYP, CFMYP and NPMYP) with different primary structures in *S. intermedius*, while there may be two types of MYPs (EGMYP and CFMYP) with different structures in *M. nudus*. Taken together, the MYPs of both sea urchins were a glycoprotein complex consisting of components with different molecular weight, and the type of MYP varied between the two species. Moreover, the structural similarities suggest that the major source of EGMYP is NPMYP, which is synthesized in NPs, instead of CFMYP synthesized in the digestive tract.

In order to elucidate whether MYPs were encoded by a single gene or multiple various *myp* genes in sea urchin, 5' region of cDNA in *S. intermedius* (Si) were performed. Two distinctive types of Si *cfmyp* (Si *cfmyp*-1 and -2) having distinctive 5' untranslated region (5' UTR) were cloned from the intestine, and the length of each clone was 356 and 181 bps, respectively. Four types of Si *npmyp* (Si *npmyp*-1, -2, -3, and -4) comprising different lengths and sequences (171, 208, 356 and 181 bps long, respectively) were cloned from the immature testis. Taken together with the previously obtained data of *fmyp* (cloned from female immature gonad) and that of *cfmyp* (cloned from intestine), the results of the present study strongly suggest the existence of multiple *myp* mRNAs encoding multiple MYP proteins in different tissues. Moreover, the N-terminal aa sequences of purified EGMYP, CFMYP and NPMYP of *S. intermedius* mostly matched the aa sequences derived from Si *fmyp*, Si *cfmyp* and Si *npmyp*. Thus, it is assumed that these *myp* might encode different types of MYPs. However, complete cDNA of multiple MYPs are needed to get concrete answer on which *myp* gene encodes which MYP protein. In addition, partial genome DNAs encoding MYP of *S. intermedius* and *M. nudus* were characterized, and five types of *myp* genes on the genome of *S. intermedius* and two types on that of *M. nudus* were obtained in the present study. In *S. intermedius*, some types of *myp* genes were one or two intron less genes. In some clones of *M. nudus*, there were 10-20 bps deletions happened in some parts of introns according the results of intron sequence alignment. Moreover, in both sea urchin, the deduced amino acid sequences in each types were similar but not identical, and certain types only shared a 95.2% identity to another type. Thus, the present data strongly suggest that the sea urchin MYPs are encoded by multiple genes instead of a single gene in each species of sea urchin.

In conclusion, this thesis confirmed the presence of multiple *myp*/MYP in two species of sea urchins, *S. intermedius* and *M. nudus*, and a novel model for synthesis and accumulation of MYP is established. Although this new model is confirmed only in limited species of sea urchins, the present research makes a significant contribution to a better knowledge to develop MYP as a biomarker of reproduction and gametogenesis in economically important species of sea urchins in Hokkaido, and gives a first step to recover depleted natural populations of sea urchins.