Studies on red color-related pigment-binding protein derived from the shell of Pacific white shrimp Litopenaeus vannamei

［課程博士・論文博士共通］

博士学位論文内容要旨
Abstract

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論文題目 Title	Studies on red color–related pigment–binding protein derived from the shell of Pacific white shrimp *Litopenaeus vannamei* (バナメイエビ殻由来の色素結合タンパク質に関する研究)		

Crustaceans like shrimps and crabs have a remarkable red color change during cooking. In addition to reflecting the freshness of crustaceans, the red color change also plays a significant role in consumer acceptability of commercial crustacean species. Studies on crustacean shell color change were mainly focused on lobsters like American lobster (*Homarus americnaus*), European lobster (*H. gammarus*), and Western Australia lobster (*Panulirus cygnus*). It revealed that this well–know red color change is caused by the releasing of pigments from denatured pigment–binding proteins, which is consist of apoprotein and astaxanthin (ATX), named crustacyanin. However, information on the red color–related pigment–binding proteins derived from other crustacean species is insufficient. To our knowledge, there has been few report on the pigment–binding proteins involved in the red color change on the shell of Pacific white shrimp, *Litopenaeus vannamei*. Therefore, this study dealt with the purification, identification, and elucidation of the structural and thermal properties of the red color–related pigment–binding protein in the shell of *L. vannamei*. In addition, specificity of the red color–related protein derived from *L. vannamei* was investigated among *H. americanus*, *Marsupenaeus japonicus*, and *Panulirus japonicus*.

In order to clarify the red color change on *L. vannamei* shell surface, the red color–related protein was purified from shell by ammonium sulfate precipitation, gel filtration and anion exchange HPLC in Chapter 2. The purified red color–related protein afforded a major single peak as analyzed by gel filtration HPLC on a TSKgel G3000SW$_{XL}$ column and a single band in native– and SDS–PAGE, indicating that this protein was a hemogeneous monomer with molecular mass of ~75 kDa and was termed as LvPBP75 (*Litopenaeus vannamei* pigment–binding protein with molecular mass ~75 kDa). Peptide mass fingerprinting (PMF) analysis revealed a protein that named hemocyanin (GenBank accession number: CAA57880) matched LvPBP75 with a nominal mass of ~74,992 Da. Absorption spectrum of acetone extract from the precipitate of heated LvPBP75 was typical of ATX with absorption maxima at 481 nm. The results suggested that the red color change on *L. vannamei* shell surface is correlated with a novel red color–related pigment–binding protein, LvPBP75, which is consist of hemocyanin and astaxanthin, but not the previous described crustacyanin in lobster shell.

In chapter 3, in order to elucidate the structural properties of LvPBP75, cDNA cloning and circular dichroism spectroscopy analysis were carried out. On the basis of the partial amino acid sequences determined by PMF analysis, a full length cDNA of 2,183 bp including an ORF of 1,986 bp that encodes 662 amino acid residues (GenBank/EMBL/DDBJ accession number KY695246) was cloned. Multiple sequence alignment indicated that LvPBP75 shows a high similar identity (~80%) with the hemocyanin or its subunits derived from the hepatopancreas of *L. vannamei*. α–Helix, β–sheet, β–turn, and random coil contents of unheated LvPBP75 were calculated to be 51.0 ± 0.216, 19.5 ± 0.262, 16.4 ± 0.216, and 13.6 ± 0.245%, respectively. Meanwhile, to investigate the thermal properties of LvPBP75, studies on the effects of temperature, ion strength, pH, and

alcohol on the red color change of LvPBP75 were carried out. As results, initial color change of LvPBP75 occurred at 30 °C and no significant changes were observed before heated at 60 °C. Within the increasing of heating temperatures, the protein color increased significantly in both the redness and yellowness scales. Color change of LvPBP75 is reversible at 30 °C with low NaCl concentrations (< 0.05 M), but irreversible when heated with high NaCl concentrations (> 0.1 M). No significant color change was detected under the pH of 6, 7, and 8. The red color values of acidic or alkali pH–treated LvPBP75 were significantly moved to red and yellow scales after heat treatments. Before heating, LvPBP75 changed to pink when 20% methanol and 30% ethanol was added. A yellow color was detected when 40% methanol and 60% ethanol was added. All samples turned to both redness and yellowness scales after heat treatments. The results suggested a novel function of hemocyanin as binding with pigment and its involvement in *L. vannamei* shell color change.

In chapter 4, in order to demonstrate the pigment–binding property of LvPBP75, tissue distribution of LvPBP75 and reconstruction of artificial LvPBP75 were carried out. It was found that LvPBP75 has the highest expression level in hepatopancreas, mediate level in heart, hemolymph, epithelium, and intestine, and the lowest in eyestalk, nerve, and muscle based on the analysis of tissue distribution. LvPBP75 was successfully expressed in *E. Coli* using the pET–44a vector system and a prominent 75 kDa protein band corresponding to His–tagged recombinant LvPBP75 (rLvPBP75) was observed in the precipitate fraction of IPTG–induced bacteria. After overnight incubation at 4 °C, the rLvPBP75 was successfully combined with ATX. However, ATX peak (~ 480 nm in acetone) was not detected in the binding experiment when using the hemocyanins derived from the hemolymph of *Megathura crenulata* and *Limulus polyphemus*, indicating that hemocyanins with oxygen transportation function do not possess the pigment–binding functions. Three–dimensional structural analysis revealed that LvPBP75 monomer possesses four spatial structural differences compared with the hemocyanin monomer which was derived from *L. vannamei* hepatopancreas. The results suggested that the pigment–binding ability of hemocyanins has species– or tissue–specificity and their unique structural features play an important role in binding ability.

In chapter 5, in order to illuminate the relationship between small molecular weight proteins (< 40 kDa) and red color change in *L. vannamei*, the < 40 kDa proteins were separated and subjected to color change experiments. As results, no significant red color change could be detected after heat treatment, indicating that *L. vannamei* shell color change was not correlated with < 40 kDa proteins. Meantime, to investigate whether the LvPBP75 is specific in *L. vannamei* or not, red color–related proteins derived from the shell of *H. americanus*, *M. japonicus*, and *P. japonicus* were purified using gel filtration and ion exchange HPLC, respectively. As results, the 75 kDa protein was correlated with the red color change on the surface of *L. vannamei*, *M. japonicus*, and *P. japonicus*, while the 22 kDa protein was correlated with the red color change in *H. americanus*. The results suggested that the red color change on *L. vannamei* surface is mainly correlated with LvPBP75 and it is not specific in *L. vannamei*, further investigation is necessary to understand the red color–related proteins among a variety of crustacean species.

This study identifies a novel red color–related hemocyanin–ATX binding protein, LvPBP75, from the shell of *L. vannamei* and strongly suggests a novel function of hemocyanin as binding with pigment and involved in *L. vannamei* shell red color change.