Carotenoproteins from the Exoskeleton and the Muscular Epithelium of the Black Tiger Prawn *Penaeus monodon*

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A blue carotenoprotein ($\lambda_{\max}=615$ nm) was purified and characterized from the exoskeleton of black tiger prawn *Penaeus monodon*. It contained free astaxanthin as the prosthetic group. Its molecular weight was found to be 126 kDa and 120 kDa by gel filtration and native-polyacrylamide gel electrophoresis (PAGE), respectively. SDS-PAGE indicated that the carotenoprotein consisted of six subunits, with three sets of two kinds of subunits of 22.4 and 21.4 kDa in a molar ratio of 1:1. The stoichiometry of carotenoid and apoprotein was 2:1. Another blue carotenoprotein ($\lambda_{\max}=625$ nm) was isolated from the muscular epithelium of *P. monodon* by collagenase treatment. The molecular weight of the carotenoprotein was determined to be 21.4 kDa by gel filtration. SDS-PAGE indicated that the carotenoprotein was composed of a polypeptide of 22.9 kDa. This carotenoprotein also contained free astaxanthin as the prosthetic group.

Key words: black tiger prawn, carotenoprotein, astaxanthin, exoskeleton, muscular epithelium, collagenase

Carotenoproteins are widely distributed in invertebrates, especially in crustaceans and are implicated in their coloration. Many studies have been done on carotenoproteins, such as their distribution,^{1–3)} spectral shift of the prosthetic groups,^{4,5)} and molecular properties of the protein moiety.^{6,7)} Some carotenoproteins shift their absorption maxima to longer wavelengths by about 160 nm without changing the whole absorption pattern when the prosthetic groups are bound to the apoprotein.^{8,9)} This phenomenon is comparable to the alteration in absorption spectrum of retinal with and without its apoproteins.¹⁰⁾ The blue carotenoprotein, α -crustacyanin, from the carapace of the lobster *Homarus gammarus* is one of the best models for such interaction between carotenoid and its associated protein.^{11–17)}

A few studies have been reported on carotenoproteins of commercially valuable aquatic animals. Muriana *et al.* reported a blue carotenoprotein ($\lambda_{\max}=640$ nm) of 280 kDa from *Penaeus japonicus*.¹⁸⁾ Since the body color of prawn affects the market price, the color development largely due to the amount of carotenoid in the diet is the major concern of those involved in prawn aquaculture.

The black tiger prawn *Penaeus monodon* is also an important species in aquaculture. The appearance of this specimen is characterized as generally blue/black banded and sometimes slightly pale in color. Our previous work has indicated that the main carotenoid in the exoskeleton of *P. monodon* is astaxanthin even if the prawns show a variety of body colors.¹⁹⁾ The color turns red when the prawn is heated or treated with organic solvent. This color change suggests the presence of carotenoid as carotenoprotein in the exoskeleton and the muscular epithelium. Therefore carotenoprotein must have an important role for expression of the body color of *P. monodon*. This encouraged us to isolate and characterize carotenoproteins in

the exoskeleton and the muscular epithelium of *P. monodon*.

Materials and Methods

Materials

Frozen specimens of black tiger prawn *P. monodon* were purchased at the Tokyo Central Wholesale Market, and stored at -80°C until used.

Purification of Carotenoprotein from the Exoskeleton

The exoskeleton of the abdominal part was peeled from the prawn and the adhering hypodermic tissues were cleanly removed. The exoskeleton (about 110 g) was then ground in an electric blender and washed for 4–5 h under continuous stirring with 10 volumes of 0.3 M boric acid adjusted to pH 6.8 with solid Tris. The suspension was then filtered through No. 2 filter paper (Advantec Toyo) and the residue was subjected to the overnight extraction of carotenoproteins with 10 volumes of 10% EDTA trisodium salt (pH 7.5) under continuous stirring. The extraction mixture was centrifuged at $9,000 \times g$ for 20 min and the supernatant fraction containing carotenoproteins was salted out with ammonium sulfate between 20 and 50% saturation. The precipitate was dissolved in 20 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and 0.1 mM EDTA, and dialyzed exhaustively against the same buffer.

The dialyzed solution was chromatographed on a DEAE-Toyopearl 650 M column (2×6.5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 6.8) at a flow rate of 2 ml/min. Proteins were eluted with a linear gradient of KCl using 150 ml each of 20 mM potassium phosphate (pH 6.8) and the same buffer containing 0.3 M

KCl. Elution profile was monitored by absorbance at 278 and 615 nm. The fractions colored blue were pooled, dialyzed against the starting buffer, and rechromatographed on the same column.

The carotenoprotein fraction was further purified by high-speed gel filtration using a TSK-G 3000SWG column (21.5 mm × 60 cm) equilibrated with 20 mM potassium phosphate (pH 6.8) at a flow rate of 3 ml/min. The gel filtration was repeated a few times until a single peak of carotenoprotein appeared.

All steps in the purification procedure were carried out at 4°C in the dark or in subdued lighting.

Purification of Carotenoprotein from the Muscular Epithelium

Carotenoproteins in the muscular epithelium were extracted by treatment with collagenase. Collagenase from *Clostridium histolyticum* (Wako Pure Chemical, 208 units/mg) was dissolved at a concentration of 0.18% (w/v) in 20 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM PMSF. To 32 abdomens without the exoskeleton (472 g) was added 720 ml of the collagenase solution and the mixture was incubated at 28°C for 2 h under gentle shaking. After incubation, the colored solution containing carotenoproteins collected by filtration was salted out with ammonium sulfate between 20 and 60% saturation. The precipitate was dissolved in 20 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM PMSF and dialyzed exhaustively against the same buffer. Further purification was done by only high-speed gel filtration under the same conditions described above. The elution profile was monitored by absorbance at 278 and 625 nm. All steps in the purification procedure were carried out at 4°C in the dark or in subdued lighting.

Molecular Weight Determination

The molecular weight was determined by high-speed gel filtration with the same conditions as for purification. The calibration curve for estimation of the molecular weight in terms of elution volume was obtained from the following standard proteins. (MW-GF-200, Sigma); β -amylase (molecular weight, 200,000 = 200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

Alternatively, native polyacrylamide gel electrophoresis using a 4–20% gradient slab gel was carried out for the carotenoprotein from the exoskeleton by the method of Davis and Ornstein.²⁰ Protein standards used were thyroglobulin (molecular weight, 669,000 = 669 kDa), ferritin (443 kDa), lactate dehydrogenase (139.9 kDa), bovine serum albumin (66 kDa), and trypsin inhibitor from soybean (20.1 kDa) (Daiichi Pure Chemical).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10–20% gradient slab gels was performed by the method of Laemmli.²¹ The following standard proteins (Daiichi Pure Chemical) were used; myosin (molecular weight, 200,000 = 200 kDa), β -galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42.4 kDa), carbonic anhydrase (30 kDa) and myoglobin (17.2 kDa). After running, the gel was stained with Coomassie brilliant blue R250 and scanned at 660 nm with a Gelman

ACD-18 densitometer to obtain the relative concentration of each subunit.

Analysis of Amino Acid Composition

The purified carotenoprotein from the exoskeleton was exhaustively dialyzed against distilled water and 0.27 mg of the protein was hydrolyzed with 3 N mercaptoethanesulfonic acid at 110°C for 24 h, according to the method of Penke *et al.*²² Amino acid composition was then analyzed using a Hitachi 835 amino acid analyzer.

N-Terminal Amino Acid Sequencing

The N-terminal amino acid sequence was determined by the method of Matsudaira²³ as follows: Proteins from the exoskeleton were separated on SDS-PAGE, electrically transferred onto an Immobilon PVDF membrane (Millipore), and stained with Coomassie brilliant blue R250. The parts of the membrane carrying the blotted bands of the carotenoprotein subunits were cut out with a clean razor. Several membranes were placed together on the Teflon seal of the cartridge block in an Applied Biosystems model 477 A protein sequencer with an on-line system

Fig. 1. A: DEAE-Toyopearl 650 M chromatography of crude carotenoprotein from the exoskeleton of *P. monodon*.

Detection was carried out at 278 nm (○) and 615 nm (●).

B: SDS-PAGE pattern of some fractions. Abbreviations used are Ma, molecular weight markers of standard proteins; C, crude extract. The numbers under the photograph are fraction numbers. The proteins later identified as the subunits of carotenoprotein are indicated by arrowheads (22.4 and 21.4 kDa). The volume of each fraction applied on a lane was 20 μ l.

model 120 A.

Analysis of Carotenoid Composition

Carotenoid was liberated by treatment with acetone from the carotenoproteins and transferred into diethyl ether according to the method of Powls and Britton.²⁴⁾ The carotenoid was dissolved in benzene and the absorption spectrum was measured. Carotenoid composition was checked by thin-layer chromatography (TLC) using a silica gel 60F₂₅₄ plate (Merck) with a mobile phase of benzene/ethyl acetate (9:1). The carotenoid was also identified by high performance liquid chromatography (HPLC) on a Superiorex-ODS column (4.6 × 150 mm) with a mobile phase of methanol/ethyl acetate/water (54:15:10) at a flow rate of 0.7 ml/min.

Determination of Stoichiometry of Carotenoid and Apoprotein

A solution containing the purified carotenoprotein from the exoskeleton was dialyzed exhaustively against distilled water for 2 days and freeze-dried. The weight of freeze-dried carotenoprotein was measured gravimetrically immediately after evacuation by a vacuum pump for 1 h. The freeze-dried carotenoprotein (1.3 mg) was treated with ace-

tone to liberate carotenoids, and the carotenoid content was determined by the method of McBeth.²⁵⁾ The molecular weight obtained by gel filtration was used for calculating the molar concentration of the carotenoprotein.

Treatment of the Carotenoprotein from the Exoskeleton with Collagenase

The purified carotenoprotein from the exoskeleton was dissolved in 20 mM phosphate buffer (pH 6.8) and treated with collagenase in the same manner as the extraction of the carotenoprotein from the muscular epithelium. After treatment, the solution was subjected to gel filtration. Blue colored fractions were collected and any change by collagenase treatment was checked by native-PAGE.

Results

Purification of Carotenoproteins from the Exoskeleton

Figure 1A shows the elution pattern from the first DEAE-Toyopearl 650 M column. Though roughly four peaks were observed by monitoring at 278 nm, there was a single peak having absorbance at 615 nm at a concentration of KCl of 0.09 M. Some fractions were analyzed for protein composition pattern by SDS-PAGE. The fractions having absorbance at 615 nm contained mainly five components including those of 22.4 and 21.4 kDa later identified as the subunits of the carotenoprotein (Fig. 1B). By repeating the ion exchange chromatography for the blue colored fractions, a single peak of the proteins appeared, however, the absorbance at 278 nm was still larger than that at 615 nm. Then further purification was done by high-speed gel filtration. Figure 2A shows the elution pattern of the proteins from the first TSK G-3000SWG column. Several peaks having absorbance at 278 nm appeared but there

Fig. 2. A: Gel filtration on TSK G-3000SWG column of the carotenoprotein from the exoskeleton.

Detection was carried out at 278 nm (○) and 615 nm (●).

B: SDS-PAGE pattern of some fractions.

Refer to the legend of Fig. 1 for abbreviations and further details.

The volume of each fraction applied on a lane was 20 μ l.

Fig. 3. A: Native-PAGE pattern of purified carotenoprotein from the exoskeleton. Ma, molecular weight markers; CE, carotenoprotein from the exoskeleton.

B: SDS-PAGE pattern of purified carotenoprotein from the exoskeleton.

Proteins applied were 10 μ g/lane.

Table 1. Amino acid composition of the carotenoprotein from the exoskeleton of *P. monodon*

Amino acid	mole%
Arg	4.8
Lys	5.2
His	1.4
Asp	11.5
Glu	9.1
Thr	5.2
Ser	7.7
Pro	5.6
Gly	7.8
Ala	9.3
Val	5.8
Ile	4.2
Leu	6.6
Cys/2	1.2
Met	0.9
Tyr	6.1
Phe	6.2
Trp	0.9

PC_{22.4}
PC_{21.4}

DKIPDFVVP
DGIPDFVVP

HC₁
HA₂

DKIPDFVVP
DGIPSFVTAG

Fig. 4. The N-terminal amino acid sequences of two polypeptides of 22.4 kDa (PC_{22.4}) and 21.4 kDa (PC_{21.4}) from the exoskeleton of *P. monodon*.

The sequences of apocrustacyanin C₁(HC₁)¹⁶ and apocrustacyanin A₂(HA₂)¹⁵ from *H. gammarus* are shown together.

was only one peak having absorbance at 615 nm. SDS-PAGE showed that the blue colored fractions contained two main components of 22.4 and 21.4 kDa (Fig. 2B). After repeated gel filtration, a single peak of the blue carotenoprotein appeared and showed a higher absorbance at 615 nm than 278 nm, suggesting that the carotenoprotein was purified to homogeneity. The native PAGE of this carotenoprotein showed one band irrespective of staining with Coomassie brilliant blue (Fig. 3A). This result also supported the homogeneity of the carotenoprotein. Finally, 8.4 mg of the carotenoprotein was obtained from 110 g of the exoskeleton.

Molecular Weight of the Carotenoprotein from the Exoskeleton

The molecular weight of this carotenoprotein was determined to be 126 kDa by the relative elution volume in gel filtration compared with the standard proteins. Alternatively, native PAGE showed only a single band corresponding to 120 kDa (Fig. 3A). SDS-PAGE of this protein showed two sharp polypeptide bands having molecular weight of 22.4 and 21.4 kDa though some minor components were also found (Fig. 3B). The molar ratio of the two subunits was about 1:1. These results indicated that the carotenoprotein contained six subunits, with three sets of two types of subunits of different molecular weights.

Amino Acid Composition of the Carotenoprotein from the Exoskeleton

The amino acid composition of the carotenoprotein

Fig. 5. Absorption spectrum of the carotenoprotein from the exoskeleton of *P. monodon* in 20 mM phosphate buffer (pH 6.8).

from the exoskeleton is shown in Table 1. The content of aspartic acid was highest followed by alanine, glutamic acid, and glycine. The tryptophan content was about 1 mole %.

N-Terminal Amino Acid Sequence of the Carotenoprotein from the Exoskeleton

The N-terminal amino acid sequences of the two polypeptides of 22.4 and 21.4 kDa in the carotenoprotein from the exoskeleton are shown in Fig. 4. Ten residues were determined in both peptides. Both peptides had almost the same sequence except the second amino acid residue, which was lysine in the polypeptide of 22.4 kDa, and glycine in that of 21.4 kDa.

Absorption Spectrum of the Carotenoprotein from the Exoskeleton

The carotenoprotein from the exoskeleton in 20 mM potassium phosphate buffer (pH 6.8) had absorption maxima at 615, 370, 320, and 278 nm. The absorbance ratio ($A_{615/278}$) was 3.67 for the purified protein (Fig. 5).

Identification of Carotenoid Prosthetic Group in the Carotenoprotein from the Exoskeleton

A carotenoid liberated from this carotenoprotein exhibited $\lambda_{\max}$ at 487 nm in benzene and gave a single red spot on TLC using a silica gel plate with a mobile phase of benzene/ethyl acetate (9:1). The R_f value was coincident with that of free astaxanthin. This carotenoid also showed the same retention time as free astaxanthin in HPLC using a Superiorex ODS column. Small amounts of *cis*-isomers of astaxanthin were also found. Therefore, this carotenoprotein was proved to contain only free astaxanthin as the prosthetic group.

One point three milligrams of the carotenoprotein which corresponds to 1.083×10^{-8} mole liberated 2.33×10^{-8} mole of astaxanthin. From this result, the stoichiometry of the carotenoid and the apoprotein was determined to be a molar ratio of 2:1.

Fig. 6. A: Gel filtration on TSK G-3000SWG column of the carotenoprotein from the muscular epithelium of *P. monodon*. Detection was carried out at 278 nm (○) and 625 nm (●). B: SDS-PAGE pattern of some fractions. Refer to the legend of Fig. 1 for abbreviations and further details. The volume of each fraction applied on a lane was 20 μ l.

Purification of the Carotenoprotein from the Muscular Epithelium

At first we failed to extract the carotenoprotein in the muscular epithelium by the same method as the extraction of carotenoprotein from the exoskeleton. Collagenase treatment enabled us to obtain the soluble carotenoprotein from the muscular epithelium with its color intact. This carotenoprotein was not retained on the DEAE Toyopearl column equilibrated with 20 mM phosphate buffer. Therefore, further purification was carried out by only high-speed gel filtration. Figure 6A shows the elution pattern of the first gel filtration of the carotenoprotein. Some fractions were analyzed for protein composition pattern by SDS-PAGE (Fig. 6B). There were several peaks having absorbance at 278 nm. The main peak under absorption measurements at 625 nm was found in fraction No. 25 to 31. A minor peak was also found around fraction No. 15 to 18. This minor peak eluting around the void volume may be caused by carotenoids liberated and bound artificially to aggregated proteins during precipitation with ammonium sulfate or subsequent dialysis, because the color of those fractions was not blue for carotenoproteins but red for free carotenoids. The retarded fractions were collected as

Fig. 7. A: Native-PAGE pattern of purified carotenoprotein from the muscular epithelium, carotenoprotein from the exoskeleton, and carotenoprotein from the exoskeleton after collagenase treatment.

The gel is not stained with Coomassie brilliant blue. Abbreviations used are CM, carotenoprotein from the muscular epithelium; CEC, a newly derived carotenoprotein from the original carotenoprotein from the exoskeleton after collagenase treatment; CE, carotenoprotein from the exoskeleton. Proteins applied were 5 μ g/lane (CM), 10 μ g/lane (CEC), 2 μ g/lane (CE).

B: SDS-PAGE pattern of purified carotenoprotein from the muscular epithelium.

Proteins applied were 2 μ g/lane.

carotenoproteins, though the absorbance at 625 nm was smaller than that at 278 nm, and subjected to the same gel filtration repeatedly. The carotenoprotein was isolated as a single peak having absorbance at 625 nm after three repetitions of the gel filtration. The yield was 3.3 mg from 32 abdominal parts (472 g).

Molecular Weight Determination of the Carotenoprotein from the Muscular Epithelium

The molecular weight of this carotenoprotein was determined to be 21.4 kDa by high-speed gel filtration. The molecular weight could not be determined by native PAGE according to the method of Davis and Ornstein²⁰⁾ because this blue carotenoprotein turned purple and gave at least three pigmented bands during running (Fig. 7A). Additional bands without the color of carotenoprotein appeared when the gel was stained with Coomassie brilliant blue. The relative mobilities of the purple bands were not consistent with the molecular size determined by gel filtration. SDS-PAGE showed a band corresponding to the molecular weight of 22.9 kDa but the band was obscure (Fig. 7B).

Identification of Carotenoid Prosthetic Group of the Carotenoprotein from the Muscular Epithelium

The carotenoid liberated from this carotenoprotein gave a single red spot on TLC using a silica gel plate with a mobile phase of benzene/ethyl acetate (9:1) and the R_f value

was coincident with that of free astaxanthin. The carotenoid had a maximum absorbance at 487 nm in benzene. From these results, this carotenoprotein was proved to have only free astaxanthin as the prosthetic group.

Comparison of the Carotenoproteins from the Exoskeleton with that from the Muscular Epithelium

The carotenoprotein from the exoskeleton gave an additional peak having absorbance at 625 nm on gel filtration after treatment with the collagenase. The additional peak of blue carotenoprotein appeared immediately after the peak of original carotenoprotein from the exoskeleton on gel filtration. The molecular size of the second peak was somewhat larger than that of the carotenoprotein from the muscular epithelium. Native-PAGE of the isolated carotenoprotein of the second peak after collagenase treatment showed a main blue band which showed lower mobility than the original carotenoprotein from the exoskeleton (Fig. 7A).

Discussion

The absorption maximum at 615 nm of the carotenoprotein from the exoskeleton was somewhat red-shifted compared with the corresponding value for the blue carotenoproteins from *P. japonicus*¹⁸⁾ and α -crustacyanin from lobster.¹²⁾ It shifted from 615 nm to around 480 nm by heating the carotenoprotein to 50°C (data not shown). This large shift suggests a strong carotenoid-protein interaction which is frequently found in blue carotenoproteins isolated from other crustaceans. The purification procedure adopted in this study for the carotenoprotein from the exoskeleton was almost the same as that for α -crustacyanin from the lobster *Homarus gammarus*.²⁶⁾ The carotenoprotein from the exoskeleton of *P. monodon* was eluted from a DEAE column at a lower concentration of KCl than α or γ -crustacyanin. This means that the carotenoprotein from *P. monodon* is less negatively-charged than crustacyanins from *H. gammarus*. The absorption ratio ($A_{615/278}$) of 3.67 is quite similar to that of crustacyanin purified from *H. gammarus*.¹²⁾ This value is consistent with the amino acid composition which shows similar abundance of tyrosine and phenylalanine to crustacyanin.¹³⁾ Some of these aromatic amino acids are thought to be important in stabilizing the binding of astaxanthin in the cavity of apocrustacyanin.¹⁶⁾ The carotenoprotein from the exoskeleton of *P. monodon* may have the same mechanism to stabilize the binding between carotenoid and apoprotein by these aromatic amino acids.

On the other hand, the carotenoprotein in the muscular epithelium of *P. monodon* was not extracted in the same way for the exoskeleton. Muriana *et al.* extracted a carotenoprotein of *P. japonicus* from the hypodermis with phosphate buffer, though the carotenoprotein obtained showed a quite large absorption maximum at 276 nm.¹⁸⁾ It is not known whether the difficulty in extraction of the carotenoprotein in the muscular epithelium of *P. monodon* is owing to its original properties or denaturation by freezing. The carotenoprotein from the muscular epithelium was not adsorbed onto a DEAE column at all. The collagenase treatment might deprive the carotenoprotein of charging effects. The absorption spectrum of

the carotenoprotein from the muscular epithelium has an absorption maximum at 625 nm quite similar to those of other blue carotenoproteins.^{4,8,9)}

The molecular weight of the carotenoprotein from the exoskeleton of *P. monodon* is rather smaller than that of α -crustacyanin (380 kDa)¹²⁾ or a blue carotenoprotein from *P. japonicus* (280 kDa).¹⁸⁾ In native PAGE, the carotenoprotein from *P. monodon* was so homogenous that it showed only one band (120 kDa). The results from SDS-PAGE indicated that this carotenoprotein contained six subunits, with three sets of two subunits of molecular weight 22.4 kDa and 21.4 kDa. A minor band of 23.1 kDa was also seen, but the appearance of this band depended on the preparation. This component might be a by-product derived from one of the two major components during purification. The sizes of two subunits are very close to those of apocrustacyanins A₁, C₁, C₂ (23 kDa) and A₂, A₃ (21.5 kDa).²⁶⁾ The amino acid composition also resembles that of α -crustacyanin.¹³⁾ The N-terminal amino acid sequences of the two subunits of 21.4 and 22.4 kDa from the exoskeleton of *P. monodon* are also quite similar to apocrustacyanin A₂¹⁵⁾ and C₁,¹⁶⁾ respectively. Thus, the carotenoprotein in the exoskeleton of *P. monodon* may be composed of quite similar apoproteins as crustacyanins. However, the quaternary structure of this carotenoprotein must be different from that of α -crustacyanin, because the subunit composition is different, and because the stoichiometry of carotenoid and apoprotein is 2:1 in the carotenoprotein of *P. monodon*. This ratio means that the carotenoprotein from *P. monodon* is composed of 6 apoproteins and only two of them contain one molecule of astaxanthin. On the other hand, α -crustacyanin is an octamer of β -crustacyanin which is composed of one set of two types of apocrustacyanin having one molecule of astaxanthin.⁶⁾ The total number of astaxanthin molecules in the carotenoprotein from the exoskeleton of *P. monodon* is the same as that of β -crustacyanin though the size of this carotenoprotein is three times as large as β -crustacyanin. Tryptophan residue is thought to be important in stabilizing astaxanthin molecules in a cavity of apocrustacyanin.¹³⁾ One tryptophan residue is thought to be necessary to stabilize one molecule of astaxanthin. The number of tryptophan residue in the carotenoprotein from the exoskeleton of *P. monodon* is rather smaller than that in α -crustacyanin.^{13,15,16)} This may explain the smaller number of astaxanthin molecules in the carotenoprotein from the exoskeleton of *P. monodon* than α -crustacyanin.

The molecular weight of the carotenoprotein from the muscular epithelium is rather smaller than that from the exoskeleton or that from the hypodermis of *P. japonicus*.¹⁸⁾ The molecular weight determined by gel filtration is very close to that of a subunit determined by SDS-PAGE, indicating that this carotenoprotein is composed of only one polypeptide chain. It is not clear why this epithelial blue carotenoprotein turned purple and gave many bands in native-PAGE. This phenomenon seems to resemble the dissociation of α -crustacyanin to β -crustacyanin which is purple and electrophoretically heterogeneous. But the molecular size of the carotenoprotein from the muscular epithelium of *P. monodon* is about 20 kDa and corresponds to one apocrustacyanin. Therefore, it is unlikely that this carotenoprotein dissociates into smaller compo-

nents. The many bands on native-PAGE of the carotenoprotein from the muscular epithelium may mean the presence of same size proteins with different charge effects formed by collagenase treatment because SDS-PAGE of this carotenoprotein showed a single band of 22.9 kDa.

Another explanation for the curious pattern of the bands in native-PAGE is variation in the molecular weight of the carotenoprotein by aggregation. Some parts of the original carotenoprotein might be deprived by collagenase treatment, resulting in changes of intact configuration. Such a structural change could have led to aggregation of the carotenoprotein by exposing its hydrophobic parts.

The carotenoprotein from the exoskeleton also underwent some change by collagenase treatment. Its molecular weight determined by gel filtration is not consistent with that determined by the method of Davis and Ornstein.²⁰ The effect of collagenase on both carotenoproteins from the exoskeleton and the muscular epithelium should be same. However, most of the carotenoprotein from the exoskeleton maintained its blue color, suggesting that these two carotenoproteins are not identical.

The carotenoid prosthetic group is free astaxanthin in both carotenoproteins from the exoskeleton and the muscular epithelium. This is consistent with the carotenoid composition of *P. monodon*.^{19,27} Different colors of *P. monodon* might be expressed by overlapping of blue carotenoproteins containing only free astaxanthin and astaxanthin esters.

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