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Carotenoid Forms in the Exoskeleton of Crayfish and Kuruma Prawn

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Abstract

Various kinds of carotenoid forms, such as hydrophobic reddish fraction, blue and orange carotenoproteins, and lipoproteins, were isolated from EDTA soluble fraction of the exoskeleton of crayfish and kuruma prawn. The hydrophobic reddish fraction was the main carotenoid form in crustaceans. The blue carotenoprotein possessed free astaxanthin alone, while astaxanthin esters along with free astaxanthin were present in the orange carotenoprotein. Three types of lipoproteins containing both free astaxanthin and astaxanthin esters were present in the exoskeleton, although the lipoproteins were the minor carotenoid forms. The lipoproteins isolated from the exoskeleton differed from the hemolymph lipoproteins in apolipoprotein compositions. The color development of crustaceans appeared to be affected by these carotenoid forms.

Carotenoid-protein complexes are widely distributed throughout the invertebrates including crustaceans and classified into two groups, carotenoproteins and carotenolipoproteins[1-4]. In the former complex carotenoids are bound stoichiometrically to a simple protein and bathochromic spectral shifts in excess of 100 nm are observed because of the interaction of protein with the carotenoid chromophore. Carotenolipoproteins contain both carotenoids and lipids, and carotenoids are simply dispersed in the lipid components of the lipoproteins. The absorption spectrum of carotenoids in the carotenolipoproteins is unaltered or weakly bathochromically shifted, distinct from carotenoproteins.

Carotenoproteins with various coloration have been isolated from the carapace and hypodermis of crustaceans[5-9]. The properties of crustacyanin with bluish coloration, which was isolated from the carapace of lobster, have been examined in detail among carotenoproteins[10-15]. Crustacyanin consists of free astaxanthin and apoproteins, and has absorption maxima at 632 nm[4]. On the other hand, carotenolipoproteins have been reported to be present frequently in eggs and ovaries of crustaceans[3,4,16]. Carotenolipoproteins in the egg and ovary have been known as ovoverdin with greenish coloration, which consists of free astaxanthin and lipovitellin, and shows absorption maxima at 640 and 460 nm[16]. Carotenolipoproteins have hardly been isolated from the carapace of crustaceans, distinct from carotenoproteins of carapace. Thus carotenoproteins play an important role for the development of carapace coloration of crustaceans, but the coloration of carapace is not necessarily blue despite of the presence of bluish crustacyanin. This suggests strongly that the coloration of carapace is influenced by the presence not only of carotenoproteins but of other carotenoid forms, namely carotenolipoproteins and carotenoids not associated with proteins.

In the present study, we found hydrophobic reddish precipitate and several lipoproteins containing carotenoids besides blue and orange carotenoproteins in the exoskeleton including carapace and hypodermis from crayfish and kuruma prawn. The results indicated that various carotenoid forms were associated with the coloration of exoskeleton from crustaceans.

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Materials and Methods

Animals

Crayfish *Procambarus clarkii* (average body weight 26.6 g) and kuruma prawn *Penaeus japonicus* (average body weight 11.8 g) were obtained from commercial dealers. These animals were transported alive to the laboratory and the exoskeleton including carapace and hypodermis was subjected to the following experiments.

Preparation of EDTA-soluble fraction

The exoskeleton was dissected from the body, washed under cold running water, rinsed, and dried with a filter paper. Then it was cut into small pieces with scissors and homogenized with 4 volumes of a 10% EDTA solution (pH 7.5) for 3 min using a Polytron homogenizer[10]. The homogenate was then stirred slowly overnight in a cold room in the dark. The homogenate was then centrifuged at 8,000 rpm for 20 min at 4℃. The pellet was reextracted with the 10% EDTA solution until no more solubilized carotenoids were detected in the supernatant. The residue was termed EDTA-insoluble fraction. The combined supernatant, EDTA-soluble fraction with reddish coloration was subjected to ammonium sulfate treatment, and the fraction precipitatied with 50% saturation of ammonium sulfate was recovered and dialyzed overnight against 150 mM NaCl-1.3 mM EDTA, pH 7.5 (saline solution). The dialysate was then centrifuged at 8,000 rpm for 20 min at 4℃, and the bluish supernatant was separated from the reddish precipitate.

Isolation of carotenoproteins and lipoproteins

The bluish supernatant was subjected to a density gradient ultracentrifugation for the separation of carotenoproteins from lipoproteins[17]. Potassium bromide was added to the bluish supernatant to the final concentration of 44.5%. The bluish solution (31 ml) with KBr was placed in a centrifuge tube and overlaid with 31 ml of saline solution. The tube was centrifuged at 35,000 rpm for 17 h. at 15℃ in a 45 Ti rotor using a Beckman L-70 ultracentrifuge. After centrifugation, fractions were collected and the density was determined by refractometry. The fractions with densities below or above 1.21 g/ml were termed lipoproteins or carotenoproteins, respectively. The carotenoproteins separated from lipoproteins were dialyzed against saline solution and were treated with ammonium sulfate to separate orange carotenoprotein from blue one. The orange and blue carotenoproteins were precipitated at 30% and 50% saturation of ammonium sulfate, respectively.

Extraction of carotenoids

Carotenoids were extracted from EDTA-soluble and insoluble fractions by the addition of acetone. After transfer to n-hexane, the carotenoids were concentrated under reduced pressure. The carotenoid content was calculated, assuming the $E^{1\%}_{1cm}$ value in acetone at 477 nm to be 2200. Carotenoid composition was analysed by thin-layer chromatography (TLC). Identification of each carotenoid was made by co-TLC with reference specimens. The TLC plates were developed by benzene-ethyl acetate 65 : 35 (v/v).

Determination of protein and lipid contents

The protein contents of carotenoproteins and lipoproteins were determined using a protein assay kit from Bio-Rad, with bovine serum albumin as a standard. Phospholipid, triacylglycerol and cholesterol were determined using enzyme-based assay kits purchased from Kyowa Medex Co., Ltd, Tokyo.

Electrophoresis

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of carotenoproteins and lipoproteins was performed according to Laemmli[18]. Electrophoresis was conducted on gradient gels (4.5-18% polyacrylamide) at 20 mA for 3 h. Samples containing 1% 2-mercaptoethanol were heated to 95℃ for 5 min. Protein bands on gels were stained with 0.25% Coomassie Brilliant Blue R-250.

Results

Carotenoid distribution in exoskeleton

The exoskeleton of crayfish possessed much more

Table 1 Carotenoid distribution in various fractions prepared from exoskeleton of crayfish and kuruma prawn

Fraction	Carotenoid content (mg/100g exoskeleton)	
	Crayfish	Kuruma prawn
EDTA soluble fraction	4.575 (23.84)*	7.842 (76.43)
Bluish supernatant after dialysis	0.408 (2.12)	0.532 (5.19)
Carotenoproteins	0.313 (1.63)	0.206 (2.01)
Lipoproteins	0.095 (0.49)	0.326 (3.18)
Reddish precipitate after dialysis	4.167 (21.72)	7.310 (71.24)
EDTA insoluble fraction	14.618 (76.16)	2.418 (23.57)
Total fractions	19.193 (100)	10.260 (100)

*Percentage of each fraction to total carotenoid contents of exoskeleton.

carotenoids than that of kuruma prawn, reflecting their color in exoskeleton. The color of exoskeleton was deep reddish purple in crayfish and light orange in kuruma prawn. Carotenoids in crayfish were not readily extracted with EDTA solution because of their hard exoskeleton. Although most carotenoids were present in EDTA-insoluble fraction of crayfish, some carotenoids were distributed in reddish fraction which precipitated after dialysis of EDTA-soluble fraction (Table 1).

Carotenoid distribution in the exoskeleton of kuruma prawn largely differed from that of crayfish. Carotenoids in kuruma prawn were readily extracted with EDTA solution and were mostly present in reddish fraction of EDTA-soluble fraction. Although carotenoids in both crayfish and kuruma prawn were not mainly distributed in carotenoproteins and lipoproteins of EDTA-soluble fraction, carotenoids were clearly detected in these fractions (Table 1). No carotenoids were present in the supernatant fraction at 50% saturation of ammonium sulfate in both crayfish and kuruma prawn.

Lipoproteins and Carotenoproteins in EDTA-soluble fraction

The bluish supernatant of EDTA-soluble fractions from the exoskeleton of crayfish and kuruma prawn was submitted to density gradient ultracentrifugation with the 1.05-1.30 g/ml density range for the separation of lipoproteins and carotenoproteins (Fig.1). Lipoproteins from crayfish and kuruma prawn were clearly separated from carotenoproteins because of their difference in density. On ultracentrifugation three lipoprotein peaks were evident with densities of 1.07, 1.11-1.12, and 1.17-1.19 g/ml in both crayfish and kuruma prawn. They were termed lipoprotein-Ⅰ, Ⅱ, and Ⅲ, respectively. Lipoprotein-Ⅰ, Ⅱ, and Ⅲ had absorption maxima at 480 and 280 nm (Fig.2). All lipoproteins in both crayfish and kuruma prawn contained carotenoids and showed an orange coloration.

The density of carotenoproteins from crayfish and kuruma prawn was larger than 1.25 g/ml, distinct from lipoproteins (Fig.1). Carotenoproteins had three absorption maxima, 620, 480, and 280 nm. This

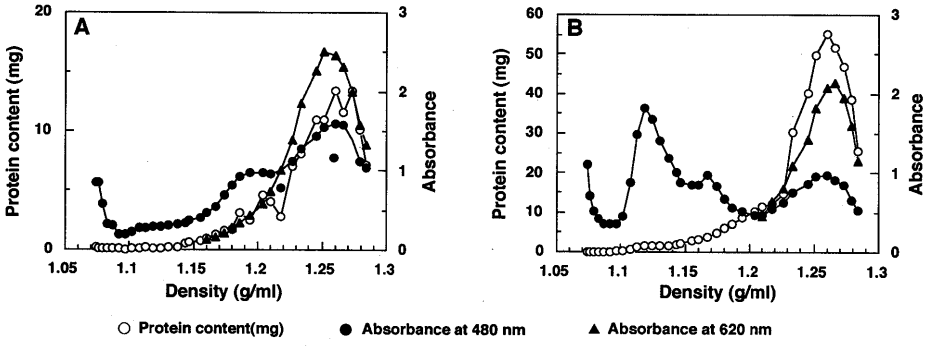

Fig. 1 Density gradient ultracentrifugation of EDTA-soluble fractions from the exoskeleton of crayfish (A) and kuruma prawn(B).

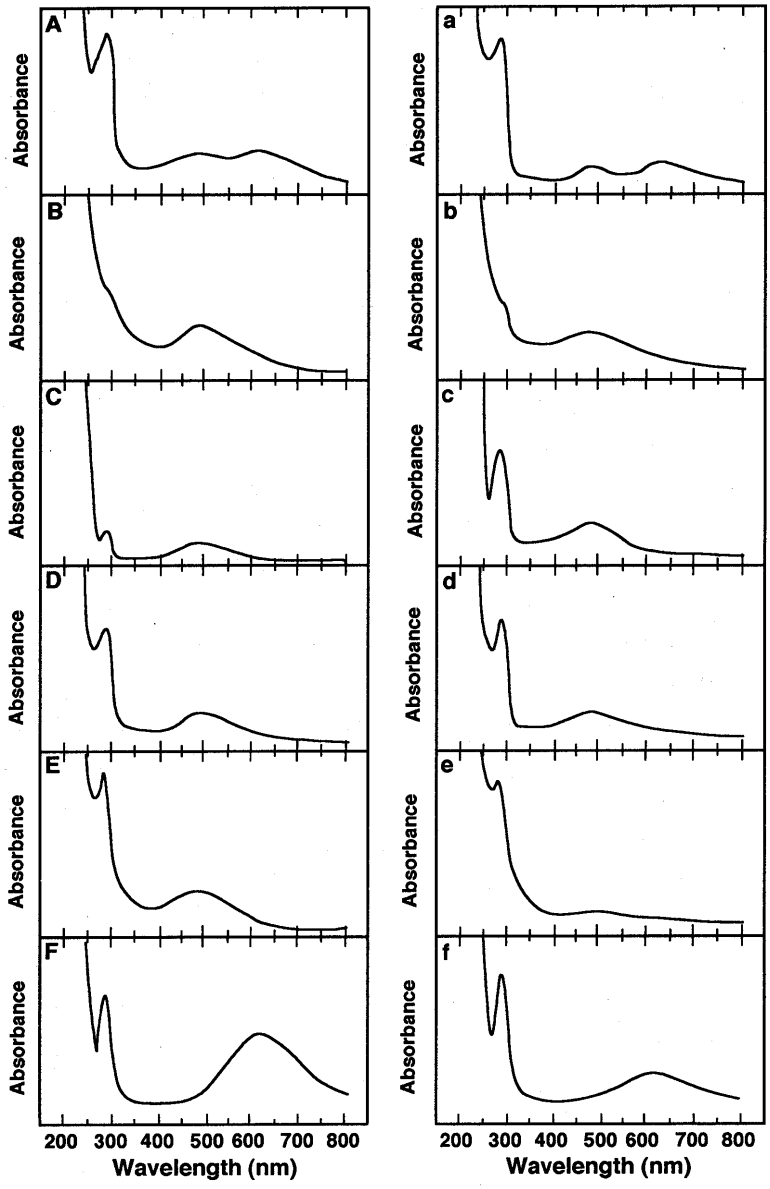

Fig. 2 Absorption spectra of EDTA-soluble fractions (A, a), lipoprotein-Ⅰ (B, b), lipoprotein-Ⅱ (C, c), lipoprotein-Ⅲ (D, d), orange carotenoprotein (E, e), and blue carotenoprotein (F, f) from crayfish (capital letter) and kuruma prawn (small letter).

suggested that carotenoproteins were colored not only bluish but also orange. Ammonium sulfate treatment enabled to separate blue carotenoprotein from orange one. The orange and blue carotenoproteins were precipitated at 30% and 50% saturation of ammonium sulfate, respectively. The former had absorption maxima at 480 and 280 nm, while the latter at 620 and 280 nm, a carotenoprotein like α-crustacyanin. (Fig.2).

Carotenoid contents of lipoproteins and carotenoproteins from the exoskeleton of crayfish and kuruma prawn are summarized in Table 2, along with their lipid compositions and densities. The ratio of lipid to protein decreased with increased densities of lipoproteins. Lipoprotein-Ⅰ, Ⅱ and Ⅲ contained phospholipid as a predominant lipid component. In EDTA-soluble fraction of crayfish carotenoids were mainly distributed in carotenoproteins, especially a blue carotenoprotein. Although carotenoids were also present in lipoproteins from crayfish, carotenoid contents in lipoproteins were much lower than those in carotenoproteins. The different carotenoid

Table 2 Protein, lipid, and carotenoid distributions in lipoprotein and carotenoprotein fractions prepared from exoskeleton of crayfish and kuruma prawn

Fraction	Content (mg/100g exoskeleton)	
	Crayfish	Kuruma prawn
Lipoprotein-I	4.823 (100)*	18.124 (100)
Protein	0.770 (15.96)	3.905 (21.55)
Triglyceride	0.091 (1.89)	N.D.
Phospholipid	3.224 (66.85)	10.700 (59.04)
Free cholesterol	0.208 (4.31)	1.954 (10.78)
Cholesteryl ester	0.495 (10.26)	1.542 (8.51)
Carotenoid	0.035 (0.72)	0.023 (0.13)
Density(g/ml)	1.074	1.074
Lipoprotein-II	6.286 (100)	104.890 (100)
Protein	3.611 (57.44)	53.865 (51.35)
Triglyceride	N.D.	1.262 (1.20)
Phospholipid	1.501 (23.88)	39.288 (37.46)
Free cholesterol	0.197 (3.13)	5.018 (4.78)
Cholesteryl ester	0.962 (15.30)	5.240 (5.00)
Carotenoid	0.016 (0.25)	0.218 (0.21)
Density(g/ml)	1.106	1.118
Lipoprotein-III	23.163 (100)	182.377 (100)
Protein	20.580 (88.85)	170.794 (93.65)
Triglyceride	0.115 (0.50)	N.D.
Phospholipid	1.495 (6.45)	9.418 (5.16)
Free cholesterol	0.228 (0.98)	0.511 (0.28)
Cholesteryl ester	0.701 (3.03)	1.568 (0.86)
Carotenoid	0.044 (0.19)	0.086 (0.05)
Density(g/ml)	1.193	1.167
Carotenoprotein(Orange)	121.738 (100)	349.206 (100)
Protein	121.673 (99.95)	349.193 (99.99)
Carotenoid	0.065 (0.05)	0.013 (0.01)
Density(g/ml)	1.273	1.260
Carotenoprotein(Blue)	119.705 (100)	486.979 (100)
Protein	119.457 (99.79)	486.786 (99.96)
Carotenoid	0.248 (0.21)	0.193 (0.04)
Density(g/ml)	1.273	1.260

*Percentage of each component to lipoprotein or carotenoprotein.
N.D.: not detected.

Table 3 Carotenoid compositions in lipoprotein and carotenoprotein fractions prepared from exoskeleton of crayfish and kuruma prawn

Carotenoid	Crayfish					Kuruma prawn				
	Lipoprotein			Carotenoprotein		Lipoprotein			Carotenoprotein	
	I	II	III	Orange	Blue	I	II	III	Orange	Blue
Astaxanthin diester	36.69	25.71	11.33	14.04	N.D.	11.30	4.39	4.85	5.88	N.D.
Astaxanthin monoester	37.41	17.14	6.67	14.04	N.D.	16.95	5.70	11.65	9.80	N.D.
Astaxanthin	17.27	47.14	78.67	59.65	90.40	62.15	85.53	79.61	67.65	94.03
Others	8.63	10.01	3.33	12.27	9.60	9.60	4.38	3.89	16.67	5.97

N.D.: not detected.

distribution was found in EDTA-soluble fraction of kuruma prawn. The carotenoid content of lipoprotein-II in kuruma prawn was comparable to that of blue carotenoprotein.

Carotenoid composition of lipoproteins and carotenoproteins

Although astaxanthin was the main carotenoid in EDTA-soluble fractions from crayfish and kuruma prawn, astaxanthin compositions differed in lipoproteins and carotenoproteins (Table 3). Astaxanthin

diester and monoester as well as astaxanthin were present in lipoproteins from crayfish and kuruma prawn. The ratio of ester types of astaxanthin to free one increased with decreased densities of lipoproteins. Astaxanthin composition of blue carotenoprotein was largely different from that of orange one. Blue carotenoprotein from crayfish and kuruma prawn had free astaxanthin alone, while astaxanthin esters as well as free astaxanthin were present in orange carotenoprotein.

SDS-PAGE of apolipoproteins and apocarotenoproteins

Apolipoprotein and apocarotenoprotein features in EDTA-soluble fractions from crayfish and kuruma prawn were analyzed by gradient SDS-PAGE (Fig.3). Apolipoprotein features differed in lipoproteins from crayfish. Lipoprotein-Ⅰ possessed two major apolipoproteins of molecular weights (MW) 67KDa and 57KDa, while lipoprotein-Ⅱ consisted of three major apolipoproteins of MW 67KDa, 57KDa, and 40KDa. Lipoprotein-Ⅲ possessed one major apolipoprotein of MW 40KDa. Apocarotenoprotein features of blue and orange carotenoproteins were complicated compared with lipoproteins. Orange carotenoprotein possessed several apocarotenoproteins with higher molecular weights compared with blue one. This suggested that different types of carotenoproteins were present in EDTA-soluble fraction from crayfish.

Apolipoprotein features of kuruma prawn were different from those of crayfish, the presence of apolipoproteins of lipoprotein-Ⅰ from kuruma prawn were uncertain because of its extremely low level. Lipoprotein-Ⅱ and Ⅲ possessed two major apoliporoteins whose molecular weights were 93KDa and 35KDa, and 56KDa and 35KDa, respectively. Although both orange and blue carotenoproteins consisted of several apocarotenoproteins, these carotenoproteins differed from one another in apocarotenoprotein features.

Discussion

Although carotenoids are responsible for the color of crustaceans, it is not necessarily clear what kinds of carotenoid forms are associated with coloration of the exoskeleton. Carotenoprotein is one of carotenoid-protein complexes and has been isolated from the exoskeleton of crustaceans[5-9]. Carotenoproteins have distinct color from parent carotenoids, because the specific interaction occurs between protein and carotenoid chromophore[4]. Carotenoproteins seem to play an important role for the development of cara-

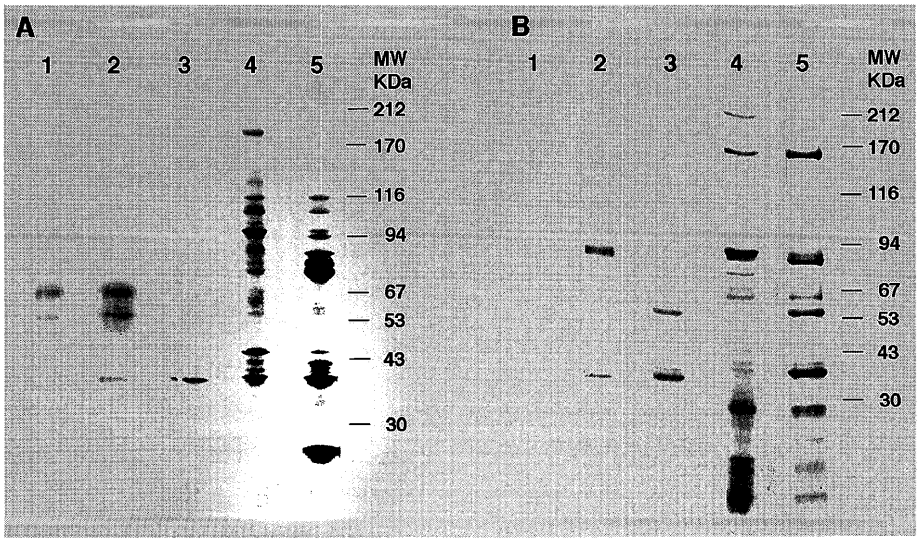

Fig. 3 Electrophoretic patterns in linear gradient of 4.5-18% polyacrylamide gelcontaining sodium dodecylsulfate of apolipoproteins and apocarotenoproteins in EDTA-soluble fractions from crayfish (A) and kuruma prawn (B).
1, Lipoprotein-Ⅰ; 2, lipoprotein-Ⅱ; 3, lipoprotein-Ⅲ; 4, orange carotenoprotein: 5, blue carotenoprotein.

pace coloration of crustaceans.

Do carotenoproteins as the sole carotenoid form exist in the carapace of crustaceans? Both blue carotenoprotein and red carotenoid fractions have recently been isolated from the muscular epithelium of the black tiger prawn *Penaeus monodon*[19,20]. The different body color of cultured black tiger prawns has been reported to be associated with the varied composition of these carotenoid fractions in the muscular epithelium[20]. The black tiger prawns with dark gray color contained much more red carotenoid fraction than those with pale blue color, while the contents of blue carotenoprotein fraction were almost the same in the prawns with different color. A red carotenolipoprotein containing astaxanthin and its esters has also been isolated from the carapace of crayfish[21]. These results suggest strongly that not only carotenoproteins but other carotenoid forms have a role to make the body color of crustaceans.

In the present study, we found three carotenoid forms from EDTA-soluble fraction of the exoskeleton of crayfish and kuruma prawn (Table 1). Although the reddish fraction which precipitated after dialysis of EDTA-soluble fraction was the main carotenoid form in both crayfish and kuruma prawn, no further characterization of reddish precipitate could be done because of its strong hydrophobicity. The reddish precipitate undoubtedly contained some proteins. Two types of carotenoproteins with blue and orange color were present in the exoskeleton of crayfish and kuruma prawn. The blue carotenoprotein contained much more carotenoids than the orange one. The blue and orange carotenoproteins differed from one another in absorption spectrum, carotenoid composition, and apocarotenoprotein composition. Three types of lipoproteins containing carotenoids were isolated from the exoskeleton of crayfish and kuruma prawn, although the lipoproteins were the minor carotenoid form. Lipoproteins have been known as carriers of lipids and other hydrophobic compounds like carotenoids. Both high density lipoprotein (HDL) and very high density lipoprotein (VHDL) have been isolated from the hemolymph of crustaceans[22]. Lipoprotein-Ⅰ, Ⅱ, and Ⅲ isolated from the exoskeleton of crayfish and kuruma prawn differed from the hemolymph lipoproteins in apolipoprotein compositions. Lipoprotein-Ⅰ, Ⅱ, and Ⅲ found in the present study seemed to be specific components in the exoskeleton of crustaceans. Lipo protein-Ⅰ, Ⅱ, and Ⅲ in the exoskeleton corresponded to low density lipoproteins, HDL2 and HDL3 in fish plasma with respect to density, respectivel y[23]. Lipoprotein-Ⅰ, Ⅱ, and Ⅲ in the exoskeleton contained both free astaxanthin and astaxanthin esters. It is of interest how these lipoproteins are synthesized in the exoskeleton.

Thus, the color development of crustaceans was affected by the hydrophobic reddish fraction, blue and orange carotenoproteins, and lipoproteins.

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