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Studies on Carotenoprotein in Aquatic Animals

IV. Carotenoid Pigments in Crayfish (*Procambarus clarkii*)

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(Figs. 1–4, Tables 1–2)

Carotenoproteins evidently have an important role in the color-formation of crustaceas, and in explaining their pigmentation the contribution of unbound-carotenoids can not be disregarded. Therefore the relationship between unbound-carotenoids and the carotenoproteins in the color-formation was taken up as the subject of the present study in order to understand better the color patterns of crayfish.

In describing the nature of pigments in the crayfish exoskeleton, the authors reported the effect of carotenoproteins upon color appearance[1]. In recent years several studies on pigmentation of marine isopods have been made with respect to the ecology[2)3)4)]. The pigmentation of the marine isopods was found to depend on the proportion of carotenoproteins and unbound-carotenoids in the pigmented layer[4)5)6)]. Although color change in those species is a transitory phenomenon, the exoskeleton of crayfish which is covered with a thick calcified layer is unable to make his color actively fit for the enviromental condition.

The metabolism of carotenoids in many species of curstacea have been studied in detail, but little is still known as basic information to understand the pigmentation mechanism. The research work reported in this paper intends to investigate the mechanism of pigmentation in the crayfish exoskeleton.

MATERIALS AND METHODS

Extraction of carotenoids: The crayfish used were collected at the Umeda fish farming in Kannabe, Hiroshima prefecture, they were freezed immediately after catching at $-20°C$ until the extraction of pigments. Blood was collected

from the heart of living crayfish. The parts of exoskeleton were separated and pulverized by a grinder after drying. Carotenoids were extracted repeatedly with acetone until no further pigment could be obtained. Then the acetone extracts were combined and concentrated under a stream of nitrogen. Adding water to the concentrate, the carotenoids were transferred to petroleum ether. The petroleum ether solution was repeatedly washed with water until freed completely of acetone, dried over anhydrous sodium sulfate and re-concentrated under nitrogen stream. The prosthetic group carotenoids of carotenoproteins prepared by the method previously stated were analyzed, after extraction with methanol-chloroform[7].

Thin-layer chromatography of carotenoids: Carotenoids were separated by thin-layer chromatography using silica gel G (E. Merck, Darmstadt). The silica gel plates of thickness 0.25 mm were activated by heating at 105–110°C for 1 hour. Carotenoids were chromatographed with the solvent systems as followed; *n*-hexane, ethylacetate (85:15 v/v); petroleum ether, ether (80:20 v/v); benzen, methanol (98:2 v/v); and benzen, hexane (2:1 v/v). The R*f* values of each pigment were compared with authentic standards. The pigmented bands on the plates were scratched and re-extracted with acetone, and the extracts were applied for the further experiment.

Column chromatography of carotenoids: The alumina and silica gel were activated by exposure to 100°C for 3 hours in an oven, and packed to the columns. The column used was approximately 1 cm in diameter and 20 cm in length. Pigments were placed on the column and eluted with an acetone-petroleum ether mixture for alumina columns, and with ether-petroleum ether mixture for silica gel columns. The eluted pigments were transferred to petroleum ether and re-chromatographed.

Identification of pigments: Partition behavior between petroleum ether and 90 % methanol was carried out according to the method of PETRACEK & ZECHMEISTER[8]. Saponification was performed with 10 % alcoholic potassium hydroxide, overnight under nitrogen at room temperature. The presence of keto groups was proved by reduction with a sodium borohydride ethanol solution to hydroxy derivatives. The concentrated HCl test was applied in order to detect the presence of epoxide and furanoid. The absorption spectra of the carotenoids were recorded in the solutious of petroleum ether (B. P. 40–60°C), ethanol, benzen, carbon disulfide, and chloroform by using a Hitachi IV-50A Automatic Spectrophotometer. All the solvents used were of special grade.

Determination of carotenoid composition: The pigments were chroma-

tographed on the thin-layer plate of silica gel, and the color density of each band was measured by an Ozumor Densitometer 82 with a filter No. 52. The relative amount of each carotenoid was given as percentage of the total.

Quantitative determination of carotenoids : Quantitative determinations were based on the specific extinction coefficient at the wavelength of maximal absorbance in petroleum ether, 2000 as the $E^{1\%}_{1\,cm}$.

Authentic carotenoids : All the separated pigments were compared with known carotenoids on thin-layer plates. α-and β-carotenes, and canthaxanthin were purchased. Echinenone from the gonad of sea urchin was isolated and purified. Cryptoxanthin and zeaxanthin were isolated from maize meal. ε-carotene, lutein, and astaxanthin were isolated from sea bream by the method of KATAYAMA *et al.*[9] Isocryptoxanthin and isozeaxanthin were prepared by the reduction of echinenone and canthaxanthin, respectively.

RESULTS

Thin-layer chromatograms on silica gel plates of extract from whole exoskeleton are shown in Fig. 1 and 2. A total of 12 carotenoid pigments were separated on a thin-layer plate by using the solvent system, *n*-hexane and ethylacetate. A dominant red band and trace of the others were separated respectively as the prosthetic group carotenoid from each colored carotenoprotein. The absorption maxima of the carotenoid fractions which were separated by thin-layer and column chromatography are given in Table 1.

Identification of carotenoid pigments : The sequence of fractions on the thin-layer plate and their number are shown in Fig. 1 and 2. The properties and identification of the resulting 13 fractions are given below.

Fraction 1–1 (β-carotene) The yellow Fraction 1 of the highest R*f* value on the thin-layer plate using *n*-hexane and ethylacetate was epiphasic by partition test. Moreover, Fraction 1 was able to separate two fractions (Fraction 1–1 and 1–2) in silica gel column chromatography by elution with petroleum ether. Fraction 1–1 agreed closely with an authentic sample of β-carotene in spectral characteristics and column chromatographic behavior, and its characteristics were unaffected by saponification.

Fraction 1–2 (β-carotene isomer) The fraction could be separated from Fraction 1 by silica gel column chromatography eluted with 3 % ether in petroleum ether. The concentrated HCl test was negative, showing the absence of both a 5, 6–epoxide and its isometic 5, 8–furanoid derivative. It had absorption

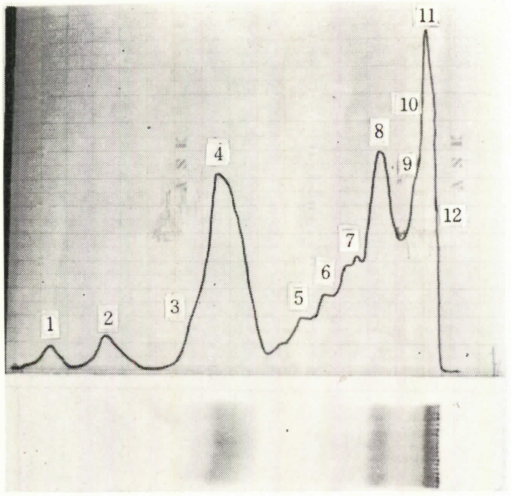

Fig. 1. Photograph of thin-layer chromatogram and its densitometry of carotenoids obtained from the exoskeleton of crayfish. *n*-hexane, ethylacetate (85 : 15).

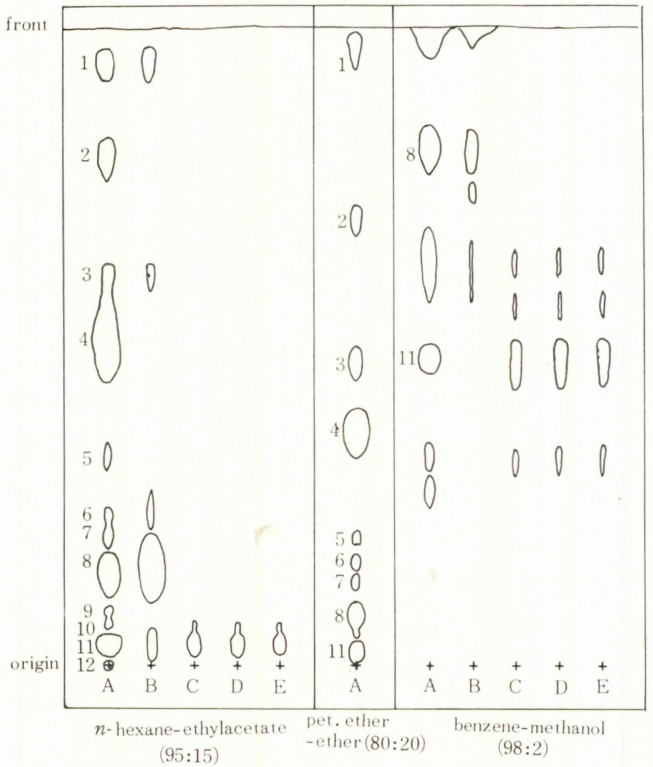

Fig. 2. Schematic chromatograms of carotenoids on silica gel plates.
A : Whole extract from the exoskeleton.
B : After saponification.
C : Prosthetic group of blue carotenoprotein.
D : Prosthetic group of purple carotenoprotein.
E : Prosthetic group of red carotenoprotein.

Table 1. Absorption maxima (nm) of carotenoids obtained from crayfish

No. of Fraction		Petroleum ether	Carbon disulfide	Chloroform	Ethanol
1-1		(428)451-478	486-517	466-496	454-480
1-2		(425)446-475	478-509	436-458-487	(428)448-478
2		457	494	477	465
	S	467	505	486	477
	R	(425)447-474		(435)459-487	(427)449-476
3		465	500	483	475
	S	460			
	R	(427)448-475		(435)459-487	449-475
4		471	507	491	482
	S	471	511	491	480
	R	(429)451-476			
5		445	477	465	455
	S	435			
	R	(425)447-475		(435)459-487	449-475
6		452	483	468	458
	S	455	488		
	R	(426)448-476		(436)460-488	(427)449-476
7		460	498	480	467～70
	S	463	497		
	R	(428)448-476		(438)460-487	(428)451-477
8		473	508	492	481
	S	473	508	492	481
	R	(429)451-476			(430)458-481
9		468	502	485	475
	S	465		493	
	R	(427)450-476		(435)461-484	450-477
10		466	510	488	472
	R	(425)445-474		(435)458-486	(425)447-475
11		469	505	489	476
	S	473	508	492	481
	R	(429)451-478			

R : reduction with sodium borohydride S : saponification

maxima at 446, 475 nm, and a shoulder at 425 nm in petroleum ether. The absorption spectra in various solvents were different from those of authentic α-, β-, and ε-carotene, and were unaffected by saponification. The absorption data agreed with β-carotene isomer presented by LEE[5], and CZECZUGA & CZERPAK[10]. These properties suggested that the fraction was β-carotene isomer. The fraction was mixed with a β-carotene fraction for the purpose of quantitative determination of the caroteuoid composition.

Fraction 2 (unknown) This orange yellow fraction clearly separated on the silica gel plate was almost entirely epiphasic in the partition test and the characteristic was altered to hypophasic by saponification. It had a single absorption maximum in petroleum ether at 457 nm. The asymmetrical absorption curve of the fraction was similar, but Rf value was separable from authentic echinenone when co-chromatographed on a thin-layer plate. The absorption maximum of the unsaponifiable pigment showed at longer side than parent pigment. Following saponification, Rf decreased and became unseparable from astaxanthin. Astacin, however, was not detected on the chromatography of the resulted product. On reduction with sodium borohydride the absorption curve was characteristic of Fraction 1-2. These characteristics suggested to be an esterified xanthophyll with keto group. The fraction had an absorption spectrum similar to data of monohydroxy monoketo-β-carotene reported by many authors, which were summerized by CAMPBELL[11]. But further difinite identification could not be done.

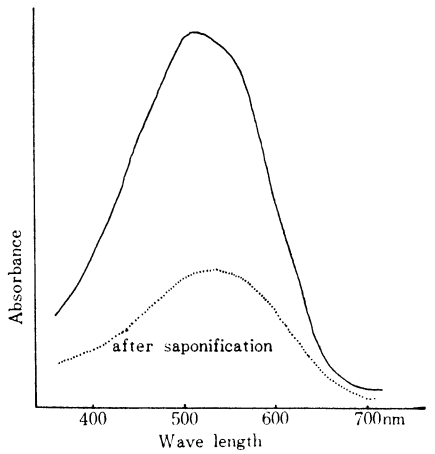

Fig. 3. Absorption spectra of unknown fraction (Fraction 2) in petroleum ether.

Fraction 3 (echinenone) The orange colored fraction. which had a similar Rf as astaxanthin ester (Fraction 4), agreed with echinenone obtained from the

gonad of sea urchin in R*f*. The absorption property and R*f* value remained the same before and after saponification. On reduction, the pigment gave a product with absorption spectra identical to those of Fracion 1–2.

Fraction 4 (astaxanthin ester) The absorption spectrum with its single symmetrical maximum suggests a structure of conjugated diketone. On partition test the fraction was epiphasic but after saponification the pigment became hypophasic. And the resulted pigment was inseparable with Fraction 8 (astacin) by co-chromatography on a silica gel plate. Following reduction of saponification product, the absorption spectrum showed β-carotene-like spectrum.

Fraction 5, 6, and 7 (unknown) These fractions developed between astaxanthin ester and astacin were separable from each other on the silica gel plate by using the solvent system of *n*-hexane and ethylacetate. The absorption spectra of these fractions were different each other, but seemed to be esterified keto-carotenoids, since the products resulted by saponification showed decreased R*f* values which were inseparable from each other. Their partition behaviors were epiphasic, but changed to hypophasic after saponification. The reduction yielded absorption spectrum identical to that of Fraction 1–2. One of these fractions suggested the possibility of such β-doradexanthin ester as shown in gold fish[12].

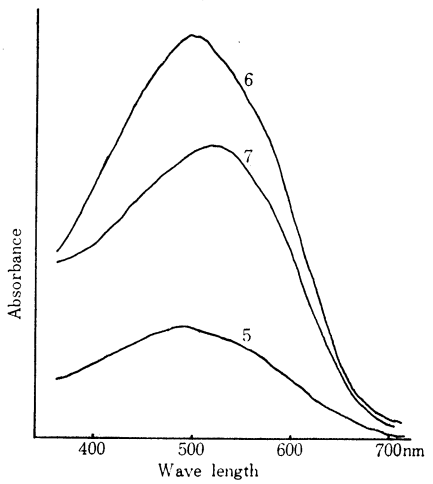

Fig. 4. Absorption spectra of unknown fractions (Fraction 5, 6 and 7) in petroleum ether.

Fraction 8 (astacin) The single symmetrical maximum at 473 nm in petroleum ether suggests a keto-carotenoid. The partition behavior was completely hypophasic before and after saponification. After saponification the R*f* value of the fraction remained equal to that of astacin. The reduction product showed an

absorption spectrum identical to that of β-carotene, and a remarkably decreased R*f* value on silica gel plate. The decrease of R*f* value seemed to depend on the formation of crustaxanthin. Therefore, the identification of the fraction was confirmed as astacin. In this fraction, the presence of traces of canthaxanthin was confirmed by the re-chromatography on silica gel plate using benzene and methanol solvent system.

Fraction 9 (*phoenicoxanthin*) The absorption maximum of the fraction was similar with that of phoenicoxanthin which was reported by KATAYAMA *et al.*[13] in prown. After reduction, the spectrum closely agreed with that of β-carotene.

Fraction 10 (*unknown*) The red fraction with lower R*f* value exibited a symmetrical absorption curve in petroleum ether. The partition behavior was entirely hypophasic. After reduction, the spectrum closely agreed with that of Fraction 1–2. The presence of keto group was suggested in the fraction.

Fraction 11 (*astaxanthin*) This red fraction had a lower R*f* on a thin-layer plate. Trace of astacin (Fraction 8) appeared sometimes when re-chromatopraphed. The absorption spectrum in petroleum ether with single symmetrical maximum at 469 nm suggested a keto-carotenoid. The strong adsorption on the alumina column was a characteristic of astaxanthin. The absorption maximum of the fraction was very similar to those reported by other authors. The partition test was hypophasic before and after saponification. The saponification increased its R*f* value, which became identical with that of astacin. The reduction resulted in a remarkable decrease of R*f* value. The identification of the fraction was confirmed as astaxanthin. Zeaxanthin was not detected in this fraction.

Carotenoid distribution in exoskeletons: The carotenoids extracted from the different part of exoskeleton were fractionated on thin-layer chromatography. As a result the carotenoid composition is illustrated in Table 2. Astaxanthin, its ester, and astacin were always major pigments in every part of crayfish exoskeleton. The carotenoid content is rich in the abdomen & tail which are less calcified than the chela. It should be noted that such exoskeleton was thinner because the calcified layer here was smaller than in other parts. As seen in Table 2, astacin was found in all parts under the chromatographic condition. Table 2 also illustrates the carotenoid content and the relative amount of each of the indivisual carotenoids. Although the color of chela showed a characteristic pattern, no remarkable difference was detected in the carotenoid composition

Table 2. Carotenoid composition of the various parts of the exoskeleton of crayfish

No.	Carotenoids	Leg	Chela		Cephalo-thorax	Abdomen & Tail
			Red	Blue		
1	β-carotene	1.6%	0.3%	0.3%	1.4%	2.0%
2	unknown	1.6%	1.6%	1.6%	3.9%	4.9%
3	echinenone	1.8%	4.7%	7.1%	4.4%	5.9%
4	astaxanthin ester	27.0%	27.7%	27.8%	28.1%	22.4%
5	unknown	3.2%	6.3%	5.8%	1.8%	1.2%
6	unknown	6.3%	4.7%	4.3%	3.5%	1.8%
7	unknown	8.1%	3.3%	11.6%	5.3%	2.9%
8	astacin	17.1%	12.5%	10.7%	15.8%	13.7%
9	phoenicoxanthin	2.7%	9.5%	6.7%	4.4%	3.9%
10	unknown	6.8%	7.6%	5.0%	4.4%	5.4%
11	astaxanthin	18.0%	19.2%	15.8%	22.8%	26.3%
12	unknown	5.9%	2.7%	3.3%	4.4%	9.8%
Carotenoid content(mg%)		12.9	18.1	16.9	26.0	28.1

comparing with the other parts.

Carotenoid distribution in blood and carotenoproteins: The carotenoid composition of blood analyzed by silica gel thin-layer chromatography was as followed; astaxanthin 65.5%, β-carotene 15.5%, astexanthin ester 3.5%, and others 15.5%. The composition of blood carotenoid showed indivisual and seasonal variations, however, astaxanthin and β-carotene were dominant in every case. The carotenoids as the prosthetic group of three carotenoproteins, blue, purple, and red carotenoproteins, isolated from the crayfish exoskeleton contained large amounts of astaxanthin and sporadic trace of other keto-carotenoids. Moreover, it was found that the carotenoid composition was identical in the three prosthetic groups as shown in Fig. 2.

DISCUSSION

The carotenoid pigments of exoskeleton were fractionated into 13 bands on thin-layer and column chromatography. The major carotenoids in every exoskeleton were astaxanthin and its ester, and their content was about 40 per cent of the total carotenoids. The astaxanthin and its ester were widely distributed in crustacean exoskeletons. It is well known that astaxanthin was the prosthetic group in the various crustacean carotenoproteins.

The oxidation pathway from β-carotene to astaxanthin in a variety of crustacea has been recognized by many authors. The presence of β-carotene

isomer in the carotene fraction and its derivatives was suggested. The absorption properties of β-carotene isomer were almost identical with those of LEE[5], and CZECZUGA & CZERPAK[10]. WOLF & CORNWELL[14] observed in a number of astaxanthin ester isomer bands on alumina column chromatography. Many unidentified bands supposed to be astaxanthin ester isomers and ketocarotenoids were separated on silica gel plate. The authors are not sure at present that the cis-isomer in the crayfish exoskeleton derives either naturally or artificially during extraction and chromatography. Whereas it is well understood that astaxanthin and its esters partially oxidized to astacin during alumina column chromatography. It may be possible that the astacin present in the exoskeleton and other tissues was either the result of partial oxidation of astaxanthin or its esters during separation, or metabolite in the pigmented layer of exosketon. Although only sporadic amounts of canthaxanthin were observed in the crayfish exoskeleton, this may be considered to be a precursor of astaxanthin from β-carotene.

The poenicoxanthin reported by KATAYAMA *et al.*[13] and MATSUNO *et al.*[15] in the exoskeleton of prown and crub, respectively, was found also in the crayfish. KATAYAMA *et al.*[12][16] reported the existence of β-doradexanthin ester in gold fish as an intermedate in the biosynthetic pathway to astaxanthin ester. The present study also suggests that thus ester might be involved in the exoskeleton of crayfish too.

The dominancy of astaxanthin in the cepharothorax, and abdomen & tail was probably due to the protein-bound carotenoids. No difference of carotenoid composition between bluish and reddish chela was observed. The variability in respect to the exoskeleton color of the crayfish might not be due to variations in carotenoid composition, but mainly to the carotenoprotein composition. On the other hand the color tone of gold fish (*Carassius auratus*) integument is influenced by the amount of astaxanthin. With an increase in astaxanthin, the reddish color increases[17]. Moreover, MIYAKE *et al.*[18] and LEE[4][5][6] observed the difference of carotenoid composition among different colored exoskeletons in crustacea.

The analysis of the carotenoid composition of the crayfish showed that no lutein was detected, although this is not uncommon in crustacea. In the hepatopancreas, however, dominantly β-carotene and a small amount of keto-carotenoids were detected[19]. This fully accords with the result presented in decapod by GILCHRIST & LEE[20]. The accumulation of some β-carotene in this organ might depend on the ingested carotenoids in diet. Since astaxanthin and other keto-carotenoids were dominant pigments in the exoskeleton, β-carotene present in the hepato-

pancreas might be converted, *via* a series of intermediates, to xanthophylls such as astacin, astaxanthin and its esters, and to protein-bound carotenoids.

It is likely that the blood acts as a transport system and temporary store of carotenoid[21]. The carotenoid constituents of the blood and the exoskeleton showed a slight similarity. Astaxanthin and β-carotene were dominant in the blood of the crayfish as mentioned above. It seemed probable that the carotenoids associate with proteins in the state of either carotenoprotein as presented by CECCALDI[22], HERRING[23], and GLLCHRIST & LEE[24] or lipoprotein which dissolves carotenoids in lipid moiety, The individual and seasonal variations in carotenoid content of blood was observed, These may be caused primarily by the carotenoids ingested as diet. In the level of the blood carotenoid of the sand crub (*Emerita analoga*) taken in various months, there appears a seasonal variation[24].

The color change of crayfish was not a transient event such as prown, especially in adults. The molting was the most important color change, and was more frequently made in young animals than in fully adult ones. The ccolorless or pale sallow thin exoskeleton of the young crayfish became densely colored as they grew older, depositing red carotenoprotein and carotenoids from the circulating system. Just after molting, the carotenoproteins from the uncalcified exoskeleton can be extracted with 0.6 *M* ammonium sulfate, while the color of highly calcified ones was extractable only by means of organic solvent. GREEN[25] suggested that the carotenoid pigment was induced to deposit in the fat body of *Daphnia* by light. Therefore the bluish carotenoproteins were suggested to be the principal pigment of the bluish exoskeleton of the crayfish, and the carotenoproteins were replaced by unbound-carotenoids deposited. It is probable that the unbound-carotenoids are concerned with the basal pigmentation of the reddish crayfish exoskeleton. However, the contribution of the red carotenoprotein could not be disregarded for the pigmentation. Therefore the color appearance of red exoskeleton in the crayfish was suggested to depend on the coexistence of red carotenoprotein and unbound-carotenoid.

The previous scientific name of crayfish, *Cambarus clarkii,* was changed to *Procmbarus clarkii* from this report.

SUMMARY

1. Astaxanthin ester, astaxanthin, and astacin were found as major pigments in the exoskeleton of crayfish.

2. Astaxanthin, which was the prosthetic group of carotenoproteins, was

found in comparatively larger quantities in the exoskeleton of cepharothorax and abdomen & tail than in those of chelae and legs.

3. A remarkable difference of carotenoid composition, however, was not found between the red and the blue chela exoskeletons.

4. The blue in the exoskeleton may depend on bluish carotenoproteins, and on the carotenoproteins replaced by unbound-carotenoids deposited as the animal older. The unbound-carotenoids seemed to be in relation with the basal pigmentation of the reddish exoskeleton.

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水産動物のカロチノプロテインに関する研究

Ⅳ アメリカザリガニのカロチノイド

中川 平介，鹿山 光
山田 久，浅川 未三

1. アメリカザリガニ甲殻のカロチノイドを薄層およびカラムクロマトグラフィーで分離同定した結果，主成分はアスタキサンチンエステル，アスタキサンチンおよびアスタシンであった．

2. 青，紫および赤色の三種のカロチノプロテインの補欠分子族はアスタキサンチンを主成分とし，それぞれのカロチノイド組成に差は認められなかった．

3. アスタキサンチンは頭胸部，尾部および尾柄部など青い部分の甲殻に比較的多く含まれる．しかし，鋏脚の濃青色部と鮮赤色部の甲殻のカロチノイド組成に殆ど差は認められなかった．

4. 甲殻の赤い色は主として非結合型カロチノイドの色により，甲殻の青い色は非結合型カロチノイドの他に青および紫色カロチノプロテインが比較的多く存在する為と考えられる．甲殻の色が成長と共に赤味を増すのは非結合型カロチノイドがカロチノプロテインと置換して沈着する為と考える．