J. Fac. Fish. Anim. Husb.,
Hiroshima Univ. (1975), 14 : 61～67

Studies on Carotenoprotein in Aquatic Animals V. Thermal Reddening of Exoskeleton of Crayfish (*Procambarus clarkii*)

Heisuke NAKAGAWA, Mitsu KAYAMA and Suezo ASAKAWA

Department of Fisheries, Faculty of Fisheries and Animal Husbandry, Hiroshima University, Fukuyama

(Figs. 1–2, Table 1)

Many explanations have been presented on the relation between color variation and protein structure[1～9]. It is well known that an hypsochromic shift of absorption maximum of bluish carotenoprotein occurs in the presence of certain denaturation reagents, by dehydration, in the state of low ion concentration or by heating[1,2,5,7,10～12]. The thermal alteration of absorption maximum of carotenoprotein was reversible when carotenoprotein was heated lower than 60°C[10,13]. When a crustacean is cooked in hot water, the exoskeleton irreversibly turns to red color from the original tint. There is a widely difused belief that heat splits the link of carotenoid-protein complex, and liberates the prosthetic group, astaxanthin, which is successively turned to astacin by oxidation[13].

However, little is known about the mechanism of the thermal denaturation of carotenoproteins. The carotenoid-protein interaction which is responsible for the spectral shift was briefly discussed and a possible mode of reddening the bluish carotenoprotein by thermal treatment is suggested in this report.

EXPERIMENTAL AND RESULTS

Material The crayfish kept in a deep-freeze cabinet were dissected. The exoskeleton was separated from muscle and epidermis as much as possible. A batch of the exoskeleton cut into small pieces was used for the analysis.

(I) Microscopic appearance of integument

The postmoult stage with relatively thin exoskeleton was selected. For sectioning the abdominal integument of that had a dark color appearance, blue was used. A piece of the integument was boiled in distilled water for 10 minutes at 100°C. The integument

was cut directly in 10 μ thickness as transverse section of the integument. Then the pigment distribution in the strata was compared with that of the original integument. The stratification in this study accorded with that of thick-calcified exoskeleton of decapoda[14]. Four main strata were observed structurally. They were epicuticle, exocuticle, endocuticle in the original integument were densely pigmented. After boiling the integument did not show a clear defined pigmented layer. Although the dark pigmented layer was restricted to the epicuticle and exocuticle of the original integument, a relatively thick layer between epicuticle and endocuticle turned uniformly orange in color after boiling.

The easiness of carotenoid extraction with acetone was compared in a piece of abdominal exoskeleton before and after boiling by means of the microscopic technique. When a piece of the original exoskeleton was extracted with acetone for overnight, a fairly strong amount of pigment remained in the pigmented strata. However, in the boiled exoskeleton the pigment was found to be completely removed.

(II) Carotenoid analysis

The exoskeleton was heated under the following conditions; boiled in water at 100°C during 5, 10, and 60 minutes, and heated on a flame at 170°C ± 10°C during 30 minutes. Carotenoids were extracted repeatedly from the pulverized exoskeleton with acetone until no further pigment could be obtained. The acetone extracts were combined. The carotenoids were transferred to petroleum ether to the extracts. The petroleum ether layer was repeatedly washed with water until freed completely of acetone, dried over anhydrous sodium sulfate, then concentrated under nitrogen stream. The carotenoid content of exoskeleton was based on the specific extinction coefficient of the extract at 470 nm in petroleum ether, 2,000 as the $E^{1\%}_{1\,cm}$. The carotenoids from the variously treated exoskeleton were extracted with acetone, then the remained carotenoids were successively extracted with the mixture of methanol and chloroform (1:1 v/v). Most of carotenoids were extracted with acetone. The remaining amount of carotenoids after the acetone-extraction process corresponded to 1.2 per cent of the total value in the original exoskeleton; to 0.2–0.3 per cent in the boiled exoskeletons, and lower than to 0.002 per cent in the exoskeleton heated at 170°C. Fig. 1 indicates that the boiling of exoskeleton gave a better extraction of carotenoids. The quantity of the extracted carotenoids seemed to be reverse proportional with the boiling time at 100°C. The results of the chemical analysis accorded with those of the microscopic observation. The sudden decrease of carotenoid content caused by heating at 170°C obviously points to a decomposition of carotenoids. Apparent descoloration was observed in the thus treated exoskeleton.

The extract with acetone was applied for thin-layer chromatography of silica gel G (E. Merck, Darmstadt) plate, about 0.25 mm thickness; solvent system of petroleum ether-ethylacetate (80:20 v/v). The chromatograms of carotenoids from the native and heated exoskeletons are shown in Fig. 2. Percentage composition of carotenoid

Fig. 1. Carotenoid content of chela exoskeleton after heating by several conditions.
A : Initial
B : Boiled for 5 minutes
C : Boiled for 10 minutes
D : Boiled for 60 minutes
E : Heated for 30 minutes at 170°C

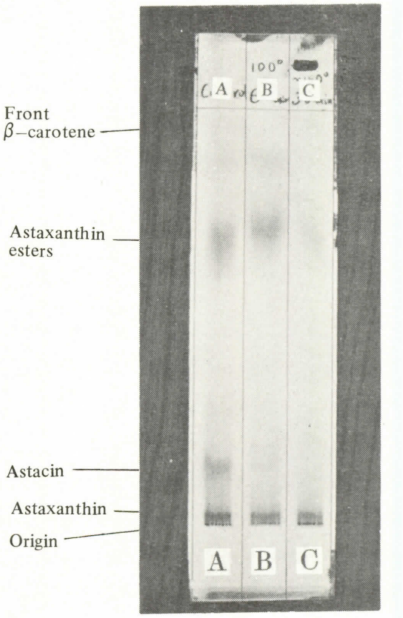

Fig. 2. Thin-layer chromatograms of the carotenoids extracted from chela exoskeletons before and after heat-treatments.
A : Initial
B : Boiled for 60 minutes at 100°C
C : Heated for 30 minutes at 170°C

was determined by measuring the color density of separated carotenoid on a thin-layer plate by an Ozumor Densitometer 82 with a filter No.52 (520 nm). The carotenoid composition is given as percentage of the total carotenoids (Table 1). The proportional changes in astacin and astaxanthin were found also in the case of heating at 170°C. As Table 1 shows, no proportional change of astaxanthin esters was observed after these treatments. Residual carotenoids extracted with the mixture of methanol and chloroform were found to consist of low R*f* components such as astaxanthin.

Table 1. Composition of carotenoid extracted from chela exoskeleton before and after heat-treatments.

No.		Initial	100°C 10 mins.	170°C 30 mins.
1	β–carotene	1.0%	0.8%	1.1%
2		3.1%	2.5%	2.7%
3	Echinenone	7.5%	9.7%	9.9%
4	Astaxanthin esters	21.7%	18.9%	19.8%
5		6.2%	10.1%	6.2%
6		5.8%	5.5%	3.4%
7		9.1%	10.5%	7.0%
8	Astacin	12.3%	11.3%	4.0%
9	Phoenicoxanthin	3.2%	2.9%	5.0%
10		8.1%	6.3%	11.0%
11	Astaxanthin	17.5%	15.6%	24.1%
12		4.5%	5.9%	5.7%

DISCUSSION

True carotenoproteins, in which carotenoids are present in stoichiometric proportions as prosthetic group, were justified by CHEESMAN *et al.*[2]. Mutual stabilization of the carotenoid and protein is one of the functions of the carotenoid-protein complex. It has been believed that striking color change of exoskeleton by boiling is caused by the splitting of astaxanthin from the blue carotenoprotein. When the blue and purple carotenoproteins obtained from crayfish exoskeleton were warmed in water at a temperature lower than 60°C, they exibited a type of reversible denaturation. On cooling off, the reddish color returned to its original color.

Assuming that the heat split astaxanthin-protein linkage and successively caused oxidation of astaxanthin to astacin, the proportion of astacin should have increased in the exoskeleton after heat-treatment. The experimental results obtained were, however, completely opposite to this assumption, showing an evident decrease of astacin after heat-treatment. Since the carotenoid was extracted rather easily from the boiled exoskeleton by means of water miscible solvent acetone, it seemed that the dissociation of carotenoid from the complex occurred by heat-treatment. No difference in carotenoid amount was observed before and after boiling in the case of extraction with hydrophobic solvent such as petroleum ether. The purified bluish carotenoproteins from crayfish exoskeleton turned into red color accompanied by an enlargement of the molecules[12]. The resulted red carotenoprotein was partially restored back after having been kept in a cold room for long period, as reported previously[12]. However, heat-treatment gave some carotenoid-free proteins. The results of electrophoretic and molecular size determinations suggested that these were apoproteins. The process gave no astacin, although astacin is also capable of yielding a carotenoid-protein complex[3]. Astaxanthin in crayfish exoskeleton seemed to be stable. There was no proportional increase of astacin in the exoskeleton after exposuring to daylight. However, astacin seemed to decompose

more preferencially than astaxanthin by heat-treatment. Hence cleavage of carotenoid-protein link leads to a considerable increase in oxidation sensitivity of carotenoid. The result that astaxanthin in the heated exoskeleton was more stable than astacin suggested the existence of a reddened carotenoid-protein complex in the heated exoskeleton. The color change of exoskeleton by heat-treatment presumably depended on the reddened carotenoid-protein complex.

The red carotenoprotein resulted from boiling was neither extracted from the powder of boiled exoskeleton with 0.6 *M* ammonium sulfate nor with dilute sodium hydroxide solution. KUHN & SÖRENSEN[15] did not succeed in extracting such a heat-treated carotenoid-protein complex which they believed to be held by calcium deposits, from the exoskeleton. The reddening of carotenoprotein by boiling did not only originate from the rupture of the carotenoid-protein complex, but also from certain structural modifications of the protein molecule or the carotenoid-protein linkage, as reported previously[16]. It has been suggested that a carotenoid-protein interaction may occur between carotenoid keto groups and basic residues of the protein in such a way as to provide a lock on the tertiary configuration[6,17]. It might be presumed that partial liberation or decomposition of the prosthetic group carotenoid by heating would lead to the enlargement of the protein molecule with color change.

These results indicate the potenciality that carotenoid-rich waste material, reddened exoskeletons of cooked crustaceans can be used for cultivation of fish whose pigmentation is important economically as diet[18].

There are pigmented layers defined clearly as outer exocuticle in the section of integument. The reddish pigmented layer is spread within the colorless strata by boiling. Colorless exoskeleton of juvenile crayfish changed to red color by heating or freeze-dry. These results suggested the presence of colorless carotenoprotein. The presence of such carotenoprotein had been suggested in prown by FOX[19]. CHEESMAN[6] had already prepared a colorless mixture of denatured ovorubin and native crustaceanin. Colorless carotenoprotein of crayfish seemed, *in vitro,* to be labile under conditions of light, heat, and dehydration. The reason is the rapid coloring under those treatments. Such colorless carotenoprotein might act as a precursor of pigmented carotenoproteins. The carotenoids were probably conveyed to the exoskeleton in the form of carotenoprotein or lipoprotein with blood, that besides astacin included all of the carotenoids[20].

SUMMARY

The carotenoid composition of crayfish exoskeleton before and after heat-treatments was determined in order to explain the mechanism of thermal denaturation of carotenoprotein.

(1) The reddening of exoskeleton by boiling resulted not only from the splitting of carotenoid-protein link, but also from some structural modifications of the protein.

(2) There was no evidence of the yield of astacin caused by heating of exoskeleton.

(3) Violent heating decomposed astacin more heavily than other carotenoids in the exoskeleton.

REFERENCES

1) JENCKS, W. P. and BUTEN, B: *Arch. Biochem.*, **107**, 511–520 (1964).
2) CHEESMAN, D. F., ZAGALSKY, P. F., and CECCALDI, H. J.: *Proc. Roy. Soc.*, B, **164**, 130–151 (1966).
3) LEE, W. L. and ZAGALSKY, P. F.: *Biochem. J.*, **101**, 9c–11c (1966).
4) KUHN, R. and KÜHN, H.: *Angewandte Chemie*, **5**, 957 (1966).
5) KUHN, R. and KÜHN, H.: *European J. Biochem.*, **2**, 349–360 (1967).
6) CHEESMAN, D. F., LEE, W. L., and ZAGALSKY, P. F.: *Biol. Rev.*, **42**, 132–160 (1967).
7) BUCHAWALD, M. and JENCKS, W. P.: *Biochem.*, **7**, 844–859 (1968).
8) ZAGALSKY, P. F., CECCALDI, H. J., and DAUMAS, R.: *Comp. Biochem. Physiol.*, **34**, 579–607 (1970).
9) ZAGALSKY, P. F. and HERRING, P. J.: *Comp. Biochem. Physiol.*, **41B**, 397–415 (1972).
10) WALD, G., NATHANSON, N., JENCKS, W. P., and TARR, E.: *Biol. Bull.*, **95**, 249–250 (1948).
11) HERRING, P. J.: *Comp. Biochem. Physiol.*, **39B**, 1039–1043 (1971).
12) NAKAGAWA, H., KAYAMA, M., and ASAKAWA, S.: *J. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **11**, 129–139 (1972).
13) FOX, H. M. and VEVERS, H. G.: in "The Nature of Animal Colours", Ist ed., pp. 62–80, Sidgwick and Jackson, London (1960).
14) DALL, W.: *Aust. J. Mar. Freshw. Res.*, **16**, 13–23 (1965).
15) KUHN, R. and SÖRENSEN, N. A.: *Ber. deutsh. chem. Gesel.*, **71B**, 1879–1888 (1938).
16) NAKAGAWA, H., KAYAMA, M., and ASAKAWA, S.: *J. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **12**, 21–30 (1973).
17) THOMMEN, H.: in "Carotenoids" (OTTO, I. ed.), Ist ed., pp. 637–668, Birkhäuser Verlagbasel und Stuttgart, (1971).
18) KAYAMA, M., NAKAGAWA, H., YAMADA, H., and MURAKAMI, Y.: *J. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **12**, 49–59 (1973).
19) FOX, H. M.: *Endeavour*, **14**, 95–104 (1955).
20) NAKAGAWA, H., KAYAMA, M., YAMADA, H., and ASAKAWA, S.: *J. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **13**, 1–13 (1974).

水産動物のカロチノプロテインに関する研究
V. アメリカザリガニ甲殻の加熱赤変について

中川　平介・鹿山　光・浅川　末三

甲殻を種々の条件下で加熱し，カロチノイド含量，及び組成の変化を調べ，甲殻の赤変との関係について論じた。

甲殻の赤変はカロチノイドと蛋白質の結合の解裂によってのみ生ずるものではなく，なんらかの蛋白質部分の変化による色素蛋白質の赤変によっても生ずると考えられる。

甲殻を加熱することによりアスタシンの増加は認められず，他のカロチノイド成分より優先的に分解を受けることを認めた。