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# Characterization of a Carotenoprotein from the Carapace of the Crab *Macropipus puber*

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An orange carotenoprotein ($\lambda_{max}$ = 480 nm) containing astaxanthin as prosthetic group was extracted and purified from the carapace of the crab *Macropipus puber*. The extraction was made possible by means of Triton X-100, yielding an orange carotenoprotein, with a molecular weight of about 56,000. Sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated only a single polypeptide of 14,000 daltons, suggesting that the orange caroteno-protein was a tetrameric form.

**Key words:** Carotenoprotein, *Macropipus puber*, Astaxanthin.

One of the ways in which carotenoids most frequently occur in animals, is bound to proteins constituting a kind of complexes so called carotenoproteins, which are widespread in the Animal Kingdom, especially in Marine Invertebrates. Crustacean carotenoproteins are the most studied ones to date (13).

These complexes have three main functions: protective coloration, stabilization of carotenes and proteins and, as a result, storage of nutrients (proteins and lipids) during the development of the eggs.

Apart from their physiological and ecological significance carotenoproteins are extremely interesting in connection to studies on visual pigments (rhodopsin, the human visual pigment, is in fact a carotenoprotein) (3) and studies of protein-small molecule interactions (4).

Carapaces are the major source from which carotenoid-protein complexes are extracted. The usual procedure to accomplish the extraction is by prolongued stirring of the pulverized carapace with a buffered solution (2, 16), but it is known that while extraction using buffer is easy in the case of crayfish, lobster and prawns (2, 10, 16, 20), is proved to be difficult in crabs. Attemps to achieve the isolation of carotenopro-

teins from carapaces of crabs failed or gave a poor yield of carotenoprotein solubilization.

In the present paper, a procedure for the extraction of the *Macropipus puber* carapace carotenoprotein involving the use of the nonionic detergent Triton X-100 is reported. The orange carotenoprotein so obtained is fully characterized.

## Materials and Methods

*Macropipus puber* specimens were bought at the local fish-market, brought alive to the laboratory and stored frozen until required.

The carotenoprotein was isolated from the carapace by the following method: carapaces were dissected, washed free from adhering tissue under cold running water, ground to a fine powder and homogenized overnight with four volumes of 5 % Triton X-100 (v/v) in 0.05 M phosphate buffer pH = 7.0, in the cold and in diffused light. The homogenate was then centrifuged at 10,000 × *g* for 15 min. This extraction was performed until the pellet was almost completely colourless. Solid ammonium sulfate was added to the total extracted material to give 20 % saturation. After 24 hours, the extract was centrifuged at 10,000 × *g* for 15 min, and the orange precipitate was removed and dissolved in 0.05 M Tris-HCl buffer, pH = 8.0.

The carotenoid-protein complex was chromatographed on a 2.3 × 90 cm column of Sephadex G-200 (Pharmacia), equilibrated and eluted with the 0.05 M phosphate buffer pH = 8.0. Fractions with maximal absorption at 480 nm were collected, dialyzed against 0.02 M Tris-CHl, pH = 8.0 buffer and then adsorbed on to a 1.7 × 3.5 cm DEAE cellulose (DE-52 Whatman) column. Elution was carried out with a linear gradient of sodium chloride (2 × 200 ml, 0-0.8 M) in the same buffer.

Purification was monitored by continuous disc gel electrophoresis using the method of DAVIES (8). Protein samples (80-100 μg) in 0.05 M Tris-HCl pH 8.8 buffer; containing 20 % glycerol, 0.01 % bromophenol blue, were applied to 10 % w/v polyacrylamide rod gels with 0.27 % w/v N,N'-methylenebisacrylamide. 0.05 M Tris-HCl, pH = 8.8 for separating gel and 0.02 M Tris-glycine, pH = 8.8 as electrode buffer were used. Gels were electrophoresed at 3 mA/tube. After running, the gels were stained with Coomassie brilliant blue R250 (0.25 % in acetic acid-methanol-water, 9:45:46) overnight. Destaining was achieved by diffusion into acetic acid-methanol-water 10:20:70.

All steps of isolation and purification were carried out at 4° C.

Determination of the molecular weight of the complex was done by gel filtration on a Sephadex G-200 column as described above, with aldolase (149,000), bovine serum albumin (62,000), egg albumin (44,000) and lysozyme (14,000) as markers.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was carried out essentially by the FAIRBANKS method (9). Protein samples (80-100 μg dissolved in the separating gel buffer, containing 20 % glycerol, 0.25 % SDS and 0.01 % bromophenol blue were incubated over a boiling water bath for 2 minutes and then applied to the 5.6 % w/v polyacrylamide rod gels with 0.21 % w/v N,N'-methylenebisacrylamide. After preelectrophoresis of the gels for 30 minutes, the samples were applied and run at 8 mA/tube using as separating gel and electrode buffer the one composed of 1 % SDS, 0.02 M EDTA, 0.004 M Tris and 0.02 M sodium acetate, adjusted to pH = 7.4 with acetic acid. Gels were stained and destained as described above. Proteins

bands were assayed quantitatively by photometric scanning on a Beckman gel Scanner. The proteins employed as standards were: ribonuclease A (13,700), β-lactoglobulin monomer (17,500), trypsin (24,500) egg albumin (44,000) and bovine serum albumin (62,000) (molecular weights according to DARNALL and KLOTZ, (7).

The purified carotenoprotein was tested for the presence of N-acetyl-glucosamine (17), for the presence of non amine sugars (15) and for the presence of lipids other than carotenoids (19).

The carotenoid was extracted from the protein by addition of acetone (14). Identification of carotenoids was made by comparison with a range of authentic carotenoids standards by means of thin layer chromatography in the system hexane-acetone (75:25)/Silicagel G, 0.02 M citric acid; and by absorption spectra measured with a Beckman Spectrophotometer UV-5260.

The extraction and hydrolysis of the carotenoids from the carapace was made as previously described (11).

The carotenoid-protein ratio was determined by using the Biuret method to estimate the protein content and by using the molar extinction coefficient for astaxanthin of 124,000 (1) to calculate spectroscopically the carotenoid content.

## Results

The orange carotenoprotein obtained at the end of the purification procedure, showed an only band on polyacrylamide gel electrophoresis by Davies method, indicating the purity of the sample.

The carotenoprotein isolated was orange in colour. The extinction measurement in a phosphate buffer pH = 7.0 gave two maxima; the first and highest maximum at 291 nm, and the second one at 480 nm.

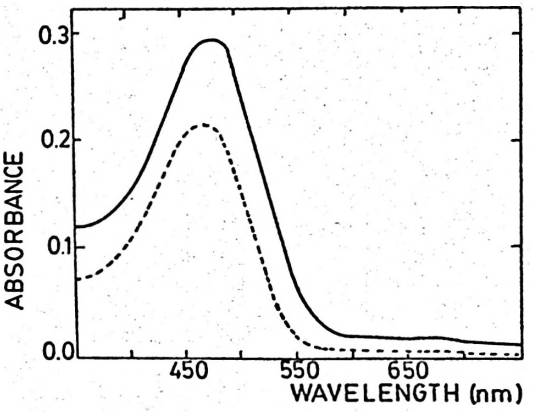


Fig. 1. *Comparative visible absorption spectra of the orange carotenoprotein in buffered solution (solid line) against its carotenoid astaxanthin, in ethereal solution (dashed line) showing a shift from 480 nm to 468 nm.*

The protein analysis by filtration on Sephadex G-200 showed a molecular weight of 56,000. A yield of 3.41 mg carotenoprotein per g dry exokeleton was found as a final result of the extraction and purification process.

The carotenoid-protein complex contained 0.885 mg lipids per mg protein, estimated by the method of SANTIAGO *et al.* (19), and 0.067 μg N-acetyl-glycosamine per mg protein, estimated by the REISSING-LELOIR procedure (17). Non-amine-sugars tested by the method of PAYNE-LATOUR (15) were not detected.

Finally, by SDS gel electrophoresis, a molecular weight of 14,000 was obtained, and by filtration on Sephadex G-100 in the presence of SDS, the same molecular weight was obtained: 14,000. These results suggest that the orange protein is a tetrameric form.

The carotenoid was released from the carotenoprotein by extraction with acetone. This carotenoid showed a shift to 468 nm in ethereal solution (fig. 1) and the characteristic shape of keto-carotenoids.

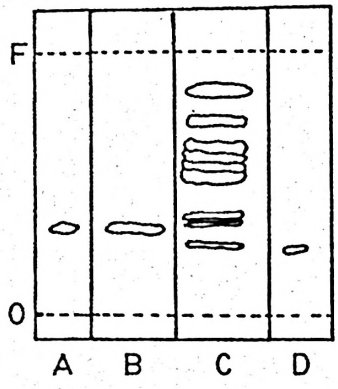


Fig. 2. *Thin layer chromatography of the carotenoid from the carapace of* Macropipus puber *in native state (C) and hydrolyzed (B), against standards of astacene (A) and astaxanthin (D).*

The carotenoid could not be distinguished from the authentic astaxanthin standard by means of spectroscopic analysis in several solvents (hexane = 466 nm; $S_2C$ = 502 nm; pyridine = 490 nm) and co-chromatography on thin layer chromatography. The amount of astaxanthin assayed spectroscopically per mg of protein was 0.187 $\mu$g.

The carotenoids extracted from the carapace of the crab were examinated by thin layer chromatography, giving several bands (fig. 2) which after hydrolysis appeared as an only band, showing spectroscopically the maxima in different solvents (hexane = 470 nm; $S_2C$ = 507 nm; pyridine = 490 nm) identical to the astacene standard, and in addition, it could not be separated from the astacene standard by thin layer chromatography (fig. 2).

## Discussion

Triton X-100 has shown to be an effective agent to accomplish the carotenoprotein extraction. The detergent that could effect the characterization of the carotenoprotein is eliminated by passing the sample through the Sephadex G-200 column, as it has been demonstrated (18).

It is noteworthy that after extraction of the carapace with 5 % Triton X-100, the pellet exhibited small blue pieces, what indicated the remaining of unsolubilized blue carotenoproteins (which turned to red after acetone treatment) in spite of such a drastic procedure of isolation. This carotenoprotein would account for the change in colour noted after boiling the carapace.

Carotenoproteins in which the protein strongly interacts with the carotenoids, presumibly by increasing the polarization of the molecule, giving rise to a bathochromic shift from the wavelength of the free carotenoid (usually orange coloured with $\lambda_{max}$ around 470 nm) to the 500-600 nm region (green, purple, and blue colour) occur more frequently. However, some orange-red carotenoproteins have been previously described from different sources, such as the eggs of the marine snail *Pomacea caniculata* (5), the carapace of the crayfish *Orconectes limosus* (6), and the cyanobacteria *Spirulina maxima* (12). In all these cases, the bathochromic shift is small, but easily detected.

The high lipid content of the orange carotenoprotein explains the difficulty of using buffered solutions as extracting agents, and the requirement of a detergent as Triton X-100 to solubilize such an hydrophobic complex.

Having been elucidated the presence of astaxanthin as prosthetic group of the carotenoprotein, as it was mentioned above, the carotenoid content in the whole carapace was studied, concluding from the appearance of an only band on thin layer chromatography after hydrolysis identified as astacene, the oxidative product of the alkaline hydrolysis of astaxanthin esters, that astaxanthin is present in the carapace of *Macropipus puber* as the main carotenoid which is

found either free and in esterified form, mostly bound to proteins constituting orange carotenoid-protein complexes.

## Resumen

Se extrae y purifica del caparazón de la nécora *Macropipus puber* una carotenoproteína naranja ($\lambda_{max}$ = 480 nm) cuyo grupo prostético es la astaxantina. La utilización del Triton X-100 permite la extracción de dicha carotenoproteína, cuyo peso molecular aproximado es de 56.000. La electroforesis en gel de poliacrilamida con SDS muestra una única banda correspondiente a una cadena polipeptídica de 14.000 daltones, lo cual sugiere que la carotenoproteína naranja está constituida por 4 monómeros.

## References

1. BUCHWALD, M. and JENCKS, W. P.: *Biochemistry*, **7**, 834-843, 1968.
2. BUCHWALD, M. and JENCKS, W. P.: *Biochemistry*, **7**, 844-859, 1968.
3. CALLENDER, R. and HONIG, B.: *Ann. Rev. Biophys. Bioeng.*, **6**, 33-55, 1977.
4. CAREY, P. R., CARRIERE, R. G., PHELPS, D. J. and SCHENEIDER, H.: *Biochemistry*, **17**, 1081-1087, 1978.
5. CLARK, R. J. H., D'URSO, N. R. and ZAGALSKY, P. F.: *J. Am. Chem. Soc.*, **102**, 6693-6698, 1980.
6. CZECZUGA, B. and KRYWUTA, S.: *Comp. Biochem. Physiol.*, **68B**, 339-343, 1981.
7. DARNALL, D. W. and KLOTZ, I. M.: *Arch. Biochem.*, **149**, 1-14, 1972.
8. DAVIES, B. J.: *Ann. N. Y. Acad. Sci.*, **121**, 404-427, 1964.
9. FAIRBANKS, B., STECK, T. L. and WALLACH, D. F. H.: *Biochemistry, N. Y.*, **10**, 2606-2617, 1971.
10. GAMMACK, D. B., RAPER, J. H., ZAGALSKY, P. F. and QUARMBY, R.: *Comp. Biochem. Physiol.*, **40B**, 295-300, 1971.
11. GÓMEZ, R., GOÑI, F. M. and MACARULLA, J. M.: *Rev. esp. Fisiol.*, **34**, 253-256, 1978.
12. HOLT, T. K. and KROGMANN, D. W.: *Biochem. Biophys. Acta*, **637**, 408-414, 1981.
13. LEE, W. L.: Carotenoproteins in animal coloration. Dowdens, Hutchinson and Ross. Stroudsburg, 1977.
14. POWLS, R. and BRITTON, G.: *Biochem. Biophys. Acta*, **453**, 270-276, 1976.
15. PAYNE, H. W. and LATOUR, J. P.: *J. Endocrynol. Metab.*, **15**, 106-114, 1955.
16. QUARMBY, R., NORDEN, D. A., ZAGALSKY, P. F., CECCALDI, H. J. and DAUMAS, R.: *Comp. Biochem. Physiol.*, **56B**, 55-61, 1977.
17. REISSIG, J. L., STROMINGER, J. L. and LELOIR, L. F.: *J. Biol. Chem.*, **217**, 959-966, 1955.
18. ROTTEM, S., STEIN, O. and RAZIN, S.: *Arch. Biochem. Biophys.*, **125**, 46-50, 1968.
19. SANTIAGO, E., MULE, S., REDMAN, H., HOKIN, M. R. and HOKIN, L. E.: *Biochim. Biophys. Acta*, **84**, 550-562, 1964.
20. ZAGALSKY, P. F.: *Comp. Biochem. Physiol.*, **71B**, 243-247, 1982.