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A CAROTENOID-PROTEIN FROM CYANOBACTERIA

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Easily solubilized carotenoid-containing proteins have been found in aqueous extracts from three genera of cyanobacteria. The three proteins have been purified, and the absorption spectra have been determined to be virtually identical with absorption maxima at 495 and 465 nm. During the purification the orange protein spontaneously changed to a red protein with a single, broad absorption maximum at 505 nm. The orange protein showed a molecular weight of 47 000 on gel filtration while that of the red protein was 26 700. Sodium dodecyl sulfate polacrylamide gel electrophoresis indicated a single polypeptide of M_r 16 000 in both the red and orange forms, but this method removed the chromophore from the proteins. The main carotenoid component of the complex was determined to be 3′-hydroxy-4-keto-ββ-carotenoid or 3′-hydroxyechinenone. The number of carotenoid molecules per molecule of orange protein of molecular weight 47 000 was between 20 and 40. The stoichiometry of carotenoid to protein seemed reasonably constant.

Introduction

This paper describes an unusual water-soluble orange pigment found during the purification of photosynthetic catalysts from cyanobacteria. This pigment behaves like other proteins, yet its absorption spectrum is like that of a carotenoid. The properties of this carotenoid-protein are distinct from those of the bulk of the carotenoids found in cyanobacteria. In very crude extracts of some cyanobacteria, there is a yellow, opalescent fraction which contains traces of chlorophyll with little or no protein. This yellow material passes through ion-exchange cellulose columns and is driven to the surface of aqueous extracts by prolonged high-speed centrifugation. This may correspond to the osmiophilic globules which are produced in other photosynthetic systems [1]. In contrast, the carotenoid-protein described in this paper is orange but transparent even at very high concentrations and may be chromatographed on DEAE-cellulose columns. While a carotenoid-protein complex can be obtained after organic solvent extraction of photosynthetic membranes as in the preparation of cytochrome *f*, much of the material tends to be insoluble or very difficult to solubilize in aqueous media [2]. Ke [2] concluded that many of these β-carotene-proteins with red-shifted absorption spectra are artifacts of the preparation not originally present in the photosynthetic membrane. In contrast, the carotenoid-protein described here is obtained without exposure to organic solvents, and the protein releases the carotenoid on exposure to ethanol or acetone. Powls and Britton [3] describe a carotenoprotein isolated from *Scenedesmus obliquus* D_3 under conditions which involve neither organic solvent nor detergent treatment. This carotenoprotein contains violaxanthin as its major chromophore, and carotenoid is present in a ratio of 1 : 1 with the protein of molecular weight 140 000. The carotenoid-protein from cyanobacteria has a higher ratio of carotenoid to protein, a different carotenoid and a smaller polypeptide.

Abbreviation: SDS, sodium dodecyl sulfate.

Materials and Methods

Spirulina maxima was acquired at Lake Texcoco, Mexico, from the commercial culture of that cyanobacterium maintained by Sosa Texcoco Co. with generous help from the staff of that company. *Aphanizomenon flos-aquae* was obtained from Lake Okoboji, IA, and *Microcystis aeruginosa* from Lake Kegonsa, WI. All three samples were homogenous as indicated by unambiguous amino acid sequences of cytochrome c_{553} isolated from them. The cyanobacteria were stored at −20°C until needed. 4 l of drained, frozen cells were thawed, diluted to 10 l with deionized water and refrozen. The freeze-thaw cycle was repeated three times to break open the cells. All subsequent steps were carried out at 4°C. Solid $(NH_4)_2SO_4$ was added to the solution to give a concentration of 20% of saturation. A green precipitate containing most of the photosynthetic membranes was allowed to settle out, and the supernatant fluid was siphoned off. Solid $(NH_4)_2SO_4$ was added to bring the solution to 45% of saturation. After 48 h, a dark-blue precipitate had settled out, and the supernatant fluid was removed and brought to 100% of $(NH_4)_2SO_4$ saturation. After 72 h, the precipitate was collected and dialyzed against three changes of 10 l of deionized H_2O. The dialysate was centrifuged at 27 000 × *g* for 5 min, and the precipitate was discarded. Whatman DEAE-cellulose (DE 22) was added to the supernatant fluid until the liquid became yellow and transparent. The DEAE-cellulose was suspended in cold, deionized water, poured into a 5 × 20 cm column and washed with 10 mM phosphate, pH 6.4, until the effluent was colorless. The column was washed with 500 ml of 0.05 M NaCl in 10 mM phosphate, pH 6.4 and then with 500 ml of 0.1 M NaCl in the same buffer. The last solution eluted the orange carotenoid-protein. The orange protein solution was diluted 10-fold with deionized water and adsorbed to a 3 × 5 cm column of DEAE-cellulose (DE 23) equilibrated with 10 mM phosphate buffer, pH 6.4. The orange protein was eluted in concentrated form with 0.5 M NaCl. The protein was chromatographed on a 4 × 80 cm column of Sephacryl 200 (Pharmacia) equilibrated and eluted with 100 mM phosphate buffer, pH 5.8. Two distinct bands appeared, an orange fraction preceding a red fraction. If the orange fraction was reloaded onto the Sephacryl 200 column, orange and red bands appeared again. The orange protein was adsorbed to a 1.5 × 5 cm DEAE cellulose (DE 52) column, eluted in a gradient of 0–0.2 M NaCl in 10 mM phosphate buffer, pH 5.8, dialyzed against distilled water, and lyophilized. Lyophilization in the presence of salt produced insoluble protein.

Determination of molecular weights of orange and red carotenoid-protein complexes from *S. maxima* was done by gel filtration on Sephadex G-75 Superfine. The column (1.5 × 90 cm) was equilibrated and eluted with 25 mM phosphate, pH 6.4, containing 100 mM NaCl. The operating pressure was 50 cmH_2O and the flow rate was 8 ml/h. The orange and red proteins could be seen as they eluted from the column. Blue dextran and horse heart cytochrome *c* were used to calibrate the column.

Acetone at room temperature, when added to a solution of either the orange or red form of the carotenoid-protein, solubilized the carotenoid while denaturing the protein. The aqueous acetone extract was evaporated under a stream of nitrogen, and the carotenoid was redissolved in a minimum volume of dry acetone and diluted 100-fold with hexane for chromatography. A 50 : 50 mixture of diatomaceous earth (Hyflo Super Cel, Johns Manville) and silica gel G (Brinkmann Instruments) was blended for 1 h in a Waring Blendor and poured into a 1.5 × 23 cm column connected to a vacuum filtration device (Fisher Scientific Co. 'Filtrator') under full vacuum. The adsorbent was packed to a depth of 9 cm, then 1 cm of anhydrous Na_2SO_4 was placed on top and hexane was added to saturate the column. The carotenoid solution was poured into the column, and the flow rate was controlled by a valve on the vacuum line. The column was washed with hexane and eluted with 100-ml portions of hexane/acetone mixtures in the ratios of 99 : 1, 97 : 3, 96 : 4, 90 : 10 and 80 : 20 at a flow rate of 5 ml per min.

Absorption spectra were measured with a Cary 14 spectrophotometer.

Results

The absorption spectra of the orange and red carotenoid-proteins of *S. maxima* are shown in Fig. 1 and are essentially the same as the spectra of these proteins from *M. aeruginosa* and *A. flos-aquae*. The

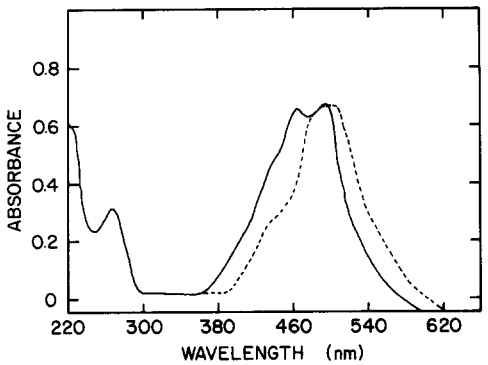


Fig. 1. Absorption spectra of orange (———) and red (- - - - -) forms of carotenoid-protein complex from *Spirulina maxima*. Samples which eluted from a Sephacryl S-200 column were used to obtain the spectra.

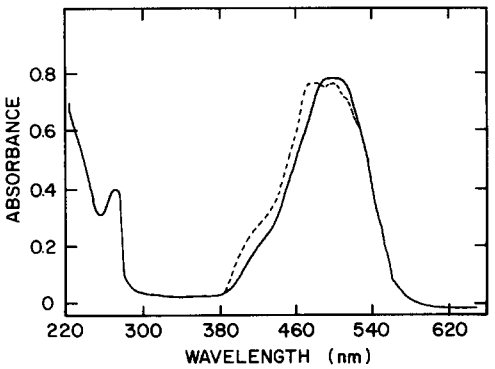


Fig. 3. Absorption spectra of red form of carotenoid-protein complex before (———) and after (- - - - -) reduction with $NaBH_4$.

orange protein shows maxima at 494, 465 and 275 nm with a slight shoulder at 440 nm. A_{495}/A_{275} is used as an index of purity and is 1.8 for a number of preparations. The absorption spectrum of the red form shows that the two maxima of the orange form are shifted to longer wavelengths and merged into a single broad peak with a maximum at 505 nm. The carotenoid prosthetic groups of both the orange and red forms are susceptible to $NaBH_4$ reduction, which causes slight shifts to lower wavelengths in the spectra of both forms (Figs. 2 and 3).

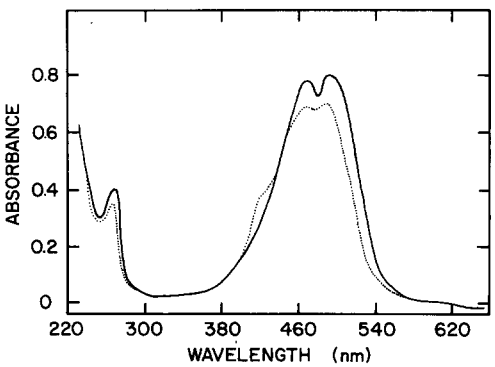


Fig. 2. Absorption spectra of orange carotenoid-protein complex from *S. maxima* before (———) and after (· · · · · ·) reduction of the carotenoid with $NaBH_4$. To an aqueous solution of the carotenoid-protein complex was added an excess of $NaBH_4$. The mixture was incubated for 15 min. The reaction mixture was passed through a Sephadex G-25 column to remove the $NaBH_4$ from the protein. The spectrum of the protein was then determined.

The orange form changes to the red form when the preparation is stored at either 25 or 4°C. Freeze-thawing the preparation accelerates the transition. The orange form appears to be quite stable at pH 5–6, but at pH 6.5 and above the red form appears rapidly. The presence of EDTA and phenylmethylsulfonyl fluoride has no effect on the orange-to-red transition, suggesting that the change is not induced by protease. We have no evidence for the conversion of the red form to the original orange form. Repeated freeze-thawing of the red form causes the appearance of a new orange form with an absorption maximum at 450 nm which is similar to that of the carotenoid dissolved in hexane. Both the red and orange forms are very sensitive to agitation in solution and form clumps which can be dispersed by SDS. This detergent, like repeated freeze-thawing, shifts the absorption maxima to 450 nm. When the carotenoid-protein is subjected to SDS-polyacrylamide gel electrophoresis, the carotenoid is separated from the polypeptide.

The molecular weights of the orange and red forms of the carotenoid-protein were determined by gel filtration on a Sephadex G-75 column. The orange form had a molecular weight of 46 700 and the red form 26 700. The orange and red proteins were the only proteins detected by monitoring the column effluent with an ultraviolet detector.

SDS-polyacrylamide gel electrophoresis [4] of the orange form showed bands at M_r 37 000 (thought to be a contaminant) and 16 000. The red form showed a single band at M_r 16 000.

Isoelectric focusing between pH 3 and 10 of the carotenoid-protein [5,6] showed that the orange and red forms had nearly identical isoelectric points at pH 4.7. The red form was slightly more acidic.

Acetone removed the carotenoid from the protein. The red form (1 mg) was dissolved in 0.4 ml of 10 mM phosphate buffer, pH 6.4, and 5 ml of acetone (–20°C) were added. The mixture was centrifuged in a clinical centrifuge for 2 min to give a clear yellow carotenoid solution and a white protein precipitate. When this procedure was repeated with the orange form, the protein precipitated by acetone retained some of its color. This protein was resuspended in buffer and precipitated again without releasing any more carotenoid. The absorption spectrum of this protein did not reveal any new features, but showed a decrease in the visible-to-ultraviolet absorbance ratio (A_{495}/A_{275}) from 1.4 to 0.33. This suggested that one-fourth of the carotenoids in the orange form were inaccessible to cold acetone. These carotenoids became accessible under protein- denaturing conditions. Since the absorption spectra of the easily removed and the tightly held carotenoids were identical, no qualitative distinction could be made about the type of carotenoid in the two pools. The spectra of the carotenoids released by the red and the orange forms of the protein were also identical.

Chromatography of the free carotenoids on the silica gel G-diatomaceous earth column produced three bands. The first carotenoid to elute, the least polar, was estimated by spectrophotometry to be 9% of the total. The second carotenoid was 86%, and the most polar carotenoid was 5% of the total. The major carotenoid shows an M_{50} (50% partition from petroleum ether) in 78% (v/v) methanol in water, indicating a carotenoid of high polarity [7]. The absorption spectrum of the principal carotenoid in hexane showed a maximum at 450 nm and shoulders at 427 and 470 nm (Fig. 4 and Table I). These values were somewhat dependent on the solvent (Table I). The carotenoid was reduced with $NaBH_4$ changing the shoulder at 470 nm into an absorption peak (Fig. 4). This suggested the presence of a ketone group in the molecule. The two minor carotenoid components showed similar absorption spectra and gave similar indications of a ketone group. Further analyses were limited to the most abundant carotenoid.

The structure of the major carotenoid was

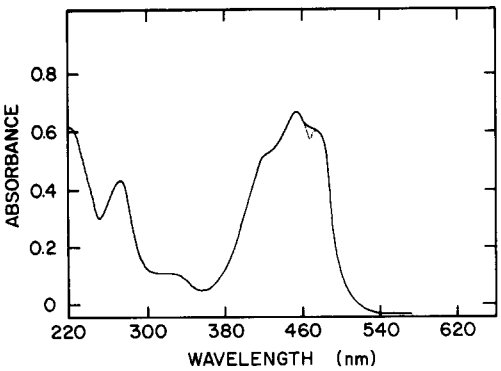


Fig. 4. Absorption spectra of carotenoid in hexane before (———) and after (· · · · · ·) reduction of carotenoid with $NaBH_4$. The free carotenoid was dissolved in methanol and an excess of $NaBH_4$ was added. After 30 min the reduced carotenoid was dried with N_2 and redissolved in hexane before the absorption spectrum was taken.

deduced by mass spectrometry (Fig. 5). The base and parent ion peak at *M/e* 566 gave the molecular weight of the carotenoid and was consistent with a carotene containing one hydroxy and one ketone group. Large peaks at *M/e* 474 (M-92) and *M/e* 460 (M-106) strengthened the identification of this molecule as a carotenoid [8]. The intensity of the peaks at high *M/e* values was suggestive of a molecule resistant to fragmentation, i.e., a molecule which could form stable radicals due to delocalization of an electron or which contained cyclic end groups. Bicyclic carotenoids have been shown to fragment preferentially within the polyene chain instead of at the end groups generating large *M/e* fragments [9]. The peak at *M/e*

TABLE I

ABSORPTION MAXIMA OF 3'-HYDROXYECHINENONE IN VARIOUS SOLVENTS

Solvent	Maxima (nm)	Shoulders (nm)
Acetone	458	475, 430 [a]
Carbon disulfide	485	507, 460 [a]
Ethanol	458	472 [a]
Hexane	450	470, 420 [a]
Methanol	458	465 [a]

[a] The shoulders on the absorption spectrum were difficult to determined accurately.

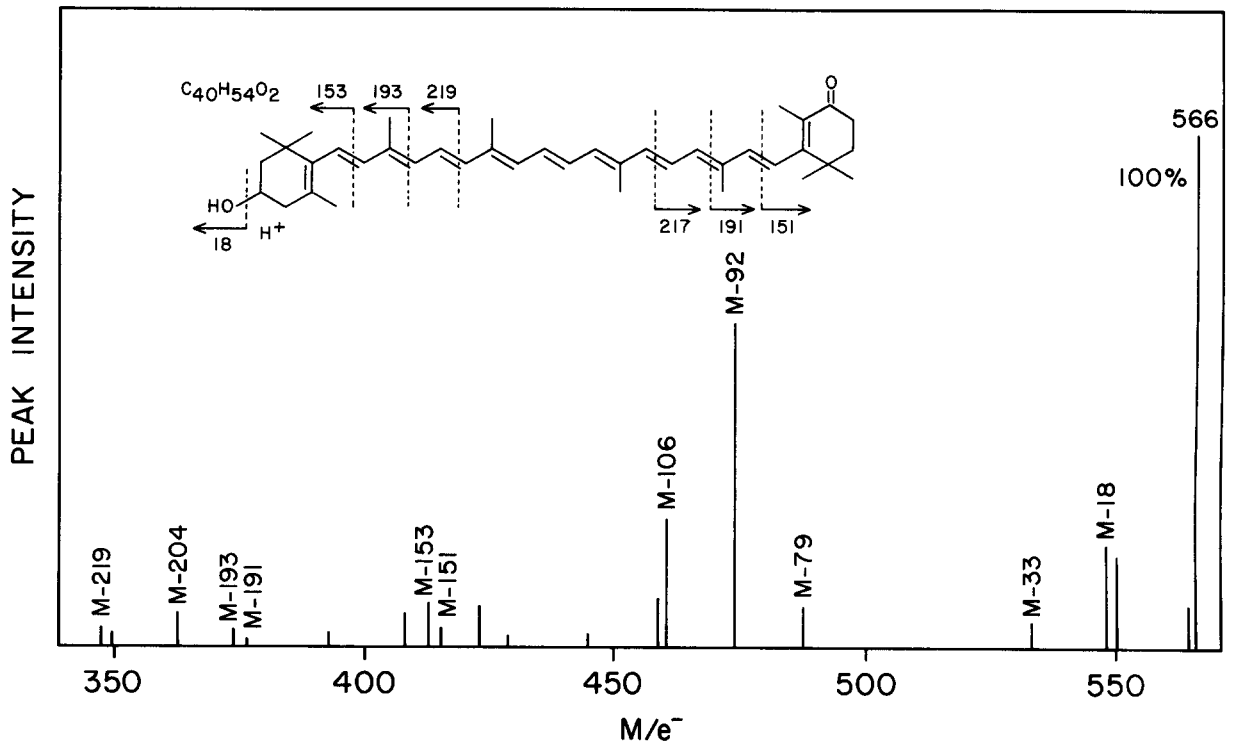
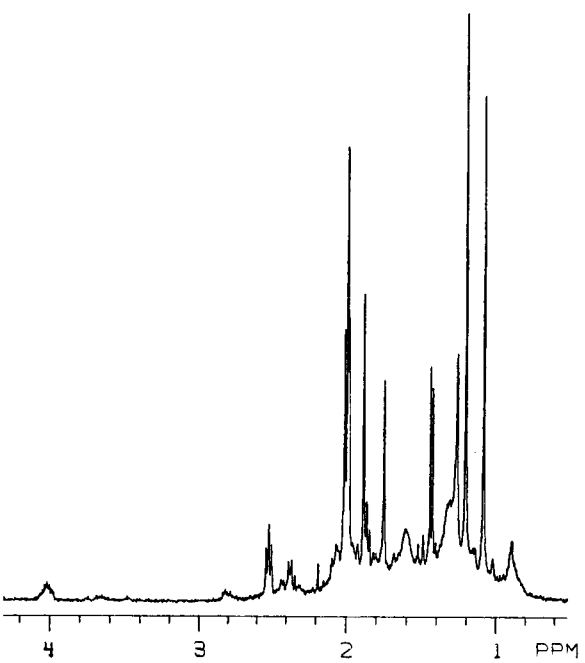


Fig. 5. Mass spectrum, structure, and fragmentation pattern of the carotenoid isolated from the carotenoid-protein complex. The mass spectrum was obtained on a CEC-21 110 mass spectrometer at 70 eV with ion source and probe temperatures of 215°C.



546 (M-18) corresponded to the loss of water from the molecule which would be expected from a hydroxylated carotenoid. Earlier evidence indicated a ketone. The mass spectrum provided evidence that the two oxygen-containing groups were at opposite ends of the molecule, since there were several pairs of peaks differing by 2 *M/e* units (M-153, M-151, M-193, M-191, M-219, M-217) which could arise from the ketone at one end and the alcohol at the other end (Fig. 5 top).

The ^{1}H-NMR spectrum of the major carotenoid (Fig. 6, Table II) was compared to tabulated chemical

Fig. 6. ^{1}H-NMR spectrum between 0 and 4 ppm of carotenoid isolated from the carotenoid-protein complex from *S. maxima*. The spectrum was obtained on a 360 MHz Nicolet Technical Corp. spectrometer. The spectrum was obtained in a deuterated chloroform solution with tetramethylsilane internal standard.

TABLE II

^{1}H-NMR CHEMICAL SHIFTS AND PROTON COUPLING FOR END GROUPS I AND II

End group	Carbon number of protons	Published values		Observed data	
		Coupling	Chemical shifts (ppm)	Coupling	Chemical shifts (ppm)
I	2	triplet	1.85	triplet	1.849
I	3	triplet	2.51	triplet	2.503
I	a,b	singlet	1.19	singlet	1.193
I	c	singlet	1.87	singlet	1.871
II	3′	multiplet	4.0	multiplet	4.011
II	4′	doublet	2.40	doublet	2.358
II	4′	doublet	2.04	doublet	2.054
II	a′,b′	singlet	1.07	singlet	1.072

shift and peak splitting data for compounds of rigorously defined structures [10]. The results of ^{1}H-NMR decoupling experiments confirmed the structural assignments (Table III). All of these data indicate the carotenoid is 3′-hydroxyechinenone.

The identification of the carotenoid allowed the use of a molar extinction coefficient of 2 500 $M^{-1} \cdot cm^{-1}$ [11] in order to estimate the stoichiometry of carotenoid to protein. The protein content of a sample of purified carotenoid-protein was determined by the method of Layne [12], and the carotenoid content estimated from the spectrum of an acetone extact done at room temperature. A value of 18 mol of carotenoid per mol of protein (M_r = 16 000) was obtained.

TABLE III

^{1}H-NMR DECOUPLING EXPERIMENTS

Position of protons decoupled		Effects in coupling	
End group	Protons	Proton affected	Transition
I	2	3	triplet to singlet
I	3	2	triplet to singlet
II	3′	4′	doublet to singlet
II	4′ [a]	3′,4′	multiplet to singlet, triplet to singlet
II	4′ [b]	4′ [a]	doublet to singlet

[a] Chemical shift 2.40 ppm.
[b] Chemical shift 2.04 ppm.

Discussion

A carotenoid-protein containing 3′-hydroxyechinenone is present in extracts from three different genera of cyanobacteria. Carotenoids may have several functions in photosynthetic membranes. They seem to protect chlorophyll against photooxidation [13,14]. They can absorb light and transfer the energy to the chlorophyll of Photosystem I to drive photosynthetic reactions [15]. Antisera prepared against lutein and neoxanthin inhibit Photosystem II reactions in membranes from a cyanobacterium [16].

The carotenoid-protein described here contains less than 1% of the carotenoid found in these cells. A very rough estimate of recovery of the carotenoid-protein suggests that it represents one molecule of carotenoid per 1 000 molecules of chlorophyll, but given the instability of the protein and the uncertainty of initial extraction, this figure is very tentative. The carotenoid-protein appears among the soluble proteins which are easily extracted when the cells are broken. If this protein is associated with the photosynthetic membrane, it is held very loosely like the phycobiliproteins. Purification from several sources has yielded a discrete orange fraction in each instance which could be purified to a constant ratio of visible-to-ultraviolet light absorbance. This suggests that these cyanobacteria make this species of carotenoid-protein with a fixed stoichiometry of chromophore to protein. It will be interesting to examine laboratory cultures at various stages of growth to see if the amount of carotenoid-protein, the ratio of carotenoid

to protein in the complex, and the degree of oxygenation of the carotenoid can be altered. Such variation is suggested by the two minor carotenoid constituents, one of which is less polar and the other more polar than the 3′-hydroxyechinenone which constitutes 86% of the carotenoid in association with this protein. We observe variable amounts of carotenoids in the form of lipid micelles in aqueous extracts from *A. variabilis* and *S. maxima*. This material resembles the osmiophilic globules in higher plant chloroplasts described by Bailey and Whyborn [17]. The amount of carotenoid in this nonprotein fraction seems to increase as the culture ages.

The carotenoid 3′-hydroxyechinenone is found in *Oscillatoria rubescens* and in an *Anthrospira* species [18]. The disposition of the carotenoid in the protein and the organization of the polypeptide in the monomeric and dimeric structures promise to be an interesting area for further study.

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