Characterisation of the carotenoprotein found in carapace shells of *Jasus lalandii* – A preliminary report

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ABSTRACT

Carotenoprotein, containing astaxanthin as the prosthetic group was extracted from the carapace shells of the lobster, *Jasus lalandii*. The material, with a molecular mass of around 400 000 Da and λ_{max} 525nm (α-crustacyanin), was readily converted under the conditions used for extraction and on standing to the subunit (β-crustacyanin) of molecular mass around 40 000 Da and λ_{max} 560 nm. The latter complex was stable up to 65°C and between pH 5 and 8. Both the large molecule and the subunit showed the reversible bathochromic shift (λmax~480 nm) after being subjected to conditions of heat above 65°C and below 85°C and pH between 3 and 10, which alter the natural protein orientation. Above 85°C and outside of the pH range, the shift was irreversible. Crustacyanin from *J. lalandii* differed from the blue crustacyanin of the *Homarus* species with respect to visible spectra (λ_{max} 632 nm and λmax 585 nm) and by displaying a hypsochromic shift between the α- and β-crustacyanin. Stability of the α-crustacyanin and affinity to DE52 also differed. Yellow protein, with a λ_{max} 409/410 nm as described for the *Homarus* species was not detected in this *J. lalandii* study, although it may have been co-eluted. The properties of the extracted complex show potential for its use as a natural water soluble food colourant or temperature sensitive indicator.

KEY WORDS

Bathochromic shift, carotenoprotein, crustacyanin, *Jasus lalandii*, lobster

1. Introduction

When lobsters are cooked, the well known colour change from the dull dark hues ideal for camouflage within a marine environment, to a bright orange red has long been attributed to the presence of carotenoproteins in the shell. This colour change is due to the release of the prosthetic carotenoid group from its encapsulating protein to reveal its natural colour (Cianci *et al*. 2002). Pioneering work on the blue carotenoproteins of the *Homarus* species has shown the predominant molecule to be α-crustacyanin (λ_{max} 632 nm). This aggregate comprises eight dimers, named β-crustacyanin (λ_{max} 585 nm) each of molecular mass around 45 000 daltons. These can be further dissociated to 2 apoproteins of around 20 000 daltons each by removal of their astaxanthin group (Zagalsky and Tidmarsh, 1985; Keen *et al.* 1991). Dellisanti et al. (2003) showed that the quaternary structure was cylindrical with the eight β-crustacyanin molecules probably having a helical arrangement.

The ability of astaxanthin to display the bathochromic shift from the λ_{max} of the free form is dependent on the specific combination of apoproteins, their spatial relationship, and the nature of the carotenoid which requires keto groups at positions 4 and 4' and the presence of methyl groups at C20 and C20' (Cianci *et al*. 2002). Durbeej and Eriksson (2003), using quantum chemical studies and *Homarus gammarus* crustacyanin confirmed this by suggesting that the colour shift is

largely due to a C4 keto group being hydrogen bonded to a histidine residue. Magic angle spinning ^{13}C NMR work provides evidence that charge redistribution plays a role in the shift mechanism (Weesie *et al*. 1995).

Investigation of other species show that the blue astaxanthin proteins (λ_{max} 634 nm) extracted from *Astacus leptodactylus* (Gomez *et al*. 1986) and *Gammarus lacustris* G.O. sars (Czeczuga and Krywuta, 1981) behaved similarly to the *Homarus* crustacyanin suggesting that the bathochromic shift is achieved in the same way even if the individual protein components differ. This was the case with two *Homarus* species which gave identical spectral patterns but where differences in the proteins were found (Zagalsky and Tidmarsh. 1985). It is therefore conceivable that the same mechanism for colour change applies to other carotenoproteins such as the dark red brown pigment found in the carapace of *Jasus lalandii,* a lobster which occurs locally along the South African south and west coasts and exhibits the noticeable colour change to bright orange-red on cooking. This natural colour change phenomenon suggests opportunities for use of the responsible compound in commercial ventures as a temperature sensitive indicator either in the reversible temperature range or to denote temperatures at which permanent colour change is achieved. Alternatively it could function as a natural water soluble colourant and antioxidant. Investigation of the *J. lalandii* complex and its behaviour under different conditions of temperature and heat was therefore undertaken to define its character and potential use.

2. Materials and methods

2.1. Raw material and extraction: Lobster carapace was obtained fresh from commercial fisheries. Preparation and extraction of the crustacyanin was based on the method described by Zagalsky (1985). The shell was removed immediately, cleaned of any attached tissue, washed in iced water and allowed to dry overnight at 0-2°C. The dry shell was ground to a fine powder at low temperature and then soaked for 18 hours, with agitation, in a solution of 0.3 M boric acid neutralised to pH 6.8 with solid Tris. Soaked shell was removed by filtration, washed with fresh borate-Tris solution and resuspended in pre-cooled 10%(w/v) EDTA, pH 7.0 using 25 g shell per 1000 g solution. The mixture was stirred continuously overnight, after which shell was removed and the purple coloured filtrate containing the crustacyanin brought to 55% saturation with ammonium sulphate. The mixture was allowed to stand at 0-2°C for around 18 hours and the resultant precipitate, the crustacyanin, was collected by centrifugation at 10 000g for 30 minutes at 5°C. The precipitate was redissolved in 50 mM potassium phosphate buffer pH 7.0, and interfering protein removed by fractional precipitation with ammonium sulphate to 15% or 20% saturation. The desired crustacyanin was then precipitated by increasing the saturation level to 55%. This material was stored at 0±2°C. A total of 13 extractions were carried out over a six month period and pooled into four batches for further use for characterisation or in food preparations.

2.2. Purification: Extracted crustacyanin from each batch was collected by centrifugation, re-dissolved in

minimal 50 mM potassium phosphate buffer pH 7.0, and dialysed against the same buffer before being subjected to chromatography on a 25 x 1.2 cm Whatman DE 52 (diethylaminoethyl cellulose anion exchanger) column. The crustacyanin and its subunits were separated by stepwise elution using buffer containing 0.1, 0.2, 0.4, 0.6 and 1.0 M sodium chloride (NaCl). Further purification was effected by re-precipitation of the fractions and repeated elution from DE52.

2.3. Characterisation: UV-visible absorption spectra were carried out on each batch to determine the maxima for the whole complex and the subunits. Size exclusion chromatography of two batches on Sephacryl S100HR and Sephacryl S300HR was used to estimate molecular mass. Columns of 95 x 1 cm in 50mM phosphate buffer, pH 7.0, and flow rate of 0.3 mL/min applied throughout and comparison to calibration standards (20 000 to 500 000 Daltons) was used to gauge molecule size. Electrophoretic separation was carried out using polyacrylamide gel in sodium dodecyl sulphate (Laemmli, 1970) and a marker kit containing a range of proteins from 12 300 - 78 000 Daltons.

2.4. Removal of prosthetic group: This was conducted according to the acetone ether method of Zagalsky (1985). The ether layer containing the carotenoid was removed and the spectrum recorded for identification (Gomez *et al.* 1986). Confirmation of identity was tested by carrying out further spectra of the extracted carotenoid in acetone, ethanol and hexane (Britton 1992).

2.5. Effect of heat and cold: The colour shift was investigated by heating the total extract up to 85°C using indirect heat in a boiling water bath. Temperature was monitored and spectroscopic and visible colour changes noted. Aliquots were removed at temperatures of 65, 70, 75, 80 and 85°C, and the time taken to revert to the starting colour measured to allow for determination of point of irreversibility. Sub zero temperature effects were examined by freezing aliquots of a combined extract in 50 mM phosphate buffer, pH 7.0 at -18, -25 and -40°C for 48 hours, thawing at 4-6°C and comparing spectra with that of the initial untreated sample. All treated aliquots were tested for their ability to still undergo a bathochromic shift after heating to 85°C post the freeze treatment. Further samples from the same source were stored at the relevant freezing temperatures for seven days before the thaw/test treatment, and allowed to stand with observation over the following two weeks.

2.6. Effect of pH: The pH of solutions of β-crustacyanin in 50 mM phosphate buffer pH 7.0, were altered by dropwise addition of base (0.1 M sodium hydroxide) or acid (0.1 N hydrochloric acid) and spectral changes and levels of reversibility were recorded.

2.7. Effect of freeze drying: A sample each of α- and β-crustacyanins were freeze dried after removal of salts by dialysis. The reconstituted dried material was tested for the bathochromic effect after heating to 85°C.

3. Results

3.1. Size and spectra: Crustacyanin was readily extracted from lobster carapace shells under the conditions used. Fractionation by ion exchange on DE52 of the extracts resulted in three fractions, each showing a different colour: one which eluted with 0.1-0.2 M NaCl was deep mauve in colour and had maxima at 560 and 278 nm (designated β-crustacyanin); one which eluted with 0.4 M NaCl and was reddish mauve with maxima at 525 and 278 nm (designated α-crustacyanin); and a remaining coloured portion, which had maxima at 480 and 278 nm, suggesting it was denatured material, required 0.6 to 1.0 M NaCl for removal. Yellow protein, with a λ_{max}409/410 nm as described for the *Homarus* species was not detected (Salares *et al.* 1977, Zagalsky & Tidmarsh 1985), though may have been co-eluted.

The crustacyanin from *J. lalandii* is thus different from the blue crustacyanins with respect to visible spectra and affinity to an anion exchange medium. Unlike the work on the *Homarus* species the subunit appeared predominant (Zagalsky and Tidmarsh 1985, Gomez *et al*. 1986) and had a maximum absorbance at a higher wavelength than the whole complex. Re-chromatography of eluted α-crustacyanin always showed the presence of some β-crustacyanin suggesting that conversion to the smaller molecule took place readily in the 50 mM buffer used during extraction and purification. These results suggest differences in the molecule composition and orientation of carotenoid and protein. Figure 1 represents typical absorption spectra for the α- and β-crustacyanin, and shows broad overlapping peaks.

Figure 1.

When subjected to gel filtration, the fraction with λ_{max}525nm, designated as α crustacyanin was shown to be the larger molecule and had a mass of around 400 kDa whilst the fraction with λ_{max}560 nm was shown to be around 40 kDa suggesting it represents the β subunit. The β subunit was the predominant fraction and conversion may have taken place during the extraction dialysis, indicating susceptibility to low ionic strength.

Sodium dodecyl sulphate polyacrylamide electrophoresis of unpurified crustacyanin and the two separated fractions was inconclusive on all samples tested. Unexplained bands suggest that dissociations may have occurred due to experimental conditions, and the method used appears to be unsuitable for size determination. However, molecules of around 20 kDa, possibly the apoproteins, were present.

Figure 2.

3.2. The prosthetic group: This was readily extracted into the ether and when compared spectrally to pure crystalline astaxanthin treated in similar fashion, gave the same spectrum with a peak at 480 nm. Spectra of the extracted prosthetic group in acetone, ethanol and hexane gave λ_{max} values of 479-480 nm, 478 nm and 472 nm respectively. Whilst these values correspond with reported maxima for astaxanthin (Britton 1992, Gomez *et al.*1986), further chemical or NMR analysis is required for conclusive identifying evidence and information as to the nature of the prosthetic group.

3.3 Effects of heat: The complex showed an initial change in the λ_{max} at around 45°C when measured spectroscopically but required heating to a minimum of 65°C to elicit a visible colour change to orange, and complete conversion required a temperature of 68-70°C (Figure 3). The bathochromic shift was demonstrable after heating to 85°C. Time for complete reversion increased with increasing temperature, taking only about three minutes after heating to 65°C, two to three hours after 75°C and up to 48 hours after treatment at 85°C. Reversion to the original state started to occur at around 45°C.

Figure 3.

A solution of α-custacyanin subjected to three cycles of heating to 70°C and followed by immediate cooling at ambient temperature showed that the colour shift ability was not compromised by this treatment. Figure 4 shows the natural spectrum, the shift from 525 to 490 nm on a third heat cycle to 70°C, and the shift after a final heating to 90°C where the maximum is at 480 nm and irreversible.

Figure 4.

Lowering the temperature to sub zero values apparently had no effect on the colour in the frozen or thawed state. On thawing the ratio of the λ_{max} at 278 and 525 nm (α-crustacyanin) and 560 nm (β-custacyanin) remained unchanged, (Table 1), and all samples satisfactorily underwent the reversible colour shift at the usual temperatures and reversion rates. However, on the further standing of these samples in 50 mM buffer, pH 7.0 at 6-8°C for 10 days, those that had been frozen showed traces of orange precipitate indicating the presence of some denatured material.due either to the low temperature or low ionic medium.

Table 1.

3.4. Effects of pH: Immediate changes in pH to below 5 or above 9 caused commencement of the colour shift (Figure 5). Full conversion to the orange was not achieved until pH 3.0 and pH 10.0 -10.5 were reached. The reaction was irreversible at pH lower than 3 or higher than 10.5 indicating denaturation of the protein. More extreme pH levels took longer to revert than those near to neutral. Further work to identify affected amino acids, and comparison with the *Homarus* model (Cianci *et al.*2002) may help with identifying the structure of the *Jasus* crustacyanin protein, and its relation to the prosthetic group.

Figure 5.

3.5. Effects of drying: Freeze drying of both α- and β-crustacyanin after dialysis to remove buffer salts appeared to have no effect on the spectra maxima of the crustacyanin molecules, or on the ability of the sample to exhibit the bathochromic shift. However, conversion of some α- to β-crustacyanin took place during the dialysis (peaks at 525 and 560 nm) and during the drying and reconstitution as seen by the flat extended peak between 525 and 560 nm (Figure 6).

Figure 6.

4. Discussion

This preliminary study showed that carotenoprotein found in *Jasus lalandii*, whilst displaying the characteristic colour changes, appears to have a different composition to that of the blue crustacyanin, including the propensity to convert to the β form. Surprisingly the λ_{max} of the larger molecule was at a lower wave length

than that of the smaller molecule suggesting differences in the prosthetic group and the protein. Confirmation of the prosthetic group by NMR, and studies on the protein-carotenoid interactions as carried out by Krawczyk and Britton (2001) could indicate whether the same orientations as found with blue α-crustacyanin pertain. Similarly, separation of the constituent peptides and comparison to the *Homarus* and *Astacus* species (Gomez *et al*. 1986, Zagalsky and Tidmarsh 1985) and analysis of the constituent amino acids (Keen *et al*. 1991) could highlight differences in the protein moiety and help explain the different behaviour in low ionic media.

The work suggests the β-complex could be exploited commercially due to its predominance, the ability to undergo reversible colour changes and the apparent stability over a wide range of temperature and pH.

5. Acknowledgements

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Captions to figures:

Figure 1: Visible absorption spectra of β- and α-crustacyanin ex *J.lalandii* eluted from DE 52 with 50 mM phosphate buffer, pH 7.0, containing 0.2 and 0.4 M sodium chloride respectively.

Figure 2: Electrophoresis in sodium dodecyl sulphate showing the breakdown to the apoprotein size molecule

Figure 3. Effect of heat on λ_{max} of alpha and beta crustacyanin

Figure 4: The visible absorption spectra of untreated and heated α-crustacyanin

Figure 5: Effect of pH on λ_{max} of β-crustacyanin

Figure 6: Effect of freezedrying and reconstitution on α-crustacyanin

Tables

Table 1: Ratio of the λ_{max} at 278 and 560 nm (β-crustacyanin) and 525 nm (α -custacyanin) after freezing at different temperatures.

Temperature °C	560:278 nm	525:278 nm
22 ±2	0.433	0.431
7 ±1	0.446	0.443
- 18 ±2	0.433	0.442
- 25 ±2	0.434	0.433
- 40 ±2	0.435	0.434

Figure 1

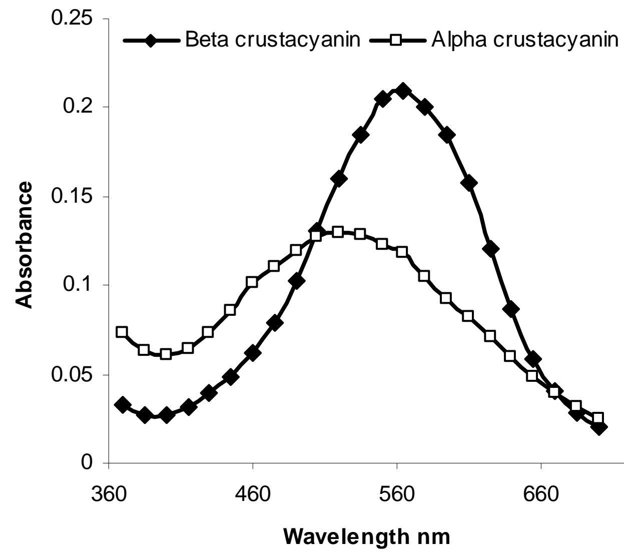

Figure 2

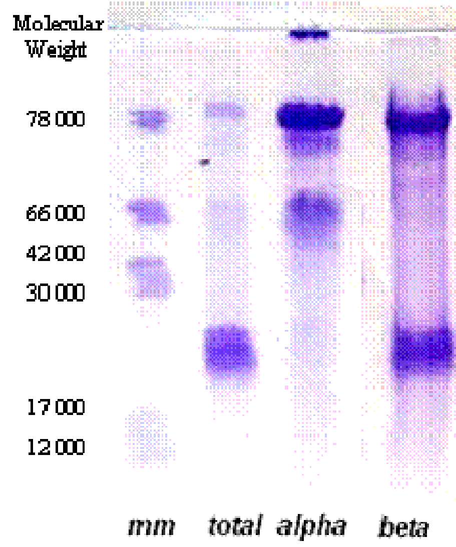

Figure 3

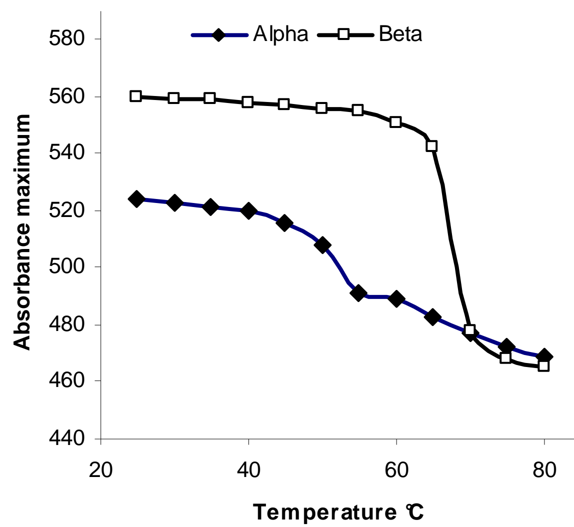

Figure 4

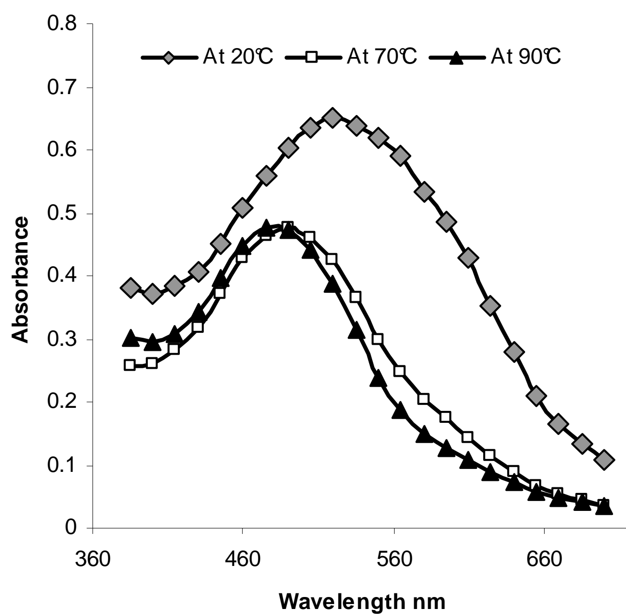

Figure 5

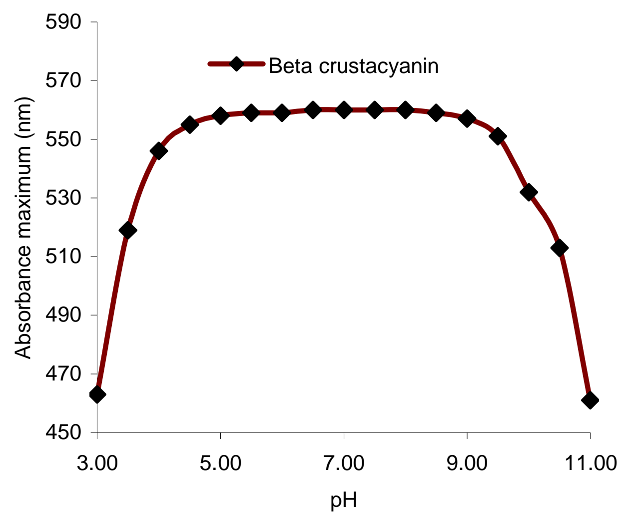

Figure 6