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Isolation and characterization of carotenoproteins from crayfish (*Procambarus clarkii*)

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Abstract

Crayfish (*Procambarus clarkii*) were separated by solid filtration into a proteinaceous fraction (PF-1) and a chitinous fraction (CF). The PF-1 and CF were used as starting materials for carotenoprotein-1 and carotenoprotein-2 preparations, respectively. Crawfish carotenoprotein-1 was obtained by controlled enzymatic autodigestion of FP-1 and carotenoprotein-2 by in situ repeated batch lactic acid fermentation. Carotenoprotein-1 has a high content of essential amino acids, ω-3- and ω-6-fatty acids and carotene (mainly astaxanthin), and constitutes an excellent nutritional source for patients with malnutrition. Carotenoprotein-2, of lower nutritional quality but with a substantial carotene content, can be used as a good protein source for animal nutrition where coloration is required, such as for poultry or for salmonid fish bred by aquaculture.
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Keywords: Carotenoproteins; Astaxanthin; Carotenoids; Crayfish; *Procambarus clarkii*; Repeated-batch fermentation

1. Introduction

While in undeveloped countries there is a high demand for food proteins, in developed countries, protein deficiency is uncommon. Current nutritional research and product development are focused mainly on products that help to reduce or control diet-related diseases, such as atherosclerosis, cancer, and renal or liver failures (Bautista, Corpas et al., 2000), as well as on special products for weight control and for hospitalised patients (Weaver, Schmidl, Woteki, & Bidlack, 1993). For that purpose, we are studying new protein sources with a high essential amino acid content, associated with other products beneficial for health, for their use as functional foods.

Crayfish (*Procambarus clarkii*) is a rich source of many valuable products, such as protein, chitin, and pigments (e.g. astaxanthin) (Guillou, Khalil, & Adambounou, 1995; No, Meyer, & Lee, 1989). Currently crayfish products are used mainly as a supplement in animal feed, or not used at all, but deposited on the land, constituting an important focus of environment pollution. However, because of its high protein content (Knorr, 1991), this by-product could be a good source of protein if appropriate processing was developed. Protein extracted from shellfish has been shown to be a good animal feed supplement (Jaswal, 1990; Meyer & Benjamin, 1987), and because of its content of the ketocarotenoid astaxanthin (3,3′-dihydroxy-ββ-carotene-4-4′-dione), one of the most powerful natural antioxidants (Chew, Park, Wong, & Wong, 1999), it could be a good protein source for human nutrition as a nutraceutical for the prevention and possible treatment of pathologies associated with the formation of free radicals and/or oxygen-active substances, such as hydroxyl-radical, superoxide or hydrogen peroxide (Tessier, Corda, & Marty, 2000).

In this work, we describe a new process, based on the direct fractionation of crayfish and in situ lactic acid production by repeated-batch fermentation, for the efficient preparation of crayfish protein concentrates (carotenoproteins), together with their characterisation.

2. Materials and methods

2.1. Samples

Whole crayfish (*P. clarkii*) and crayfish by-products (heads-thorax-, and claws) were obtained from Astria

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Lebrija S.L. (Lebrija, Seville, Spain), and Alfocan S.A. (Villafranco, Seville, Spain), mixed together and used as starting material.

2.2. Sample treatment

The crayfish by-products were fractionated by "solid-filtration" (applying pressure over a stainless steel filter of 1 mm pore size), into two fractions: a proteinaceous fraction (PF_1) and a chitinous fraction (CF). The CF comprised the material retained by the filter, and the PF_1 the material passing through the filter, as shown in Fig. 1. The PF_1 and CF were used as starting materials for carotenoprotein-1 and carotenoprotein-2 preparations, respectively, as shown in Fig. 2.

2.3. Analyses

Protein concentration was determined by HPLC amino acid analysis after hydrolysis with 6 M HCl in the presence of 1% phenol, and under vacuum atmosphere at 110 °C for 20 h. The amino acid composition of the hydrolysed fractions was determined by reversed-phase HPLC analysis of 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) derivatives according to the method described by Ward (2001), with α-aminobutyric

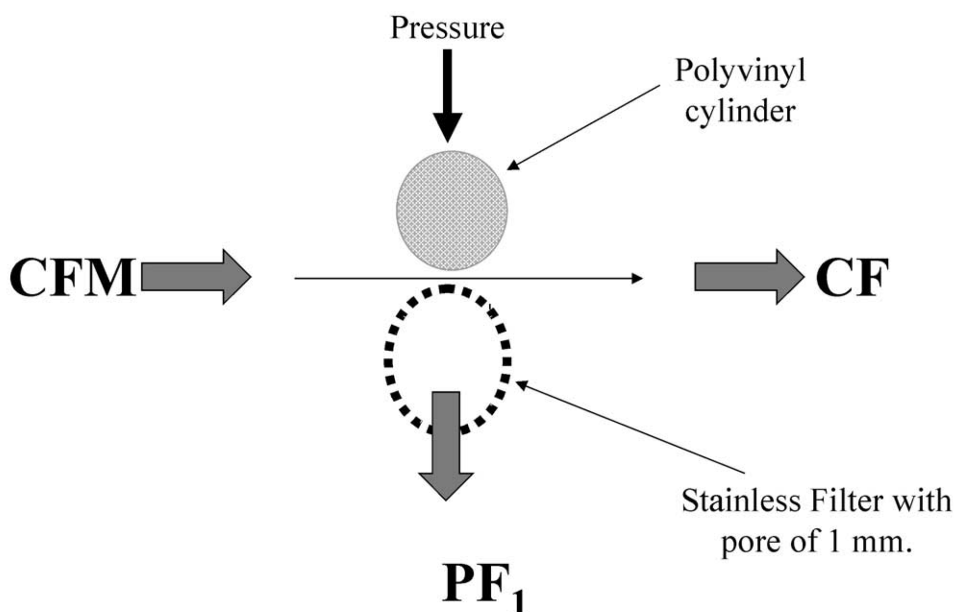

Fig. 1. Diagram of the solid filtration process.

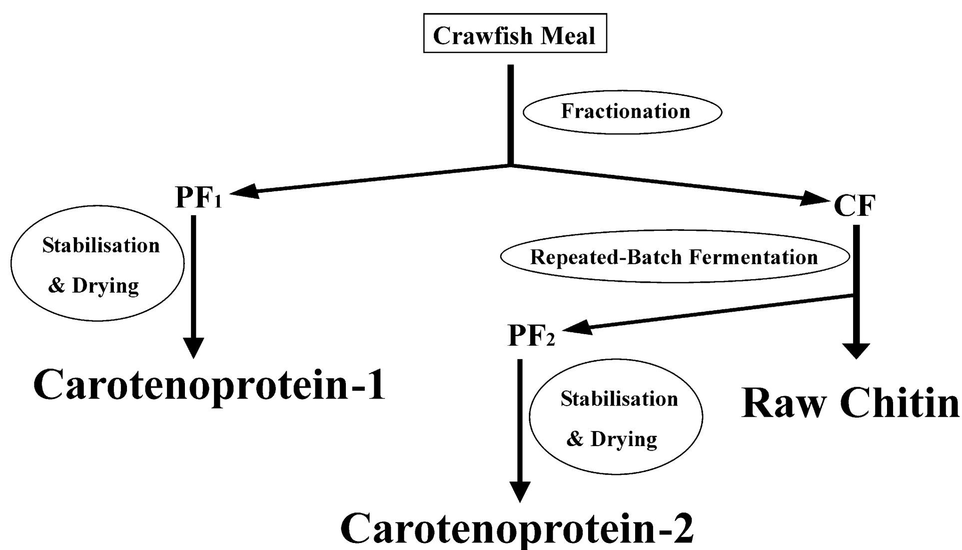

Fig. 2. Schematic flowsheet for production of carotenoproteins from crayfish (*Procambarus clarkii*). PF_1: proteinaceous fraction obtained by solid filtration. CF: chitinous fraction. PF_2: proteinaceous fraction obtained by repeated-batch fermentation.

acid as internal standard. Separation of the AQC derivatives was performed on a Water Nova Pac C18 column (150×3.9 mm i.d., 60 Å, 4 μm). Detection was by fluorescence. Losses of amino acids sensitive to acid hydrolysis, especially methionine and cysteine, were considered for accurate quantification.

Total nitrogen was estimated by the Kjeldahl method (AOAC, 1990). Nitrogen content of chitin was also estimated by the Kjeldahl method, after the sample (2–3 g, dry weight) had been purified of its calcium carbonate and protein by boiling with acid and alkali, respectively (Black & Schwarz, 1954). The chitin content was calculated by multiplying chitin nitrogen by 14.5, assuming that pure chitin contains 6.9% nitrogen (No et al., 1987).

Total carotenoids were extracted with ether:chloroform (1:2), dried, redissolved in acetone, and quantitated spectrophotometrically at 474 nm, using, as extinction coefficient value, $E^{1\%}_{1\,cm} = 2105$.

Moisture, ash, and total fats were determined in accord with the standard AOAC methods (AOAC, 1990).

Non-proteinaceous nitrogen-containing substances (NPN-CS) (such as nucleotides, free N-acetylglucosamine, and N-acetylglucosamine oligosaccharides) were determined by HPLC methods (Domar, 1993; Ferreira, Mendes, Gomes, Faria, & Ferreira, 2001).

2.4. Repeated-batch fermentation

Repeated-batch fermentation was conducted according to the procedure described by Bautista, Chico, and Machado (1986) with the lactic acid bacterium, *Lactobacillus paracasei* strain A3, supplied by Dr. G. Hall (Loughborough University, Leicestershire, UK). *L. paracasei* was maintained on dextrose-agar slopes stored at 4 °C. For the starter culture, a loopful of cells was transferred from a slope of MRS agar into 50 ml of sterile MRS broth (Difco) and incubated at 30 °C for 24 h. The inoculum for fermentation was prepared by transferring 50 ml of the starter culture into 1l sterile fermentation broth supplemented with 1 g/l casein peptone, which was incubated statically until growth reached a transmittance of 50%. Inoculum prepared according to this procedure yielded a cell concentration of approximately 10^9 cfu/ml.

Minced CF (0.1 kg) was thoroughly mixed with 10 l of fermentation medium containing 50 g/l dextrose and 1 g/l yeast extract in a 15 l fermenter, pasteurised at 70 °C for 30 min, and inoculated with 1 l of the prepared inoculum. Batch cultures were done at 30 °C without pH control (free pH evolution) and with slow stirring (50 rpm). After the initial glucose concentration had dropped below 0.2 g/l, stirring was stopped, the culture allowed to sediment, and 75% of the culture supernatant was harvested. An equivalent volume of pasteurised fermentation medium (with 60 g/l glucose), giving a final glucose concentration of 45 g/l, was added to the fermenter, and the second cycle was started. The process was repeated a second time. After the glucose concentration had dropped to 0.2 g/l, the fermentation products were harvested by filtration through a metal filter of 0.5 mm. Two fractions were obtained: a solid fraction comprising crude chitin, retained on the filter, and a liquid fraction. The latter was filtered through a 0.45 μm membrane, and the filtrate, free of bacteria, precipitated at its pI, constituting the starting material for the preparation of carotenoprotein-2.

2.5. Controlled enzymatic autodigestion

The PF1 was adjusted to pH 6.0 with acetic acid (1.0 M), and maintained at 37 °C, to allow free breakdown of the proteins by the proteases (endo- and exoproteases) present in the PF1, until the pH reached a constant value of 4.5±0.1 (approximately 10–12 h). After autodigestion, the mixture was maintained at 90 °C for 60 min to inactivate the proteases and dried in a fluidised-bed dryer at 60 °C.

2.6. Size-exclusion chromatography

Soluble proteins were separated, using an Äkta Purifier (Pharmacia), by size-exclusion chromatography on a Sephacryl S-200 HR column (1.6 cm×40 cm) equilibrated in 100 mM sodium phosphate buffer, pH 7.0, with 250 mM NaCl. Previously, the column was calibrated with the following standard proteins: α-chymotrypsinogen (25 kDa), cytochrome *c* (12.2 kDa), ribonuclease (13.7kDa), carbonic anhydrase (31 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), and lipoxygenase (108 kDa). Samples were eluted at 0.2 ml/min, and proteins were detected by absorbance at two different λ: 280 and 215 nm.

2.7. Electrophoresis

SDS-PAGE was performed by the method of Laemmli (1970). The gel system, containing 0.2% (w/v) SDS, consisted of a 15% polyacrylamide resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). The length of the resolving and stacking gels were 10 and 2.5 cm, respectively, with a gel thickness of 1.0 mm. Electrophoresis was performed at a constant current of 25 mA. Protein bands were stained by immersion of the gel in a 0.05% (w/v) Coomassie G-250 solution, in 50% methanol–10% acetic acid solution. Molecular masses were determined using the low molecular weight standard kit from Pharmacia: ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), and albumin (67,000). Native electrophoresis was carried out in 8% (w/v) acrylamide gel but omitting SDS and β-mercaptoethanol from all buffers.

3. Results and discussion

3.1. Carotenoprotein preparation

In a previous paper (Bautista et al., 2001), we described the preparation of crayfish chitin by a two-step process, based on sedimentation-flotation and semi-solid state fermentation. In the first step, CFM was fractionated by a sedimentation-flotation process, according to the procedure described by Bautista, Parrado, and Machado (1990), into two fractions: a proteinaceous fraction (PF), recovered by flotation, and a chitinous fraction (CF), which sediments at the bottom of the reactor. The process was carried out in a reactor that facilitated mixing, flotation and sedimentation, and allowed easy recovery of both fractions. The process yield was 83.5%, with a recovery of 53.4 and 30.1% as PF and CF, respectively, referred to dry weight. Some 16.5% of material was lost during the flotation-sedimentation fractionation process. This material comprised soluble substances, such as proteins, peptides, free amino acids, nucleotides, and oligonucleotides, originating from the proteolytic and nucleolytic enzymes present in the digestive tract of the animals (Bautista et al., 2001).

In an attempt to minimise losses during the fractionation process, we have adapted a separation process used in the salmon industry for the recovery of meat bound to the fish skin (Alfocan, 2001), based on the application of pressure over a stainless steel filter of 1 mm pore size, as shown schematically in Fig. 1. Two fractions were obtained: a proteinaceous fraction (PF_1), passing through the filter, and a chitinous fraction (CF), which is retained by the filter. The process yield at this stage is 96.3%, with a recovery of 69.4 and 26.9% as PF_1 and CF, respectively, as shown from the results presented in Table 1. This procedure improves the protein recovery and shows a reduced loss of products (3.8%), allowing the recovery of soluble products which are lost in the flotation-sedimentation process.

Carotenoprotein-1, obtained from PF_1 by autodigestion, until the pH reached a constant value at 4.5 ± 0.2, was stabilised at 90 °C for 1 h (protease inactivation) and dried in a fluidised-bed dryer at 60 °C. Chemical characterization of carotenoprotein-1 (Table 2) shows that the carotenoprotein-1 obtained from crayfish by this new process has a high protein content: 63.4% as true proteins (precipitated with 10% TCA) and 8.9% as free aminoacids and short peptides. The other components—total fats, NPN-CS (such as nucleotides, free N-acetylglucosamine, N-acetylglucosamine-oligosaccharides, and chitin), and total carotenoids (mainly astaxanthin)—represent 9.8, 4.3, and 0.6%, respectively.

CF has the following chemical composition: 15.9% protein, 0.7% total fat, 27.9% chitin, and 51.3% inorganic minerals (ash), expressed as dry weight; it is similar to that reported previously for acetic acid fermentation (Bautista, Cremades et al., 2000). As this result shows, a considerable amount of protein (15.9%) remains bound to the chitinous fraction, and must be removed for the preparation of high-quality chitin and to improve the global recovery of protein.

These bound proteins are currently extracted in alkaline medium (40–50% NaOH solution) at high temperatures for several hours (Roberts, 1998). Protease treatment has also been tried (Gagné & Simpson, 1993), though its application is limited. The alkaline procedure is efficient, but there are important drawbacks regarding amino acid modifications (Cheftel, 1985; Parrado, Bautista, & Machado, 1991) and environmental problems. The modification of amino acids in highly concentrated alkaline media can reduce the nutritional properties of the proteins (Cheftel, 1985; Parrado, Millan, Hernandez-Pinzón, Bautista, & Machado, 1993), and the disposal of the effluents causes considerable environmental deterioration. Enzymatic procedures, based on the use of proteases, do not have these disadvantages: they do not modify amino acids, and are environmentally friendly. However, enzymatic deproteinisation is at present an

Table 1
Product recovery by solid fractionation

CFM[a] (kg)	PF1 (kg)	CF (kg)	Yield (%)
623.4	430.8	180.8	98.1
507.3	358.5	132.0	96.7
603.1	421.7	157.0	95.9
480.7	327.2	125.0	94.1
553.6±70.2	384.5±49.9	148.7±25.4	96.2±1.7
(100%)	(69.4%)	(26.9%)	(96.3%)

[a] CFM, crayfish meal.

Table 2
Chemical composition of carotenoprotein-1 and carotenoprotein-2

	Carotenoprotein-1	Carotenoprotein-2
Moisture (%)	6.5±0.8	7.1±0.5
Total nitrogen×6.25 (%)	75.2±1.9	n.d.
Protein (%)[a]	63.4±1.5	39.8±4.9
Free AAs and short peptides (%)[b]	8.9±0.4	7.6±0.5
Nucleotides (%)	3.9±0.2	n.d.
Total fat (%)	9.8±0.6	3.2±0.3
Total carotenoids (µg/g)	610±10.2	198±6.3
Chitin (%)	0.4±0.1	0.5±0.1
Ash (%)	4.8±0.3	31.2±2.7
Others (%)[c]	13.6±2.9	48.9±1.9

Results are expressed on a dry basis.
[a] Determined by HPLC amino acid analysis in the precipitate, after precipitation with 10% TCA.
[b] Determined by HPLC amino acid analysis in the supernatant, after precipitation with 10% TCA.
[c] Determined as difference: [100−(protein + free AAs & short peptide + nucleotides + total fat + total carotenoids + Chitin)]

expensive process, due to the high cost of enzymes, and the processes so far assayed are not as efficient as the alkaline one. After protease treatment, a significant amount of protein (2.3–4.8%) remains bound to the exoskeleton (Gagné & Simpson, 1993). Protease action is restricted by steric hindrance of protease access to substrate bound to the exoskeleton support. Different approaches aimed at overcoming this problem are under investigation. One of these is the use of fermentation processes (Zakarias, Hall, & Shama, 1998).

Lactic acid was produced in situ with glucose as carbon source in a repeated-batch fermentation process, using *L. paracasei*, according to the procedure described by Bautista et al. (1986). CF was thoroughly mixed with sterile fermentation medium containing dextrose (50 g/l) and yeast extract in a 15 l fermenter, pasteurised, and inoculated with 1 l of the prepared inoculum. Batch cultures were done at 30 °C without pH control (free pH evolution) and with slow stirring (50 rpm). After the initial glucose concentration had dropped below 0.5 g/l, and a lactic acid production of 62 g/l was reached (see Fig. 3), stirring was stopped, the culture was allowed to sediment, and 75% of the culture supernatant was harvested and processed as described below. An equivalent volume of pasteurised fermentation medium (with 60 g/l glucose), giving a final glucose concentration of 45 g/l, was added to the fermenter, and a second cycle was started. The process was repeated once.

After glucose had dropped to 0.2 g/l, the fermentation products were harvested by filtration through a metal filter of 0.5 mm. Two fractions were obtained: a solid fraction comprising crude chitin, retained by the filter, and a liquid fraction. The latter was filtered through a 0.45 μm membrane, and the filtrate, free of bacteria, precipitated at its pI, constituted the starting material for the preparation of carotenoprotein-2. The treatment of CF, supplemented with dextrose, by fermentation with *Lactobacillus* has two effects that occur simultaneously during the fermentation process: demineralisation, due to the production of lactic acid, and deproteinisation, due to the hydrolytic action of exoproteases excreted into the fermentation medium by the microorganism. The chitinous product (raw chitin) obtained by repeated-batch fermentation (two cycles) shows a significant degree of demineralisation, reflected as a mineral content (Ca + P + K + Mg) of 0.62%, and a low content of protein (1.93%). It is important to note that the degree of deproteinisation achieved by this procedure is greater than that by the direct use of proteases, probably due to the demineralising action of the acid, which helps enzyme access to the exoskeleton-bound proteins. The proteins obtained from the liquid fraction, after separation of the chitinous fraction (raw chitin), do not show the drawbacks of protein obtained by alkaline deproteinisation, but do show the same advantages as those obtained by enzymatic treatment. These proteins, obtained by acid precipitation at the pI (pH 4.3 ± 0.1), were stabilised at 90 °C for 1 h (protease inactivation) and dried in a fluidised-bed dryer at 60 °C. The solid material obtained constituted the carotenoprotein-2. We are aware that the fermentation medium used can be expensive, but cheaper fermenta-

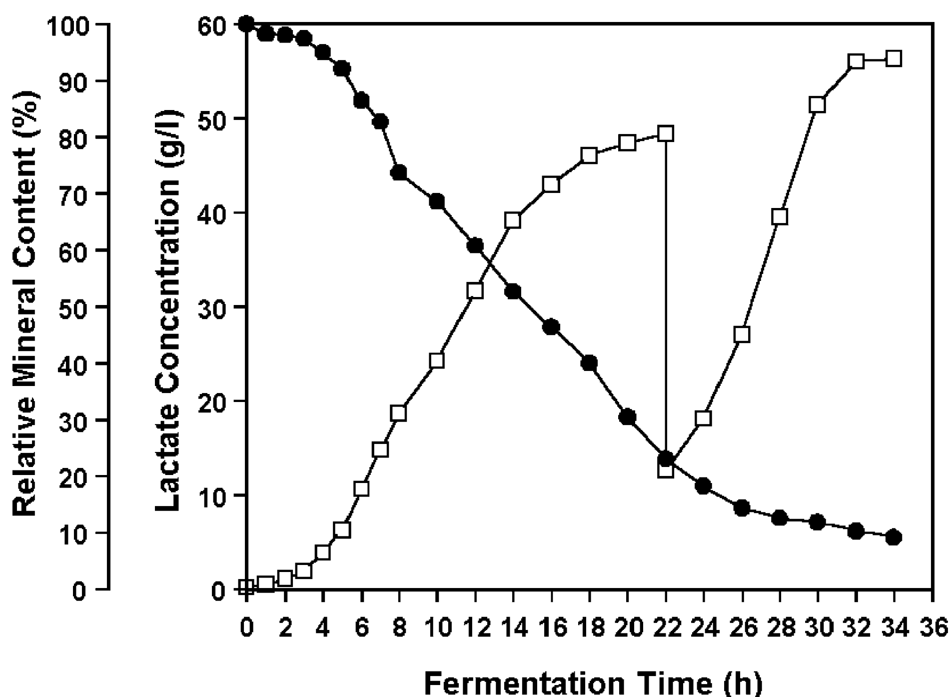

Fig. 3. Lactate production (–□—□–) by Lactobacillus paracasei, and CF demineralisation (–•—•–).

tion sources, such as whey, cassava starch, or lignocellulose, instead of dextrose or glucose, might offer a commercial route for the deproteinisation and demineralisation of crayfish exoskeleton, or chitinous fraction.

3.2. Carotenoprotein characterisation

As shown in Table 2, carotenoprotein-1, a proteinaceous product of pink-red colour, with a protein content (determined by amino acid analysis) of 63.4% and a content of free amino acids and short peptides of 8.9%, constitutes a good protein source for both animal (Jaswal, 1990; Meyer & Benjamin, 1987) and human food (Alvarez-Ossorio et al., 2002), but the main feature of this fraction is its amino acid composition, as will be discussed later. The amino acid profiles and the essential amino acid score of carotenoprotein-1 are presented in Fig. 4. As these results show that carotenoprotein-1 is an excellent protein source. It contains all the essential amino acids, accounting for 46.6% of the total amino acid content, and is a good starting material for the preparation of high-quality products that require a high content in essential amino acids, such as the diets for patients with cancer-anorexia-cachexia syndrome (Cohen & Lefor, 2001) and renal failure (Freund, 2001). Its composition (Fig. 4) meets all the FAO requirements for nutritional purposes. The high content in essential amino acids, and a considerable content in ω3- and ω6-fatty acids (15.6%), total fat (4.8%), and carotene (mainly astaxanthin) (610 μg/g of product, bound to protein, which stabilises these antioxidants (Cremades et al., unpublished results), make this product an important protein source, as a nutraceutical (or functional food), for the nutrition of patients needing protein of high-quality, rather than large amounts, and/or an antioxidant source, as in the case of cancer, AIDS, hepatic and renal patients, and the elderly (Cohen & Lefor, 2001; Freund, 2001; Tessier et al., 2000).

Carotenoprotein-2 is a proteinaceous product of red-brown colour with a lower amino acid profile quality than carotenoprotein-1 (see Fig. 4). Because of its high contents of glycine and proline (14.9 and 11.1%, respectively), it can be considered a mainly collagen-like product, probably derived from the digestion of cuticle proteins and the membranous layer that covers the inside of the crayfish exoskeleton. However, due to its

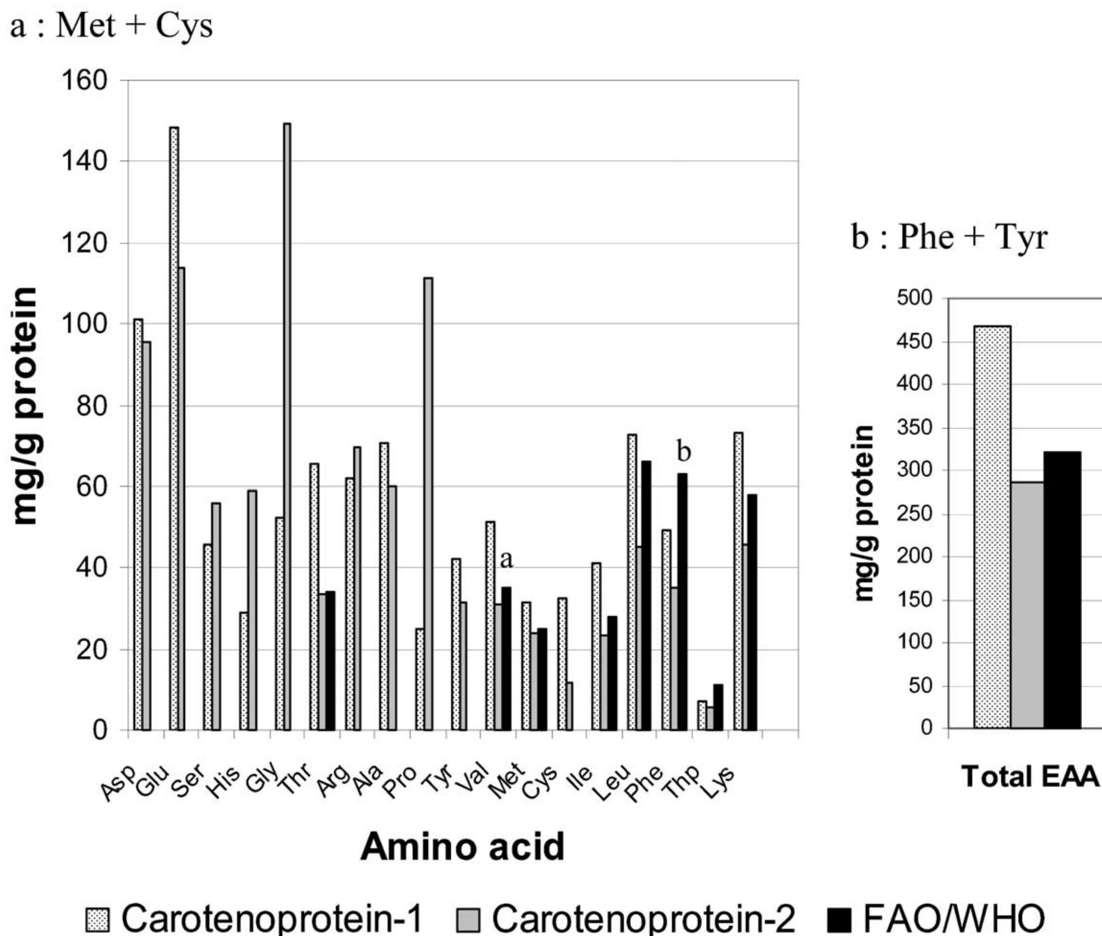

Fig. 4. Carotenoprotein-1 and carotenoprotein-2 amino acid profiles compared with FAO/WHO requirements.

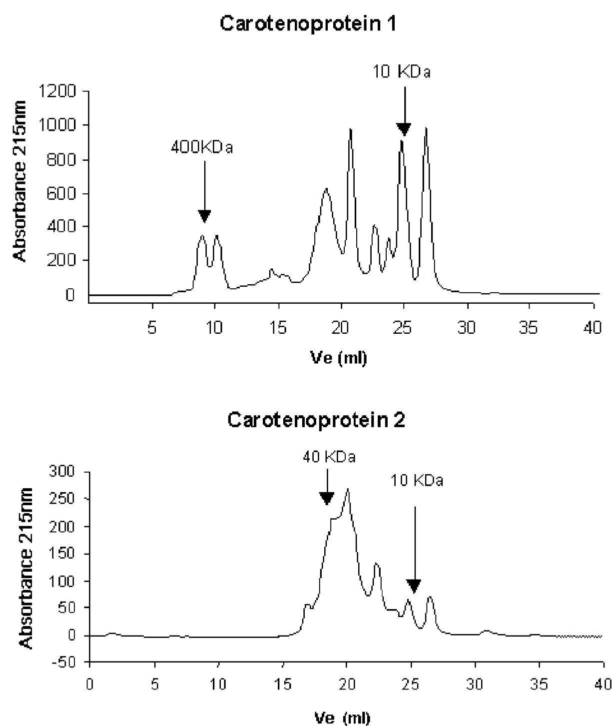

Fig. 5. HPLC size exclusion chromatogram of carotenoprotein 1 and carotenoprotein 2, on a Sephacryl S-200 HR column (1.6 cm×40 cm).

content of protein (39.8%), free amino acids + short peptides (7.6%), and carotene (97.9 μg/g), it can be used as a good protein source for animal nutrition where coloration improvement is required, such as for poultry or salmonid fish (Johnson, Villa, & Lewis, 1980; Lee, 1990)

The analysis, by HPLC size-exclusion chromatography, of soluble proteins present in carotenoprotein-1 and carotenoprotein-2 (Fig. 5) shows that carotenoprotein-1 is characterised by a series of peaks spread out over a large range of molecular weight from less than 10 kDa to more than 400 kDa, corresponding to sarcoplasmic and some myofibrillar-soluble proteins—for example, actin (42 kDa) and myosin heavy chain (200 kDa) and its aggregates (400 kDa). Carotenoprotein-2 is constituted by two main peaks of 40 kDa and 22 kDa, followed by a series of peaks corresponding to low-molecular-weight compounds, such as oligopeptides, and free amino acids, originating from the action of bacterial proteases. The native PAGE and PAGE-SDS electrophoretic analyses confirm these results.

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