Food Chemistry 112 (2009) 310–315

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Amino acid profile and enhancement of the enzymatic hydrolysis of fermented shrimp carotenoproteins

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ARTICLE INFO

Article history:
Received 16 January 2008
Received in revised form 2 May 2008
Accepted 20 May 2008

Keywords:
Carotenoproteins
Astaxanthin
Carotenoids
Enzymatic hydrolysis
Protease
Lipase

ABSTRACT

Amino acid profiles of carotenoproteins extracted from fermented and non-fermented shrimp waste were analysed. Fermented carotenoproteins were hydrolysed with a protease and a combination of a protease and a lipase. Essential amino acids in fermented and non-fermented carotenoproteins were 49% and 47%, respectively, with respect to total amino acids. The highest carotenoprotein hydrolysis (900 and 66 mg/g soluble protein and total carotenoids, respectively) was obtained by a combination of 15 proteolytic units together with 10 lipolytic units. The most efficient treatment using only protease was obtained with 15 proteolytic units (852 and 48 mg/g of soluble protein and total carotenoids, respectively). A relatively protein-free form of astaxanthin derived from shrimp waste carotenoproteins may be of interest for applications in salmon culture, and in natural health products and cosmetics. Furthermore, fermented carotenoproteins could be used in human and animal diets due to their high essential amino acids concentration.

1. Introduction

Carotenoproteins are stable complexes in which carotenoids are bound to a high density lipoprotein (Lakshman & Okoh, 1993). Amongst these protein–pigment complexes is astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), giving blue to green colours in crustaceans (Zagalsky, Eliopoulos, & Findlay, 1990). Astaxanthin in crustaceans are mostly esterified to fatty acids, for example, Guillou, Khalil, and Adambounou (1995) reported that in shrimp (*Pandalus borealis*) waste silage, astaxanthin is found as diester, monoester and free forms (76%, 20% and 4%, respectively, relative to total astaxanthin). Astaxanthin also produces a red–orange colour in cooked crustaceans because it had completely or partly been separated from the protein moiety (Schiedt, Bischof, Glinz, & Packer, 1993).

Astaxanthin is a xanthophyll mainly used to provide the red–orange colour to the muscles of salmon produced by aquaculture (Meyers, 1994). Salmonids cannot synthesise astaxanthin, thus they acquire it from external sources such as microalgae or diet added with pigments (Lorenz & Cysewski, 2000; Shahidi & Synowiecki, 1991). The demand for natural sources of astaxanthin has been growing due to health considerations over the use of synthetic astaxanthin (Lorenz & Cysewski, 2000; Meyers, 1994). Moreover, due to astaxanthin's intense red–orange colour and the lack of allergic reactions to its natural form, this carotenoid has being considered for cosmetic applications (Arad & Yaron, 1992; Oshima, 1998). In addition, astaxanthin is used as a natural supplement for human consumption due to its antioxidant capacity, approximately 10 times higher than other carotenoids such as zeaxanthin, lutein, canthaxanthin and β-carotene, and 500 times higher than α-tocopherol (Shimidzu, 1996).

Nur-E-Borhan, Okada, Watabe, and Yamaguchi (1995) demonstrated that astaxanthin is the major carotenoid present in carotenoprotein from shrimp. Carotenoproteins of 120 kDa (*Litopenaeus monodon*) (Nur-E-Borhan et al., 1995), 280 kDa (*Litopenaeus japonicus*) (Muriana, Ruíz-Gutiérrez, Gallardo-Guerrero, & Mínguez-Mosquera, 1993), and 265 kDa (*Litopenaeus vannamei*) (Armenta-Lopez, Guerrero, & Huerta, 2002) have been reported from shrimp. Previous studies reported the use of marine and land animal proteases to extract carotenoproteins from crustacean waste, recovering nearly 80% of total protein and carotenoids from shrimp waste when trypsins from bovine, porcine and cod sources were used (Cano-Lopez, Simpson, & Haard, 1987; Simpson & Haard, 1985). Moreover, similar results were obtained when microbial proteases were used (Armenta-Lopez et al., 2002). Thus, proteins from shrimp waste can be hydrolysed using proteases and the recovery of astaxanthin and protein hydrolysate may be feasible (Gildberg & Stenberg, 2001).

In relation to amino acid profile of carotenoproteins Cremades et al. (2003), reported that a carotenoprotein isolated from lactic acid fermented crayfish (using *Lactobacillus paracasei* strain A3)

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doi:10.1016/j.foodchem.2008.05.075

had a high content of essential amino acids (47% relative to total amino acids) suggesting that this protein may be used to prepare diets for human patients that suffer diseases such as cancer–anorexia–cachexia syndrome and renal failure.

Since the red–orange colour of astaxanthin increases as it is separated from the protein moiety (Armenta-Lopez et al., 2002; Guillou et al., 1995; Schiedt et al., 1993), a relatively protein-free astaxanthin produced by enzymatic hydrolysis of carotenoproteins may be of interest for commercial applications such as for salmon culture (Lorenz & Cysewski, 2000; Meyers, 1994) and for ingredient of natural health products (Arad & Yaron, 1992; Oshima, 1998). Therefore, the aim of this work was to enhance the production of astaxanthin and soluble protein through the hydrolysis of carotenoproteins from shrimp waste using a commercial protease, and to assess a novel enzymatic treatment that combined a protease with a lipase. Furthermore, this study aimed to study the amino acid contents of lactic acid fermented and non-fermented shrimp carotenoproteins.

2. Materials and methods

Shrimp processing discards were obtained from *Litopenaeus vannamei*, caught in the Gulf of Mexico. These discards were stabilized by lactic acid fermentation, using *Pediococcus pentosaceus* following the process described by Armenta-Lopez et al. (2002) to produce freeze dried carotenoproteins (Fig. 1). This freeze dried carotenoprotein was subjected to two enzymatic treatments: (1) using the commercial protease Savinase™ (EC 3.4.21.62), and (2) a mixture of Savinase™ and the commercial lipase Lipolase™ (EC 3.1.1.3). The enzymes were kindly supplied in solution by Novozymes (Franklinton, NC, USA), produced from non-pathogenic microorganisms (*Bacillus clausii* and *Aspergillus oryzae*, for the protease and lipase, respectively) classified as non-toxic, and used in detergent formulations to remove protein and oil-based stains. All reagents were from Sigma Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.1. Protease hydrolysis

Protease enzymatic activity was determined by the Kunitz method that uses a solution of 1% casein as substrate (Kunitz, 1947); the casein was dissolved in 50 ml of a 0.05 M Na_3PO_4 buffer by stirring the solution for 20 min at a boiling temperature in a bain-marie; the pH was adjusted to 7. Nine millilitres of the substrate were added to 9 test tubes (triplicates of sample, blank, and control) which were incubated at 40 °C in a bain-marie. Protease (0.15 ml) and 0.05 M Na_3PO_4 buffer (0.15 ml) were placed in sample and blank test tubes, respectively, and incubated for 20 min at 40 °C. Two millilitres of a 2% trichloroacetic acid (TCA) solution was added to all tubes to stop the enzymatic reaction. Enzyme (0.15 ml) was added to the control tubes after the TCA addition. After stirring, 1.5 ml were placed in vials and then centrifuged at 252 g; the absorbance of the supernatant was measured at 280 nm. Absorbance averages were used to obtain the absorbance of the enzymatic assay (absorbance of enzymatic assay = absorbance of sample – absorbance of control). Results were interpolated into a tyrosine calibration curve ($r^2 = 0.9992$). One proteolytic unit (PU) was defined as the amount of protease required to release 1 μmol of free tyrosine/min at 40 °C.

The absorbance of the reaction mixture (supernatant) was measured at $\lambda = 280$ nm to follow the carotenoprotein hydrolysis applying 5, 10 and 15 PU to the following enzymatic system: 10 mg of lyophilised carotenoprotein were placed in screw-capped test tubes containing 10 ml 0.05 M sodium phosphate buffer (1 mg carotenoprotein/ml buffer as final concentration). The enzymatic hydrolysis was assessed at pH 6, 7, 8, 9 and 10, by adjusting the sodium phosphate buffer pH. The enzymatic system was stirred for 24 h at 40 °C at 80 rpm. Soluble protein and total carotenoids were analysed after 24 h of hydrolysis. Carotenoprotein hydrolysis at pH

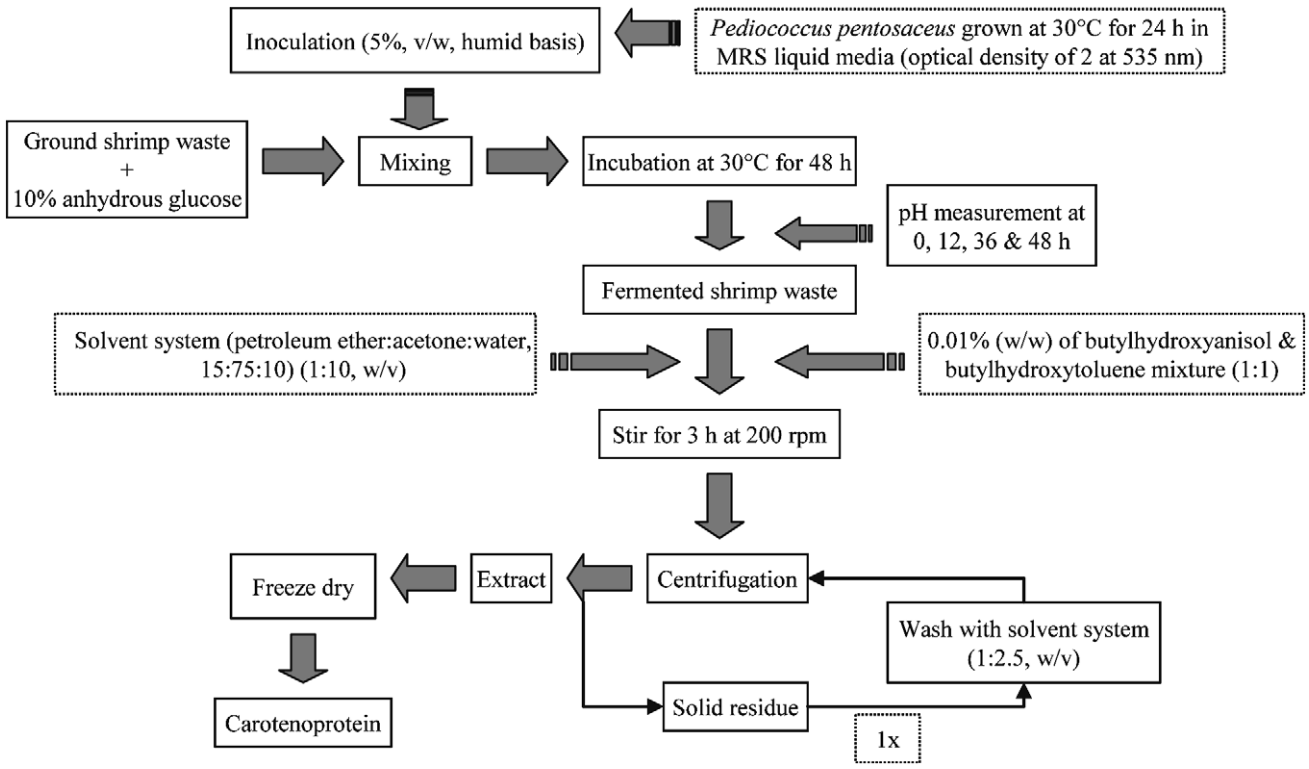

Fig. 1. Process followed to ferment shrimp waste and to extract carotenoprotein Armenta-Lopez et al. (2002).

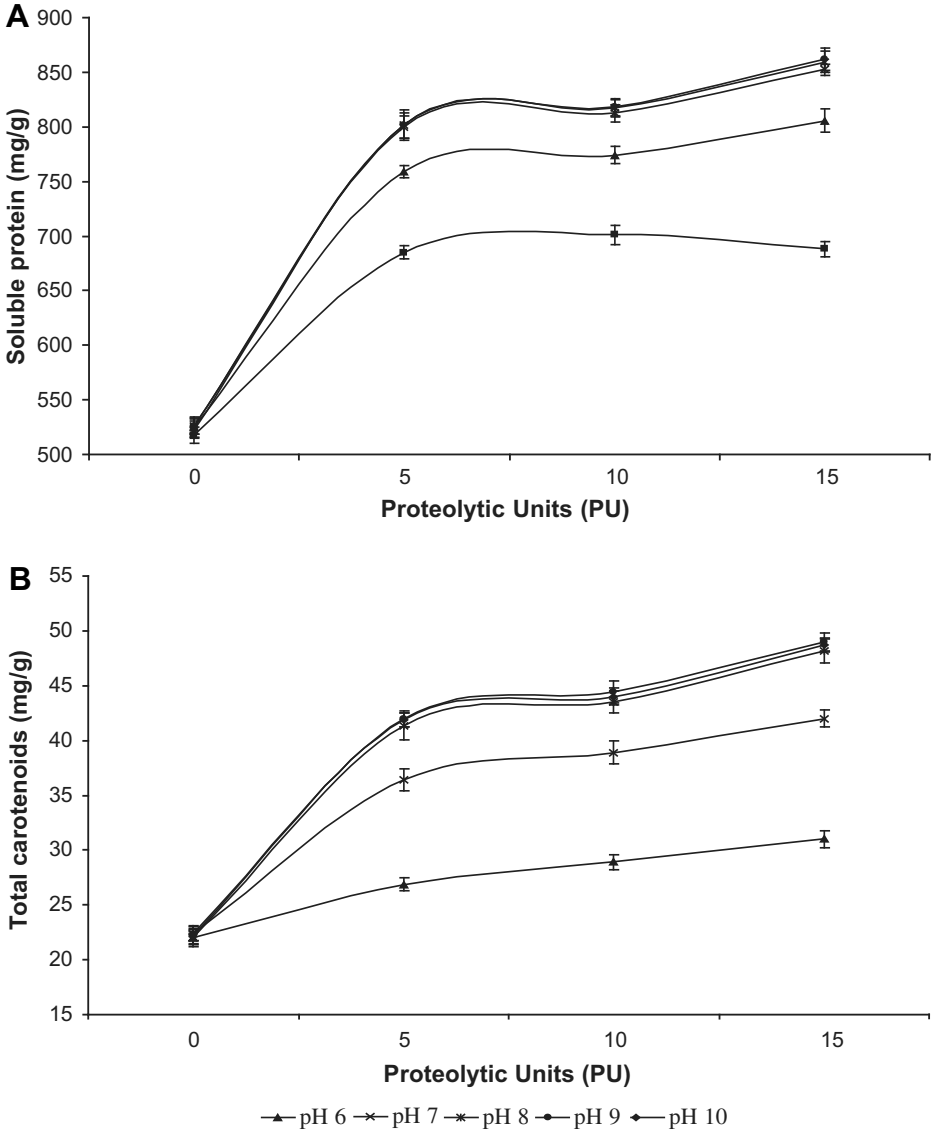

Fig. 2. Soluble protein (A) and total carotenoids (B) obtained from the enzymatic hydrolysis of carotenoproteins at different pH for 24 h at 40 °C ($n = 6$).

8 was further followed at 12 and 24 h by analysing soluble protein and total carotenoids.

2.2. Protease–lipase hydrolysis

Enzymatic activity for lipase was determined following the method reported by Kiran, Hari, Suresh, Karanth, and Divakar (2000) by using a tributyrin emulsion in phosphate buffer as the lipase substrate. One lipolytic unit (LU) was described as the amount of lipase required to produce 1 μmol of butyric acid/min at 40 °C. Four protease–lipase treatments at fixed 15 PU, were tested (15 PU:0 LU, 15 PU:5 LU, 15 PU:10 LU and 15 PU:15 LU) at pH 8 and 40 °C for 24 h, analysing soluble protein and total carotenoids at 12 and 24 h. Both protease and protease–lipase enzymatic treatments were compared to controls (treatment with no added protease or protease–lipase).

2.3. Soluble protein and total carotenoids

Soluble protein (mg/g carotenoprotein) was determined using the method reported by Peterson (1977) that uses a calibration curve of seroalbumin (0–300 mg/l). One millilitre of reagent A (made of equal parts of distilled water, copper–tartrate–carbonate solution, 10% solution of sodium dodecyl sulfate, and 0.8 N NaOH solution) was added to samples, or reference of seroalbumin, and reacted for 10 min at 25 °C; reagent B (0.5 ml of Folin–Ciocalteu reagent diluted 1:1 with distilled water) was added and the samples vortexed. Samples were placed in darkness for 30 min at 25 °C and the absorbance was measured at 750 nm. Tartrate–carbonate solution was a mixture of two solutions: 12.5 g of Na_2CO_3 dissolved in 62.5 ml of distilled water, and 0.125 g of $CuSO_4$ and 0.25 g of potassium tartrate were dissolved in 62.5 ml of distilled water.

Total carotenoid concentrations (mg/g carotenoprotein) were analysed using the spectrophotometric method reported by Armenta, Burja, Radianingtyas, and Barrow (2006) and Sachindra, Bhaskar, and Mahendrakar (2005), which uses the following formula:

$$\text{Total carotenoid}\,(\text{mg/g}) = \frac{A_{477\text{nm}} \times V_{\text{extract}} \times DF}{0.2 \times W_{\text{sample}} \times 1000}$$

where $A_{477\ \text{nm}}$ is the absorbance at 477 nm; V_{extract} is the volume of the extract; DF is the dilution factor; 0.2 is the $A_{477\ \text{nm}}$ value of 1 μg/ml astaxanthin standard; and W_{sample} is the weight of the sample.

2.4. TLC and HPLC analysis of astaxanthin

Forms of monoester, diester and free astaxanthin in supernatants were separated by TLC according to Sachindra et al. (2005), where the spots corresponding to each astaxanthin form were scrapped-off the TLC developed plate, suspended in acetone, filtered and concentrated. This concentrate, dissolved in petroleum ether, was purified twice by TLC. Free astaxanthin standard (kindly supplied by Roche, Basel, Switzerland), and TLC prepared monoester and diester of astaxanthin, were used for peak identification by high performance liquid chromatography with diode array detector (HPLC-DAD). Supernatants from enzymatic reactions were analysed by HPLC-DAD to determine astaxanthin percentage relative to total carotenoids measured spectrophotometrically. A Waters HPLC system was fitted with column and pre-column Water Symmetry C_{18} (Milford, MA, USA), and a mobile phase of acetonitrile/chloroform/methanol/water/propionic acid (71:22:4:2:1 by volume) was used under isocratic conditions with a flow of 1 ml/min.

2.5. Amino acid profiles of carotenoproteins

Amino acid contents in lactic acid fermented and non-fermented carotenoproteins were analysed in an amino acid analyzer Beckman model 6300 (Palo Alto, CA, USA) with Beckman buffers E, F and D, ninhydrin reagent diluted with deionised water, and Beckman regeneration buffer. All buffers were stored at 4 °C. Pre-hydrolysis for cysteine and methionine analysis, as well as all hydrolysis procedures were carried out as described by Gehrke, Wall, Absheer, Kaiser, and Zumwalt (1985). Samples were hydrolysed with HCl, and NaOH. Samples were prepared for acid hydrolysis by mixing 25 mg freeze dried carotenoprotein with 12.5 ml 6 N HCl in hydrolysis tubes with Teflon screw-caps. Air was removed from the solution by nitrogen purging, placing the tubes in an ultrasonic bath. The hydrolysis was carried out at 110 °C for 24 h, samples were then freeze dried, dissolved in 5 ml dilution buffer (0.2 N sodium citrate, pH 2.2) and filtered through 0.45 μm membranes (Gelman Acrodisc GHP No. 13). The filtered samples were injected to the amino acid analyzer.

As tryptophan is almost entirely destroyed during acid hydrolysis, its quantification was performed in basic conditions. The procedure was the same as the acid method, but 6 N NaOH was used instead 6 N HCl. Cysteine and methionine determination of samples was carried out by treating them first with performic acid, prior to protein hydrolysis, to form the acid derivatives (cysteic acid and sulphonated methionine, respectively). Twenty-five milligrams freeze dried of carotenoprotein in hydrolysis test tubes were placed in an ice bath. Performic acid was prepared by mixing 1 ml 30% H_2O_2 and 9 ml 88% formic acid; the mixture was left standing for 1 h at room of temperature. Before use, the mixture was cooled down in an ice bath; 10 ml cold performic acid were added with gently stirring to the carotenoprotein samples, and refrigerated at 0 °C for 14 h. One millilitre of cold 98% HBr was added while slowly stirring, and the tubes placed in ice for 5 min, then left standing at room temperature. It was freeze dried and hydrolysed by the HCl procedure, already described.

All enzymatic treatments were replicated six times. Data obtained for soluble protein and total carotenoid concentrations were subjected to the analysis of variance and Duncan's Multiple Range Test. Comparisons between carotenoprotein amino acid contents of fermented and non-fermented shrimp wastes were performed using student *t*-tests. Differences between individual means were deemed to be significant at $p < 0.05$. Statistical analyses were carried out using a SPSS package, version 13.0 (Chicago, IL, USA).

3. Results and discussion

3.1. Protein hydrolysis

Savinase™ was chosen for this study due to, as shown in a previous study (Armenta-Lopez et al., 2002), its high efficacy in carotenoprotein hydrolysis, producing >700 and >35 mg/g of soluble protein and total carotenoids, respectively. Considering that carotenoid oxidation mechanism is associated with unsaturated fatty acids reactions, rapidly occurring around 0 °C, enzymatic assay temperature was fixed at 40 °C because at this temperature a minimum carotenoid oxidation takes place (Simpson and Haard, 1985).

Fig. 2 shows a higher carotenoprotein hydrolysis ($p < 0.05$) at pH 8, producing up to 852 mg/g soluble protein and 48 mg/g total carotenoids when the treatment of 15 proteolytic units (PU) for 24 h at pH 8 was applied. This treatment (15 PU for 24 h, pH 8) also render up to 6% and 14% more soluble protein and total carotenoids, respectively, as compared to the same PU and time, but at pH 7 treatment. Moreover, 15 PU for 24 h, pH 8 produced up to 62% and 119% more soluble protein and total carotenoids, respectively, with respect to the control (no enzyme added). No significant difference ($p < 0.05$) with respect to hydrolysis amongst pH 8, 9 and 10 was observed (Fig. 2). The optimal hydrolysis from Savinase™ in detergents occurs at a pH 8–8.5 (Novozymes).

Table 1 shows that with 15 PU, either at 12 or 24 h, high carotenoprotein hydrolysis was observed (>840 and >46 mg/g of soluble protein and carotenoids, respectively, $p < 0.05$). No significant differences were observed between 12 h and 24 h of hydrolysis ($p < 0.05$) within each enzymatic treatment. Therefore, the fastest and most effective treatment was applying the treatment of 15 UP for 12 h at a pH 8.

Soluble protein concentrations were higher than those found in previous studies on shrimp waste hydrolysis, reporting 560 mg/g with cod trypsin at 4 °C for 48 h, pH 7.7 (Cano-Lopez et al., 1987), and 800 mg/g using bacterial proteases and trypsin (Simpson and Haard, 1985). Furthermore, using Savinase™ on fermented carotenoproteins, the yield of soluble protein increased by 60% as compared to hydrolysis with proteases extracted from shrimp wastes (Armenta-Lopez et al., 2002). Therefore, in order to obtain an extensive carotenoprotein hydrolysis, our results suggest using the treatment that includes 15 PU for 12 h at 40 °C, pH 8.

The high carotenoprotein hydrolysis achieved with Savinase™ (852 and 48 mg/g of soluble protein and total carotenoids, respectively) may be related to the capacity of this enzyme to attack pro-

Table 1
Soluble protein (mg/g) and total carotenoids (mg/g) obtained from the proteolysis of carotenoproteins at pH 8 for 0, 12 and 24 h at 40 °C[AB]

Treatment			
PU[C]	Time (h)	Soluble protein	Total carotenoids
0	0	524.88 ± 5.36[a]	21.35 ± 1.40[a]
0	12	529.11 ± 13.21[a]	22.14 ± 0.92[a]
0	24	525.78 ± 12.85[a]	22.00 ± 1.90[a]
5	0	529.11 ± 12.14[a]	22.90 ± 0.84[a]
5	12	703.90 ± 9.54[b]	33.70 ± 1.74[b]
5	24	729.60 ± 14.70[c]	37.30 ± 1.24[c]
10	0	530.30 ± 11.32[a]	21.62 ± 0.79[a]
10	12	740.80 ± 7.96[c]	40.90 ± 1.52[d]
10	24	772.53 ± 11.00[d]	42.50 ± 0.86[d]
15	0	526.65 ± 8.75[a]	21.08 ± 1.28[a]
15	12	843.66 ± 9.20[e]	46.30 ± 1.05[e]
15	24	852.22 ± 8.90[e]	48.18 ± 0.93[e]

[A] Data expressed as means of six replicates ± standard deviations.
[B] Different letters within a column denote significant differences ($p < 0.05$).
[C] Proteolytic units.

tein–lipid complexes. Due to this property, Savinase™ is commercially used in biodegradable detergents (Novozymes).

3.2. Protease–lipase hydrolysis

As a protease was used together with a lipase, carotenoproteins were severely hydrolysed with the treatments including 15 PU:15 LU and 15 PU:10 LU for either 12 or 24 h, producing 900 and 66 mg/g of soluble protein and total carotenoids, respectively ($p < 0.05$, Table 2). In relation to the use of a protease solely (15 PU for 12 or 24 h), the 15 PU:10 LU treatment increased up to 6% and 38% soluble protein and total carotenoid content, respectively. Also, the protease–lipase treatment increased up to 71% and 200% soluble protein and total carotenoid concentration, respectively, as compared to the control.

Table 2
Soluble protein (mg/g) and total carotenoids (mg/g) obtained from the proteolysis–lipolysis of carotenoproteins at pH 8 for 0, 12 and 24 h at 40 °C[AB]

Treatment				
PU[C]	LU[D]	Time (h)	Soluble protein	Total carotenoids
0	0	0	523.84 ± 7.25[a]	22.00 ± 1.02[a]
0	0	12	528.00 ± 14.55[a]	22.95 ± 1.85[a]
0	0	24	520.82 ± 18.40[a]	21.90 ± 1.70[a]
15	0	0	524.04 ± 17.80[a]	22.30 ± 1.68[a]
15	0	12	839.66 ± 9.95[b]	44.77 ± 2.08[b]
15	0	24	844.28 ± 8.80[b]	47.82 ± 1.55[b]
15	5	0	520.62 ± 14.00[a]	22.12 ± 1.10[a]
15	5	12	841.18 ± 8.10[b]	49.49 ± 1.00[b]
15	5	24	847.00 ± 15.26[b]	55.87 ± 1.29[c]
15	10	0	526.43 ± 13.89[a]	22.15 ± 0.95[a]
15	10	12	895.07 ± 16.70[c]	65.08 ± 0.97[d]
15	10	24	900.72 ± 13.60[c]	66.11 ± 1.04[d]
15	15	0	525.00 ± 11.03[a]	22.48 ± 1.79[a]
15	15	12	903.61 ± 10.80[c]	64.20 ± 2.10[d]
15	15	24	896.65 ± 10.70[c]	66.24 ± 0.88[d]

[A] Data expressed as means of six replicates ± standard deviations.
[B] Different letters within a column denote significant difference ($p < 0.05$).
[C] Proteolytic units.
[D] Lipolytic units.

The carotenoprotein hydrolysis increased when a protease together with a lipase was used, probably because lipase solubilised the lipids contained within the lipoprotein moiety of the carotenoprotein complex. This may have facilitated the protease access to apoprotein subunits that form carotenoproteins. Consequently, because each apoprotein unit contains up to two astaxanthin molecules (Nur-E-Borhan et al., 1995; Schiedt et al., 1993), total carotenoid yield increased as the protein was more efficiently hydrolysed. In addition, as crustacean astaxanthin is mostly esterified to fatty acids (Guillou et al., 1995), ester bond hydrolysis by lipases can improve free astaxanthin extraction (astaxanthin with no esterified lipids). Potentially, astaxanthin can be bound to other molecules by imine and ester bonds (Fig. 3). However, not all bonds may simultaneously occur due to steric hindrance.

Astaxanthin had a maximum light absorption at 474 nm, and 5 min retention time in the HPLC analysis; it was approximately 90% of the total carotenoids shown in Fig. 2, and Tables 1 and 2. It was found that within this 90% of astaxanthin, approximately 50%, 30% and 20% were diester, monoester and free forms of astaxanthin, respectively. A previous study reported that astaxanthin is the main carotenoid (>80%, including diester, monoester and free forms) in carotenoproteins (Nur-E-Borhan et al., 1995; Zagalsky, Eliopoulos, & Findlay, 1990). Guillou et al. (1995) reported that in shrimp (*P. borealis*) waste silage, astaxanthin is found as 76% diester, 20% monoester and 4% free forms. The difference with our results may be attributed to the fact that we used a different shrimp (*Litopenaeus vannamei*) processing discard.

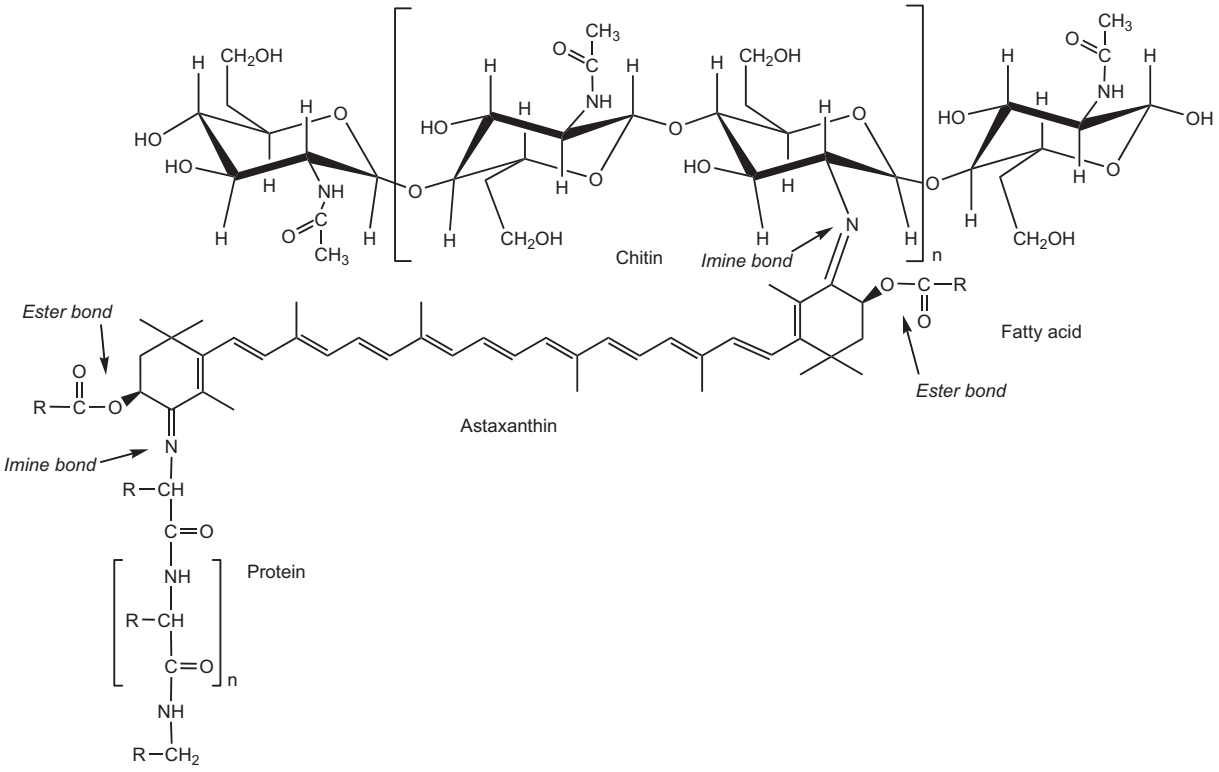

Fig. 3. Possible chemical bindings of astaxanthin with other molecules in shrimp (not all bindings may simultaneously occur due to steric hindrance).

Table 3
Amino acid profiles of shrimp carotenoproteins (mg/g of carotenoprotein)[AB]

Amino acid	Non-fermented carotenoprotein	Fermented carotenoprotein
Alanine	45.4 ± 0.7[a]	45.0 ± 0.6[a]
Arginine	58.0 ± 0.6[b]	46.2 ± 0.5[a]
Aspartic acid	129.5 ± 1.5[a]	126.7 ± 1.4[a]
Cysteine	14.0 ± 0.5[a]	13.0 ± 0.5[a]
Glutamic acid	142.6 ± 1.4[a]	142.2 ± 1.6[a]
Glycine	36.2 ± 0.5[b]	31.2 ± 0.4[a]
Histidine[C]	23.0 ± 0.3[a]	32.1 ± 0.4[b]
Isoleucine[C]	40.2 ± 0.6[a]	39.8 ± 0.5[a]
Leucine[C]	112.3 ± 1.4[a]	110.1 ± 1.2[a]
Lysine[C]	99.9 ± 0.8[a]	99.2 ± 0.7[a]
Methionine[C]	20.2 ± 0.3[a]	20.4 ± 0.3[a]
Phenylalanine[C]	42.6 ± 0.4[a]	51.3 ± 0.6[b]
Proline	25.3 ± 0.3[a]	25.6 ± 0.3[a]
Serine	32.3 ± 0.3[a]	40.0 ± 0.4[b]
Taurine	1.9 ± 0.2[a]	2.1 ± 0.1[a]
Threonine[C]	38.4 ± 0.3[a]	55.7 ± 0.4[b]
Tryptophan[C]	19.4 ± 0.2[a]	36.0 ± 0.3[b]
Tyrosine	21.7 ± 0.2[a]	35.5 ± 0.3[b]
Valine[C]	47.0 ± 0.5[b]	37.2 ± 0.4[a]
Total amino acids	949.9 ± 11.0[a]	989.3 ± 10.9[b]
Sum EAA[D]	443.0 ± 4.8[a]	481.8 ± 4.8[b]
Sum NEAA[E]	506.9 ± 6.2[a]	507.5 ± 6.1[a]

[A] Data expressed as means of three replicates ± standard deviations.
[B] Different letters within a row denote significant differences ($p < 0.05$).
[C] Essential amino acids in adults.
[D] Essential amino acids.
[E] Non-essential amino acids.

3.3. Amino acid profiles of carotenoproteins

Both carotenoproteins, lactic acid fermented and non-fermented, were rich in aspartic and glutamic acids, and the essential amino acids leucine and lysine (128, 142, 111 and 100 mg/g, respectively) (Table 3). Fermented carotenoproteins were a significant source ($p < 0.05$) of the essential amino acids histidine, phenylalanine, threonine and tryptophan (32, 51, 56 and 36 mg/g, respectively). The essential amino acid valine was found in a higher concentration (47 mg/g) in carotenoprotein from non-fermented shrimp waste. The non-essential amino acids serine and tyrosine were found in higher concentrations in carotenoprotein of fermented wastes (40 and 36 mg/g, respectively), while glycine was at a higher concentration (36 mg/g) in non-fermented waste carotenoprotein (Table 3).

The higher concentration of essential amino acids such as histidine, phenylalanine, threonine and tryptophan in carotenoproteins obtained from fermented wastes, was possibly due to debris of lactic acid bacteria, the starter used for shrimp fermentation. The total amount of essential amino acids found in fermented carotenoproteins was 49% of total amino acids. This amount agreed with that reported by Cremades et al. (2003) in a study on carotenoproteins obtained from fermented crayfish waste.

Since astaxanthin increases its red–orange colour as it is separated from the protein moiety (Armenta-Lopez et al., 2002; Guillou et al., 1995; Schiedt et al., 1993), enzymatic splitting of the astaxanthin and protein moieties from carotenoproteins may affect the commercial uses of this carotenoid. For example, the relatively protein-free natural astaxanthin may be of interest for salmon culture due to health considerations over the use of synthetic astaxanthin (Meyers, 1994; Lorenz and Cysewski, 2000). Furthermore, natural astaxanthin may be of interest for its use in natural health products due to its lack of allergic reactions (Arad and Yaron, 1992; Oshima, 1998) and its high antioxidant capacity (Shimidzu, 1996). The red–orange colour developed by astaxanthin bound to a reduced protein amount may also be of interest for use in cosmetics (Arad and Yaron, 1992; Oshima, 1998). Thus, high astaxanthin recovery, together with highly soluble protein recovery, from fermented shrimp wastes may add value to the shrimp industry. Our study found that astaxanthin was in the forms of 50% diester, 30% monoester and 10% free astaxanthin. Fatty acids such as eicosapentaenoic acid and docosahexaenoic acid could be found in esterified astaxanthin of shrimp waste (Guillou et al., 1995), which could increase the nutritional value of the pigment. Finally, due to the high content of essential amino acids in the fermented lactic acid carotenoprotein, the latter may be considered as an excellent protein source to food and feed products.

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