Research Article Carotenoprotein from by-product of banana shrimp (*Penaeus merguiensis*) extracted using protease from viscera of rainbow trout: antiradical and angiotensin I-converting enzyme inhibitory activity

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Abstract

Carotenoprotein as a marine bioactive compound was recovered from banana shrimp by-product using protease from rainbow trout viscera at various levels (5 and 15 units/g shrimp shell). The yield of carotenoprotein increased with increasing enzyme concentration up to 51.6%, which was consistent with increasing degree of hydrolysis. Protein and lipid content of the hydrolysate was 79.6 and 9.4 percent, respectively. The hydrolysate had a high nutritional value with a high amount of essential amino acids (465 mg/g) compared to the control (254 mg/g). The carotenoprotein exhibited a dosedependent increase in antiradical activity measured by two in vitro assays, with an efficiency of 94 and 57 percent for DPPH and ABTS scavenging activities, respectively. Protease-extracted carotenoprotein showed higher ACE inhibiting effect than the control at different concentrations (p<0.05). The results from this study showed that carotenoprotein from banana shrimp have both radical scavenging activity and antihypertensive properties and a high content of essential amino acids, and could be good source for value-added nutritive food ingredients.

Keyword: Rainbow trout viscera, Protease, Banana shrimp, Carotenoprotein, Radical scavenging, Activity, ACE inhibitory

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Introduction

Shrimp has become an important product in the world fishery economy, both due to its volume (about 20% of the world's seafood market) and wide geographical distribution. Annual shrimp fishery and production of aquaculture in the world is over 8 million tons (FAO, 2017). Depending on species and processing methods, solid (head. waste legs, shell) generation during processing of shrimps ranges from 50 to 60% of total body weight (Saini et al., 2018). Typically, these by-products are discarded to the environment without any use or treatment (Prameela et al., 2017). Shrimp by-products could be interesting source of chitin, protein, other nutritive components and carotenoids with functional or biological properties, including antiradical activity (Nwe et al., 2014; Senphan et al., 2014; Hajji et al., 2018). Converting shrimp byproduct to value added compounds can help solve the problem of its disposal and create extra revenue, which can contribute to develop seafood-based economy. Generally carotenoids in crustaceans are bound to a high-density lipoprotein. These compounds, which have a stable complex structure, are called carotenoprotein (Shahidi and Brown, 1998). Today there is an increasing interest in the use of carotenoprotein in food industry because of its natural origin, nontoxicity and being a vitamin A precursor (Hamdi et al., 2017, 2018).

Different methods/techniques have been used to extract bioactive products such

as carotenoid and carotenoprotein from crustacean by-products. These include organic solvents (Hamdi et al., 2018), fermentation (Cremades et al., 2001; Bueno-Solano et al., 2009), vegetable oils (Sowmya and Sachindra, 2012), and supercritique fluid extraction (Razi Parjikolaei et al., 2017). Enzymatic hydrolysis using enzymes from different sources has also been investigated as an alternative method for extracting bioactive compounds (Chakrabarti, 2002; Thiansilakul et al., 2007; Babu et al., 2008; Kishimura et al., 2008; Senphan et al., 2014; Sila et al., 2014; Poonsin et al., 2018). To reduce the cost of commercially available enzymes, one possibility is to use protease extracted from sources such as fish viscera for carotenoprotein extraction. Alkaline proteases extracted from fish digestive system (intestine, pyloric caeca, etc.) cleave peptide bonds (Kishimura et al., 2008; Poonsin et al., 2018) and via hydrolysis process can improve specific properties of protein, such as amino acid content, antiradical and antihypertensive activities compared to the raw material. The aim of the present study was to determine amino acid profile, functional properties, and bioactive potential (radical scavenging activity and antihypertensive activities) of carotenoprotein extracted from banana shrimp with partially purified protease from rainbow trout (Oncorhynchus mykiss) digestive system. The research aims to increase the value of seafood side streams and reduce environmental problems.

Materials and methods

Chemicals/enzyme

2,2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) di-ammonium salt (ABTS), 2,4,6-Tris(2-pyridyl)-s-triazine and 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 3-(2-pyridyl)-5–6diphenyl-1,2,4- triazine-4',4"disulphonic acid sodium salt (ferrozine) were reagent grade and obtained from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Merck Co. (Darmstadt, Germany).

Preparation of shells from Banana shrimp

processing Shrimp by-product (including heads, shells and tails) of banana shrimp (Penaeus merguiensis) were provided fresh by a local shrimp in processing plant Hormozgan Province. Iran. Samples were transported on ice (sample to ice ratio 1:2) to Seafood Processing Laboratory in marine sciences department at Tarbiat Modares University of Iran within 3h after production. The byproducts were dried by flowing hot air (Memmert, Schwabach, Germany) for 6h at 60°C, and eventually turned into small particles (0.1 mm) using a Panasonic MX-GX1571 blender (Japan) and kept in dark (4° C).

Extraction of alkaline protease from rainbow trout digestive tract Sample preparation

Rainbow trout with an average weight of 650g was purchased from a fish farm in Mazandaran Province, Iran. The fish were immediately eviscerated and the digestive system, including intestine and pyloric caeca were separated and used for protease extraction. The samples were cut into pieces and homogenized with three volumes of cold acetone to remove the fat. The homogenate was filtered on Whatman No. 4 paper and the residue was dried under vacuum condition at room temperature for 6h. To prepare the protease extract, the acetone-dried residue was mixed with 10 mM trisbuffer (pH 8.0) at a ratio of 1:30 (w/v) and shaken for 3h at 4°C. The alkaline crude protease extract (CPE) was using refrigerated recovered a centrifuge (Hettich, Universal 320R, Germany) centrifuging the homogenate at 7250g for 30 min (Senphan et al., 2014).

Ammonium sulfate precipitation

The CPE was subjected to ammonium sulfate precipitation at 40-60% w/v saturation by slow continuous stirring in a cold room (Michail *et al.*, 2006). Initial experiments in our laboratory showed that using 40-60% saturation resulted in enzyme with higher purity and efficiency compared to other ranges of saturation (0-20%, 20-40% and 60-80%). After dialyzing against extraction buffer, the precipitate was lyophilized and referred to as partially purified alkaline protease.

Protease activity assay

Activity of the alkaline protease was measured as described by Lassoued *et al.* (2015), using casein as the substrate. The assay was performed at pH 8 and

55°C. A blank was similarly conducted except that the enzyme was added immediately after addition of trichloroacetic acid (5% w/v). One unit of enzymatic activity was defined as the amount of enzyme capable of releasing 1 umol of tyrosine per min per mg soluble protein of the protease extract. The protein content was determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. All measurements were run in triplicate.

Extraction of the carotenoprotein

Enzymatic extraction of carotenoprotein was performed according to a slightly modified version of Simpson and Haard method (1985). Ground shrimp byproducts (50g) were blended with five volumes of water (pH 8.0) and preincubated at 55°C for 20 min prior to adding crude protease extract. Two levels of protease activity (5 and 15 units/g shrimp shell) were used. The mixture was shaken in a shaker at 200 rpm and 55°C for 120 min. The mixture was heated by microwave for 5 min at >90°C to inactivate temperature enzyme and centrifuged at 8000g for 10 min. The supernatant was lyophilized (Christ, Alpha 1-2 LDplus, Australia) and the dry matter was referred to as carotenoprotein. The control was made in the same way, but without addition of enzyme.

Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the ratio (%) of α -amino nitrogen with respect to total nitrogen of carotenoprotein, was determined using formol titration method as describe by Taylor (1957):

DH (%) =
$$\frac{(B2 - B1) \times \text{atomic weight of nitrogen}}{\text{amino nitrogen assayed by formol titration } \times \text{TN}} = \frac{\text{AN} \times 100}{\text{TN}}$$

B1 = ml 0.1 N-NaOH consumed by sample at certain time.

B2= ml 0.1 N-NaOH consumed by blank.

TN= total nitrogen assayed by micro Kjeldahl method.

Determination of chemical composition

Moisture and ash content were determined after heating in an oven at 103 and 550°C, respectively, until constant weight. Total protein was measured by Kjeldahl method (AOAC, 1990) and the lipid content was determined by the Soxhlet method. Chitin determined content was according to the method of Spinelli et al. (1974) as modified by Simpson and Haard (1985). Dried shrimp waste (2g) was mixed with 30 mL of 2% (w/v) NaOH at 25°C for 6h. After filtration, the residue was shaken with 15 mL of 1M HCl for 30 min at 25°C, filtered and washed with deionized water and then homogenized with cold acetone (-20°C) for 3 min to remove the pigment. The mixture was then washed and dried at 60°C for 24h using an oven (Memmert,

Schwal	oach, Germany	y) and the	dried	determined according to Saito and		
matter was referred to as "chitin".				Regier (1971) and Simpson and Haard		
				(1985), with slight modification. The		
Total carotenoids				carotenoid content was calculated as		
Total	carotenoid	content	was	astaxanthin using the equation given by		
				Saito and Regier (1971):		

Carotenoid content ($\mu g/g$ sample) =

 $A468 \times volume of extract \times dilution factor$ $0.2 \times weight of sample used in gram$

Where 0.2 is the A468 of 1 g/ml standard astaxanthin.

Determination of colour

Spectral reflectance of samples was measured using colourimeter a (micromatch spectrophotometer 181/3, Sheen Instruments, UK) calibrated against black and white tiles. CIE L*, a* and b* system was used to determine colour parameters values where L* defines lightness and varies between 0 (absolute black) and 100 (absolute white), a* measures greenness (+a*) or redness (-a*) and b* measures blueness (+b*) and yellowness (-b*). Colour of the sample was calculated using the following equation:

 $\Delta E_{ab}^{*} = [(\Delta L^{*})^{2} + (\Delta a^{*})^{2} + (\Delta b^{*})^{2}]^{\frac{1}{2}}$

Amino acid analysis

Amino acid composition of extracted carotenoprotein was determined according to the method of Gehrke et al. (1985). The samples were hydrolyzed in 6M HCl for 24h at 110°C and analyzed using HPLC (Knauer, Germany) with a fluorescence detector (RF-530, Knauer). Then samples were with o-phthaldialdehyde derivatized (OPA) and analyzed using a C18 column (Knauer) at a flow rate of 1 mL/min with fluorescence detector (RF-530, Knauer, Germany).

Molecular weight

Molecular weight distribution of the carotenoprotein was determined by fast protein liquid chromatography system (ÄKTA. Amersham Biosciences. Sweden) on a SuperdexTM 10/300 GL column at a flow rate of 0.5 mL/min, which separates the sample with a molecular weight range from 100 to 7000 g/mol. A molecular-weight calibration curve was plotted from the average elution volume of the following standards: Cytochrome c (12327 Da), Aprotinin (6511 Da), Vit B12 (1355 Da) and Cysteine (121.16 Da). The chromatographic data were recorded and analyzed by Unicorn software.

Antiradical activity

Different concentrations (0.25, 0.5, 1.0 and 2.5 mg/mL) of the carotenoprotein were prepared for determination of radical scavenging activity.

DPPH radical-scavenging capacity

The DPPH radical scavenging activity was determined according to the method of Thiansilakul *et al.* (2007). The sample (1.5 mL) was mixed with 1.5 mL of 0.15 mM DPPH in 95% methanol and allowed to stand in dark at room temperature for 30 min. Absorbance of the resulting solution was measured at 517 nm using a spectrophotometer. A blank was made in the same way except that distilled water was used instead of carotenoprotein. For the control sample, ethanol was used instead of DPPH ethanol solution. Radical scavenging ability (RSA, %) was calculated as follows:

RSA % = (Absorbance _{blank} - (Absorbance _{sample} – Absorbance _{control})) \times 100

ABTS radical scavenging activity

ABTS radical scavenging activity was evaluated as described by Senphan *et al.* (2014). The working solution was prepared by mixing equal quantities of 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. After 12h, sample (150 μ L) was mixed with 2850 μ L of ABTS solution. Absorbance was then measured at 734 nm using spectrophotometer after 2h. The activity was calculated as follows:

RSA % = (Absorbance _{blank} - (Absorbance _{sample} – Absorbance _{control})) \times 100

ACE-inhibiting activity

Angiotensin-converting enzyme (ACE) inhibitory activity of sample was investigated by method of Sentandreu and Toldrá (2006). This procedure is based on the reaction between ACE and o-aminobenzoylglycyl-p-nitro-Lphenylalanyl-L-proline (Abz-Gly-Phe-(NO2)-Pro) as a fluorescent substrate. The fluorescence was measured using excitation and emission wavelengths of 355 and 405 nm, respectively (Fluorescence Spectrometer 3000. Perkin Elmer, UK).

Statistical analysis

All experiments were conducted with three replicates. The obtained data were subjected to one-way analysis of variance (ANOVA) to determine differences. Duncan's test was used for comparison of means to determine the significant difference between samples within 95% confidence interval using SPSS version 22.0 (SPSS Statistical Software, Inc., Chicago, IL, USA).

Results

Chemical composition and colour

Protein was the major component (on a dry matter basis) both in shrimp byproduct and in carotenoprotein (CP) followed by chitin, ash and lipid. Protein content of CP (79.6%) was higher than that in shrimp by-product (52.5%, p<0.05). Furthermore, lipid content of carotenoprotein was significantly higher than that in shrimp shell. Colour values of samples are shown in Table 1.

Compositions	Shrimp by-product	Carotenoprotein
Protein (% dry wt)	52.48±0.15	79.57±0.36
Fat (% dry wt)	4.35±0.01	9.39±0.90
Ash (% dry wt)	$18.40{\pm}1.07$	11.22±0.42
Chitin (% dry wt)	27.01±0.96	0.56±0.00
Carotenoid (µg/g)	16.54±1.09	55.96±0.00
Colour		
L*	52.7±3.32	34.9±1.01
a*	10.1±0.65	19.9±0.50

Table 1: Proximate composition of banana shrimp	by-product and carotenoprotein recovered with
rainbow trout proteases, mean ± standard	l deviation.

Amino acid composition

Amino acid profile of carotenoprotein extracted with or without rainbow trout protease treatment is shown in Table 2. Carotenoprotein obtained from banana shrimp by-product had higher total amino acid (978 mg/g) and total essential amino acid (465 mg/g) contents than those found in the control (637 and 254 mg/g, p<0.05).

Molecular weight

Molecular weight distribution profile of carotenoprotein obtained with or without protease from rainbow trout viscera is shown in Figure 1.

Antiradical activity of carotenoprotein

DPPH and ABTS radical scavenging activity of lyophilized carotenoprotein is presented in Figure 2. As described for the DPPH assay, ABTS radical scavenging activity gradually increased as carotenoprotein concentration increased from 0.25 to 2.5 mg/mL. At 2.5 mg/mL, ABTS radical scavenging activity was significantly higher than that in the control (13% higher, p<0.05).

	Carotenoprotein			
Amino acid	Control	Trout protease		
	Control	extracted		
Asp	77.92	122.43		
Glu	95.48	149.18		
Asn	0.41	0.58		
His ^A	16.34	31.87		
Ser	40.08	54.19		
Gln	1.09	0.19		
Gly/Arg	43.68	62.60		
Thr ^A	34.41	57.54		
Tyr	88.03	128.57		
Ala	13.41	22.22		
Aba	22.29	15.70		
Met ^A	12.46	27.89		
Val ^A	32.20	62.79		
Phe ^A	41.77	75.41		
Ile ^A	30.26	63.67		
Leu ^A	53.89	94.29		
Lys ^A	33.54	58.39		
Total amino	637 26	1027 51		
acids	037.20	1027.31		
Total EAA ^B	254.87	471.85		
Total NEAA ^C	382.39	555.66		

 Table 2: Amino acid profiles of shrimp carotenoprotein (mg/g of carotenoprotein).

^A Essential amino acids in adults

^B Essential amino acids

^C Non-essential amino acids

ACE-inhibiting activity

ACE inhibitory activity of carotenoprotein at different concentrations is shown in Figure 3. The results showed that carotenoprotein recovered with rainbow trout proteases had significantly higher ACE inhibitory effect compared to that in the control, reaching an IC50 value of 3.87 mg/mL.



Figure 1: Molecular weight distribution profile of carotenoprotein recovered with or without rainbow trout protease.



Figure 2: Antiradical activities of carotenoprotein recovered from banana shrimp by-product at different concentrations. (a) DPPH radical scavenging activity (b) ABTS radical scavenging activity. Error bars show standard deviation.



Figure 3: ACE inhibitory activities of carotenoprotein recovered from banana shrimp by-product at different concentrations.

Discussion

Effect of protease concentration on carotenoprotein recovery from shrimp by-product

At the same hydrolysis time, no difference significant was found between the yield of carotenoprotein at a protease concentration of 5 unit/g and that of the control (41.4 and 36.2%, respectively) but a higher yield was obtained when the fish protease concentration was increased to 15 unit/g (51.6 %, p < 0.05). In addition, the effect of enzyme concentration on DH of carotenoprotein showed the same trend with an increase in enzyme level from 5 to 15 unit/g. Senphan et al. (2014) reported an increase in shrimp enzyme concentration (5 to 30 units/g shrimp shells), resulted in a significant increase in extracted protein from pacific white shrimp shells (Penaeus vannamei, p < 0.05). Also, Tunisian barbel (*Barbus*) *callensis*) proteases (1.0 units/g protein) vielded less than 12% recovery of carotenoprotein from deep-water pink shrimp (Parapenaeus longirostris) processing waste after 8h of hydrolysis (Sila et al., 2012). Overall, the results indicated that rainbow trout protease had a dose-dependent ability to increase yield recovery and DH value from shrimp processing by-product. Senphan et al. (2014) found that actin, totally disappeared Sodium dodecvl in sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) when protease from hepatopancreas was used.

Chemical composition

Protein content of CP (79.6%) was higher than that in shrimp by-product (52.5%, *p*<0.05). Furthermore, lipid carotenoprotein content of was significantly higher than that in shrimp shell but ash and chitin contents of carotenoprotein was lower than those in the by-product (p < 0.05). Senphan *et al.* (2014) reported protein content in Pacific white shrimp waste to be 43.89%. As shown in Table 1, the results in our study showed that extracted carotenoprotein had a higher protein content than that in freeze-dried carotenoprotein recovered from shrimp shell with the treatment of barbel trypsin (71.09% protein and 16.47% lipid, Sila et al., 2012). Ash and chitin content of carotenoprotein were similar to those reported by Chakrabarti (2002). Accordingly, protease from rainbow trout digestive system could effectively hydrolyze banana shrimp by-product to facilitate release of protein as main component and co-extract fat or lipoprotein, while insoluble undigested nonprotein substances were retained.

The colour of carotenoprotein

Colour has a major effect on overall acceptability of a product. Carotenoprotein had a dark orange colour, with a*-value of 19.9 and was both more red, darker (L*= 34.9) and more yellow (b*= 29.2) than shrimp by-product (p<0.05, Table 1.). Natural astaxanthin is usually found either bound to proteins, or esterified with fatty acids in diester, monoester and free forms (Armenta and Guerrero-

Legarreta, 2009). Higher a*- and b*values in the samples showed that astaxanthin with protein bound to it was recovered to high level from banana shrimp by-product.

Amino acid composition

The nutritional value of a food depends on the type and amount of amino acids available for bodily functions (Bueno-Solano et al., 2009). Carotenoprotein obtained from banana shrimp byproduct had higher total amino acid (978 mg/g) and total essential amino acid (465 mg/g) contents than those found in the control (637 and 254 mg/g, p < 0.05, Table 2). Both carotenoproteins obtained with fish enzyme and that of the control had high content of glutamate (131.41 and 95.48 mg/g), aspartate (113.77 and 77.92 mg/g) and tyrosine (114.51 and 88.03 mg/g), respectively. These results are in agreement with the results of Yuan et al. (2018) on hydrolysates from oriental shrimp (Penaeus chinensis) with commercial trypsin. Likewise. Klomklao et al. (2009) found that the carotenoprotein extracted from black tiger shrimp (Penaeus monodon) waste treated with the aid of Bluefish proteases was also rich in these amino acids.

Molecular weight

The molecular weight distribution profile of the carotenoprotein obtained with or without protease from rainbow trout viscera is shown in Figure 1. Analysis of peptide size distribution indicated that the major part of the samples was composed of different peptides with molecular weight ranging from approx. 300 to 3000 Da. As expected, the protein in the sample that was recovered with the aid of protease was more hydrolysed with a higher of small amount peptides than carotenoprotein recovered without enzyme (p<0.05). Slizyte et al. (2016) found that peptides in FPH of salmon molecular backbones had weight between approx. 5500 to 6500 Da. Small peptides have a high potential for absorption in intestine (Roberts et al., 1999).

Antiradical activity of carotenoprotein DPPH radical-scavenging capacity

Protease-extracted carotenoprotein showed increasingly DPPH free radical scavenging activity with increasing concentration (Fig. 2a). The activity was significantly higher than that of the control (non-hydrolyzed) at the higher concentrations (*p*<0.05). At 2.5 mg/mL. concentration of scavenging activity of control was 60.4%, whereas that of the carotenoprotein was 94.2%. These results are in accordance with those reported by Senphan et al. (2014) and Sila et al. (2014) on carotenoprotein. In addition, both the DH and type of enzyme may affect hydrogen-donating ability (Shavandi et al., 2017). As shown in Table 2, the carotenoprotein extracted by means of rainbow protease has a high content of tyrosine, methionine, lysine and histidine that possibly contribute to antiradical activity (Chen et al., 1996).

ABTS radical scavenging activity

Protease-extracted carotenoprotein has higher ABTS scavenging activity than the control sample (p < 0.05). This is presumably enzymatic due to hydrolysis of banana shrimp protein, resulting in production of various antioxidative peptides (Kittiphattanabawon et al., 2012). As described for the DPPH assay, ABTS radical scavenging activity gradually carotenoprotein increased as concentration was increased from 0.25 to 2.5 mg/mL. At 2.5 mg/mL, the ABTS radical scavenging activity was significantly higher than that of the control (13%)higher, *p*<0.05). Antiradical activity of carotenoprotein is due to presence of antiradical peptides, as well non-protein as antiradical compounds such as carotenoids (55.96 µg/g sample) which act as electron donors reacting with free radicals (such as ABTS and DPPH) leading to formation of more stable products and terminating radical chain reactions.

ACE-inhibiting activity

The results showed that carotenoprotein recovered with rainbow trout proteases had significantly higher ACE inhibitory effect compared to that of the control, reaching an IC50 value of 3.87 mg/mL. As the same shrimp by-product was used for extraction of carotenoproteins, the difference in ACE inhibitor activity is suggested to be due to the ability of proteases to break down the protein of raw material into ACE-inhibiting peptides (Slizyte *et al.*, 2016). There was no significant difference between the ACE inhibitory activity of 100 and 250 µg/mL. ACE inhibitory activity of hydrolysates depends on the cumulative effect of different peptide chains instead bioactive of а single peptide (Ambigaipalan and Shahidi 2017). Several ACE inhibitory peptides have been recovered from different protein sources (Bougatef et al.. 2008: Geirsdottir et al., 2011; Lin et al., 2012). IC50 value for banana shrimp by-product hydrolysate is lower than hydrolysates from frozen shrimp (Ambigaipalan and Shahidi 2017). defatted salmon backbones (Slizyte et al., 2016) with an IC50 more than 4.8 mg/mL, whereas it is higher than those of wild and farmed cod hydrolysates (Jensen et al., 2013).

Rainbow trout protease was found to be efficient in hydrolysis and recovery of carotenoprotein complexes from banana shrimp by-product with a maximum yield of 51.6%. The enzyme-aided hydrolysate had protein, fat. and pigment content that were higher than that of the shrimp by-product. It also had good nutritional profiles due to high contents of essential amino acids. The hydrolysate from banana shrimp by-product also showed in vitro radical scavenging activity and ACE-inhibiting effect with IC50 of 3.87 mg/mL. The carotenoprotein exhibited potential bioactive properties that could be a potential source of natural antiradical and value-added nutritive ingredients in various foods, which may offer a new field for improved use of shrimp processing by-product.

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