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pH-Solubilization Process as an Alternative to Enzymatic Hydrolysis Applied to Shrimp Waste

Julio H. Córdova-Murueta^{1,*}, Fernando L. García-Carreño¹, María de los Ángeles Navarrete-del-Toro¹

¹ Centro de Investigaciones Biológicas del Noroeste, S. C. (CIBNOR), Instituto Politécnico Nacional 195, Col. Playa Palo de Santa Rita Sur, La Paz, B.C.S. México 23000. PO Box.

* Corresponding Author: Tel.: +52.612 1238484; Fax: +52.612 1238403;
E-mail: jcordova@cibnor.mx

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Abstract

Waste materials derived from shrimp processing signify 40 to 45 % of the weight of the catch or harvest. For Biotechnology, waste materials are secondary material, as contrast of raw materials, to generate value-added products, such as chitin, protein, and pigments. We used the pH-shift and enzymatic autohydrolysis processes to obtain protein concentrates from the cephalothorax of whiteleg shrimp (*Penaeus vannamei*). Protein recovery by auto-hydrolysis and pH-shift was 83.3 % (sum of two fractions) and 87.5 respectively. Functional properties from protein concentrates were assayed. The protein concentrates obtained were: precipitated protein at pH=4.0 (PP4), remaining soluble protein at pH=4.0 (SP4), and hydrolyzed protein (3HP). Precipitated protein (PP4) had 50 % foam stability; soluble protein (SP4) 31.5 %, and hydrolyzed protein had no foam stability. Emulsion capacity of the protein concentrates was not different from egg albumin (control protein). Hydrolyzed protein had higher emulsion stability than the other protein concentrates. Protein concentrates from the pH-shift process had good balance of amino acids. Histidine and valine was not detected in hydrolyzed protein. The pH-shift process demonstrated to be an alternative for protein hydrolysis to recover protein from shrimp cephalothorax.

Keywords: Shrimp head waste, protein, pH-shift, hydrolysis.

Introduction

In Mexico, 1, 345,000 mt of shrimp from fisheries and 123,000 mt from aquaculture are processed yearly (CONAPESCA, 2004), which generates a large amount of shell and head waste. For commercial purpose, one of the most important shrimp species is the whiteleg *Penaeus vannamei* (synonymous *Litopenaeus vannamei* Boone, 1931). The cephalothorax (head portion) of shrimp accounts for approximately 400–450 g/kg, representing a huge potential waste and environmental pollution. It also represents a cheap and abundant source of materials, such as protein, chitin, and astaxanthin (Armenta-López *et al.*, 2002; Duarte-De-Holanda and Netto, 2006). Protein hydrolysis is one of the most used technique to recover protein from several sources (Fox *et al.*, 1994; Heu *et al.*, 2003; Ibrahim *et al.*, 1999; Limam *et al.*, 2008; Mizani *et al.*, 2005; Wenhong *et al.*, 2008; Wenhong *et al.*, 2009).

The solubilization-precipitation (pH-shift) process (Hultin *et al.*, 2005) has been mostly used to obtain proteins from species of low commercial value (Kim *et al.*, 2003; Kristinsson and Liang, 2006; Palafox *et al.*, 2009). By pH-shift process, muscle

protein is separated from bones and other components at pH 2–4 or 11–12, followed by isoelectric precipitation. Then the precipitated protein is collected by centrifugation to obtain the protein isolate. This method can be used to easily separate the protein from other components of shrimp cephalothorax (shells, lipids, and pigments). Differences in protein solubility are essential for various procedures of isolation of individual proteins and groups of proteins from foodstuff (Kristinsson, 2001). In this study, we propose the pH-shift process as an alternative to the enzymatic hydrolysis to obtain protein concentrates from shrimp- cephalothorax waste.

Materials and Methods

Raw Material

Two batches of shrimp cephalothorax (S-C) of recently killed shrimp were used; one batch was obtained from shrimps ponds at CIBNOR (research institute), La Paz, B. C. S., Mexico and the second one from a local seafood processor. The raw material was kept in ice for about 2 h, then ground and sieved

into a paste (2-mm mesh, Retsch/Brinkmann grinder, Westbury, NY), and then stored at -20°C until used.

Biochemical Analysis

Protein content was analyzed with a nitrogen analyzer (FP-528, Leco, St. Joseph, MI); EDTA (95.7 mg/g N) was used as the standard with a Kjeldahl conversion factor of 6.25. The protein content of S-C paste was calculated by subtracting from the total nitrogen in the sample the nitrogen content in chitin, according to the following equation: $P = 6.25(N_T - N_{CH})$, where P is the protein content in S-C paste, N_T is the total nitrogen in the sample and N_{CH} is the nitrogen content of the chitin present in the sample.

The chitin content in the S-C paste was measured by the method of Synowiecki, (Mizani *et al.*, 2005). Moisture, ash, and lipids of the S-C paste were determined by standard methods. (AOAC, 1995) Freeze-dried samples were used for preparing amino acid profiles by pre-column fluorescence derivatization using *o*-phthalaldehyde and high performance liquid chromatography as described in Vázquez-Ortiz *et al.* (1995).

Protein Solubility Curve

Thirty grams of S-C paste were mixed with 270 mL cold distilled water and homogenized for 20 s in a kitchen blender. Then, 11 subsamples from this homogenate were adjusted to pH values from 2 to 12 using 2 N HCl (0.005-0.170 mL/10 mL of homogenate) or 2 M NaOH (0.004-0.088 mL/10 mL of homogenate) and centrifuged for 20 min at $10,000\times g$ at 4°C (Eppendorf 5804R centrifuge, Hamburg, Germany). Supernatant was recovered and assayed for quantitation of soluble protein by the Biuret method (Torten and Whitaker, 1964). Based on the solubility curve pH=4.0 and pH=7.0 treatments were selected for pH-shift processing. This procedure was performed for the two S-C batches processed.

pH-Shift Processing

For protein solubilization, three samples of 120 g S-C paste (from each batch) were mixed with 480 mL cold distilled water and kept at $2-4^{\circ}\text{C}$ on an ice bath to prevent auto-hydrolysis; pH was then adjusted to 7.0 with 2 N HCl (~1.2 mL) and centrifuged at $10,000\times g$ at 4°C for 20 min. The pellet was kept at 0°C . Soluble protein in the supernatant was assayed by the Biuret method. The soluble protein ratio was calculated by dividing the protein content in the supernatant by the protein content in the homogenate. Then, the pH of the supernatant was adjusted to pH 4.0 (precipitation point of most of the proteins in the homogenate) by adding 2 N HCl (~0.90 mL/100 mL supernatant) to precipitate the dissolved proteins. Then, the mixture was centrifuged at $10,000\times g$ at 4°C for 20 min. The sediment was recovered and the

supernatant was assayed for protein quantification by the Biuret method. Prior to freeze-drying, the two recovered fractions were labeled, as follows: the precipitated protein (at pH 4.0), PP4; and the remaining soluble protein in the supernatant at pH 4.0 (SP4). The protein yield was calculated as the ratio of recovered protein for each fraction to the protein in the raw material.

Protein Recovery by Auto-Hydrolysis

Endogen enzymes achieved auto-hydrolysis of the protein in the S-C paste. Three 500-mL samples of S-C paste from each batch, containing 20 g protein were stirred continuously with a propeller in a water bath at 50°C . The pH was adjusted and kept at 8.0 by adding 1 M NaOH during reaction (Córdova-Murueta *et al.*, 2003). The degree of hydrolysis (DH%) was calculated according to equation: $1/$

$$\text{DH}\% = 100 \left(\frac{B N_B}{I/\alpha} \right) \left(\frac{1/Mp}{1/h_{tot}} \right),$$

$$\text{Where: } N_B = 1.0, I/\alpha = 1.13, h_{tot} = 8.6$$

After 25 min, the DH% reached 3 % (~5.0 mL 1 M NaOH added). The reaction was stopped by immediate cooling to $2-4^{\circ}\text{C}$ on an ice bath and then centrifuged 20 min at $10,000\times g$ at 4°C . The supernatant was freeze-dried for further analysis and kept at -20°C .

Analysis of Proteins by SDS-PAGE

Protein in samples from auto-hydrolysis were analyzed by SDS-PAGE (Laemmli, 1970) using a 7.5–15 % acrylamide gradient gel. Soluble proteins obtained by the pH-shift process at different values of pH were analyzed in 12 % acrylamide gels. Samples were mixed (1:1) with loading buffer containing 0.125 M Tris-HCl pH=6.8, 4 % SDS, 20 % glycerol, 0.2 M dithiothreitol (DTT), and 0.02 % bromophenol blue. The sample with buffer was heated for 5 min in a boiling water bath. Molecular weight standards were loaded into the same gel. Electrophoresis was done in a temperature-controlled (2°C) vertical electrophoresis device (SE 260, Hoefer, San Francisco, CA) at 15 mA per gel. After electrophoresis, gels were stained with a solution containing 0.5 % (w/v) Coomassie brilliant blue R 250, 40 % (v/v) methanol and 7% acetic acid, for at least two h. Then gels were destained with 40 % (v/v) and 7 % (v/v) solution.

To evaluate the presence of proteolytic enzymes in the S-C before and after auto-hydrolysis and after freeze-drying, subsamples of the S-C paste were analyzed by substrate-SDS-PAGE (García-Carreño *et al.*, 1993) in 12 % acrylamide gels. Briefly, each sample was mixed with loading buffer (1:1) without DTT nor boiling and loaded into the gel. After electrophoresis, the gels were immersed in 3% (w/v)

casein as substrate in 50 mM Tris-HCl, pH 8, and incubated for 30 min at 4°C, then the temperature was raised at 25°C and maintained for 90 min. After incubation with the substrate gels were washed with distilled water and stained and destained as above. Clear bands over blue background revealed the presence of proteolytic enzymes.

Functional Properties

Foam capacity and foam stability were measured by the Rudin method (Wilde and Clark, 1996) with a slight modification. Briefly, 40 mL of water solution containing 33 g/L protein was homogenized with a 44-mm diameter impeller at high speed for 1 min at 25°C. The foam and liquid volumes were recorded. Whippability was expressed as proportion of increased foam volume. Foaming stability was expressed as the proportion of the foam volume remaining after resting the sample for 60 min at 25°C. Egg albumin was used as control because of its known foaming capacity (Matringe *et al.*, 1999).

Emulsifying capacity was determined by slight modification of the method of Swift (Hill, 1996). Briefly, 50 mL of the water solution containing 33 g/L protein was added to 7.75 mL (15.5 %) sunflower seed oil. The mixture was homogenized for 30 sec at high speed at 25°C with a 44-mm diameter impeller and then centrifuged for 5 min at 5000×g at 25°C (Eppendorf 5804R). Emulsifying capacity was calculated as the ratio of the volume from the emulsion formed and the initial mixture. Emulsion stability of each sample was determined by heating the emulsion for 30 min at 80°C. Emulsion volume was measured before and after heating. Emulsion stability is expressed as a proportion of the remaining emulsifying capacity after the heating period. Egg albumin was used as control. All measurements of functional properties were done at pH=7.0 (maximum solubility).

Statistical Analysis

The data is expressed as mean values of triplicate samples (\pm S.D.). One-way ANOVA with Tukey's Honestly Significant Difference (HSD) procedure was used to assess significant differences ($P<0.05$) with software Statgraphics Plus

(STATGRAPHICS®, 2000).

Results and Discussion

Proximate Composition and Amino Acid Content

The chitin N present in the ground S-C of the both batches processed was almost 50 % of the total nitrogen. This data was useful for correctly quantifying protein content in ground S-C when the factor total N \times 6.25 is used (Table 1). Protein in ground S-C (dry weight) was 29.3 %. Protein content of the hydrolysate protein (3HP) was the highest, 71.5 %, compared with the two fractions obtained from the pH-shift process (PP4 and SP4; Table 1). The total content of essential amino acids was 236, 335, and 221 mg/g protein for PP4, SP4, and 3HP treatments respectively. The remaining soluble protein at pH 4.0 (SP4) contained 29.5 % more essential amino acids than the precipitated fraction (PP4) and 34 % more essential amino acids than the (3HP) protein hydrolysate (Table 2).

The protein content of ground S-C was about half of the previously reported value for whiteleg shrimp *P. vannamei* S-C (Wenhong *et al.*, 2009), where the protein content was quantified from total N \times 6.25. In the present work, N content from chitin in the S-C accounted for ~50 % of total nitrogen. This explains why we obtained lower protein values for shrimp cephalothorax waste than other authors.

The protein recovered from the ground cephalothorax was 83.3 (\pm 0.6)/100 g of protein for the auto-hydrolysis method and 87.5 (\pm 2.0) g/100 protein for the pH-shift method. The yield for the pH-shift process was considered the sum of the PP4 and SP4 fractions. One hundred g (dry weight) of S-C paste contained 29.3 g protein, from this, auto-hydrolysis at pH 8.0 yielded 24.4 g (83.3 %) and pH-shift yielded 25.6 g (87.5 %), which is the sum of 10.8 g of the precipitate at pH 4 (PP4) and 14.8 g of the remaining soluble protein at pH 4.0 (SP4).

Auto-hydrolysis is a suitable method to solubilize/recover protein, but it is important to limit the hydrolysis extent on the raw material to get mid size peptides. An appropriate strategy to stop the enzymes action is also needed. SDS-PAGE of proteins from ground S-C sampled for 1 h of auto-hydrolysis showed that, at 25 min; 3 % DH, peptides

Table 1. Proximate composition of cephalothorax from *Penaeus vannamei* and fractions obtained by pH-shift and auto-hydrolysis (g per 100 g dry matter). Mean values (\pm standard deviation) of triplicated samples from two different batches of ground S-C

	S-C	PP4	SP4	3HP
Total N	9.5 \pm 0.02	9.6 \pm 0.1	10.9 \pm 0.2	11.9 \pm 0.3
Chitin N	4.8 \pm 0.1	-	-	-
Protein	*29.3 \pm 0.5	58.7 \pm 0.3	67.9 \pm 0.2	71.5 \pm 2
Lipids	14.9 \pm 0.2	20.6 \pm 0.3	0.34 \pm 0.07	NM
Ash	14.5 \pm 0.2	2.6 \pm 0.1	17.3 \pm 0.3	NM

S-C = Ground shrimp cephalothorax; PP4 = precipitated protein at pH=4; SP4 = remaining soluble protein after precipitation at pH=4; 3HP = 3 % hydrolyzed protein. NM = not measured. *Calculated as (total N - chitin N) \times 6.25.

>97 kDa diminished, and after 1 h other bands had diminished intensity and some new ones appeared, meaning that endogenous enzymes hydrolyzed some proteins from S-C (Figure 1).

The zymogram (Figure 2) showed that all enzymatic activity bands displayed in the sample previous to the auto-hydrolysis process remained after the re-suspension of the freeze-dried protein (Figure 2, lane 4), which means that if enzymes are not denatured, the hydrolysis will continue after rehydrating the freeze-dried protein hydrolysate (3HP).

Protein Solubility and pH

The pH value of maximum solubility of the raw material is the most important parameter to control when using the pH-shift process. This method is based on isoelectric precipitation of the proteins after

being solubilized at either alkaline or acid pH (Kristinsson and Liang, 2006). In this process, we obtained two fractions of proteins after precipitation because not all proteins from the ground S-C were precipitated at pH 4.0. The solubility of proteins from the ground S-C was the lowest at pH 4.0. One point of high solubility occurred at pH 2.0, but also at pH 7.0–12.0 with no significant difference as observed in the solubility curve (Figure 3). These observations were supported by electrophoresis (Figure 4). Then pH 7.0 was selected for further processing. At pH 4.0, protein bands were detected in the electrophoresis gel. This observation support the fact that not all soluble proteins at pH 7 were precipitated when the pH was changed to 4.0.

Figure 5 shows the SDS-PAGE fractions of proteins obtained at the different pH values. Only in the precipitated proteins (PP4), proteins larger than 97 kDa were observed (lane 4). The myofibrillar proteins

Table 2. Side chain hydrophobicity and amino acid content of several proteins (expressed as mg/g protein)

AA	[†] SdCh	[‡] S-C	[‡] PP4	[‡] SP4	[‡] 3HP
*His	2.1	17	8	16	nd
*Ile	12.4	67	31	49	40
*Leu	10.1	114	55	84	57
*Lys	6.2	137	79	86	47
*Phe	11.1	76	33	55	46
*Thr	1.8	62	28	43	31
*Val	7.0	4	2	2	nd
Ala	3.1	118	59	60	107
Arg	3.1	93	48	44	27
Asp	2.2	153	69	111	76
Glu	2.3	218	109	132	113
Gly	0.0	108	66	38	56
Tyr	12.0	59	26	42	24
Pro	10.8	141	84	54	94

[†]Side chain hydrophobicity as reported by Sikorski (2002).

[‡]Explained in Table 1 foot note. *Essential amino acids. AA = amino acids. nd = non detected.

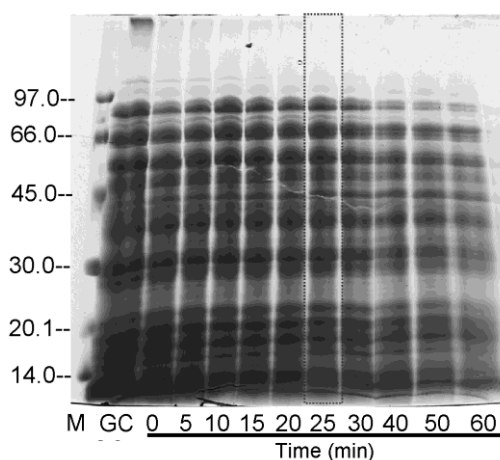


Figure 1. SDS-PAGE of proteins from autohydrolysis of ground cephalotorax. GC = ground shrimp cephalotorax, M = molecular weight markers, lane numbers indicate hydrolysis time (in minutes) Dotted box indicate the lane with protein at 3% degree of hydrolysis.

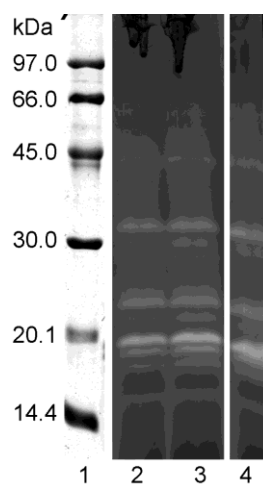


Figure 2. Zymograms of extracts from ground cephalothorax of whiteleg shrimp (*Penaeus vannamei*). Clear bands over dark background demonstrate the active enzymes. Lane 1) molecular weight markers; lane 2) enzymes before autohydrolysis; lane 3) enzymes after autohydrolysis, lane 4) enzymes after re-suspend the freeze-dried protein concentrate produced by autohydrolysis.

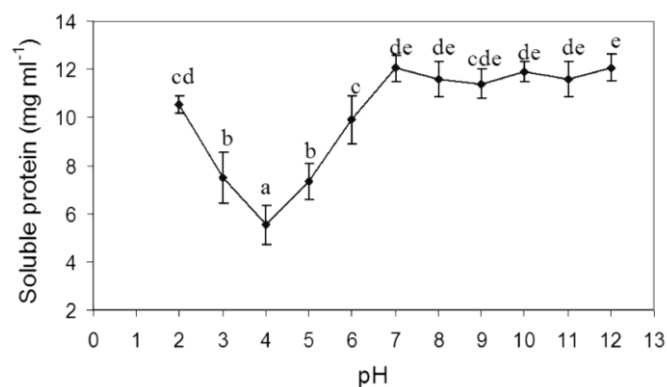


Figure 3. Effect of pH on solubility of proteins from ground cephalothorax of whiteleg shrimp (*Penaeus vannamei*). Different letters indicate significant differences ($P < 0.05$, Tukey's HSD test).

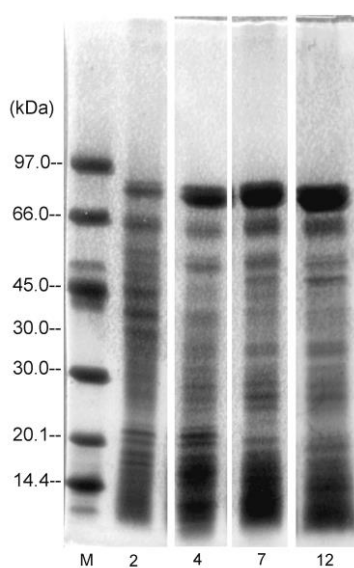


Figure 4. Gradient 7% to 15% acrylamide SDS-PAGE of soluble proteins. Lane M) molecular weight markers; lanes 2, 4, 7 and 12) correspond to the respective pH value for protein solubilization.

were not clearly distinguished; they appear to be less abundant than sarcoplasmic proteins in the cephalothorax region of shrimp or might be partially hydrolyzed before processing. Sarcoplasmic proteins are mostly enzymes; which includes proteases. This phenomenon was observed in the two batches processed. In the raw S-C paste, there is a mixture of proteins having different isoelectric points; the solubilization curve (Figure 3) showed only the average solubility values of all the proteins in the homogenate and not all-soluble protein precipitated under this condition. Additionally, some food proteins do not precipitate at their isoelectric point and other precipitating strategies, such as increased ionic strength by adding salt are needed (Li-Chang, 1996).

Functional Properties

The solubility of proteins is one of the most important parameters for protein functionality and as it was observed that the major solubility of proteins was at pH 7.0-12.0, we used pH 7.0 for functional properties evaluation. Also the hydrophobicity of amino acids contained in proteins is an important

factor for functional properties such as foaming and emulsion, but it is not possible to predict the conformation and behavior of a protein in solution on the basis of its average hydrophobicity because most hydrophobic amino acid residues of a globular protein are buried in the interior of the molecule, which gives the molecule a different surface hydrophobicity (Sikorski, 2002). (Table 3). This observation could explain the lower emulsion capacity observed in the soluble protein at pH 4.0 (SP4) than in the precipitated fraction (PP4) (Table 3).

The precipitated protein (PP4) showed the same emulsion capacity as albumin (stability of 23.3 %). The soluble protein, at pH 4.0 (SP4), showed no difference in foaming capacity when compared to albumin, which makes it a candidate for food applications where foaming capacity is desirable. Also, there are applications where protein with low foaming capacity is needed, which make the hydrolyzed protein (3HP) concentrate a good candidate. Differences observed in the functional properties of the protein concentrates obtained demonstrate that the separation process is important to allow the selection of some fractions with particular

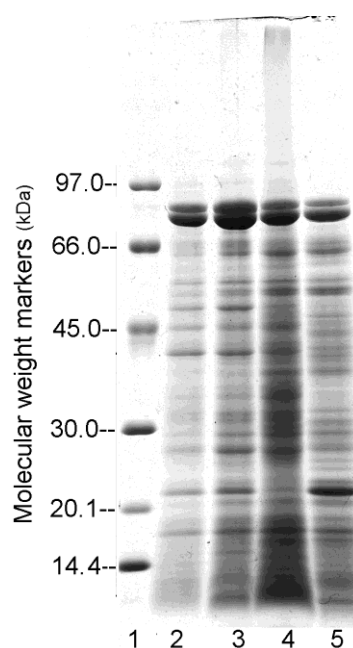


Figure 5. SDS-PAGE of proteins from ground cephalothorax (S-C) and the recovered fractions by the pH-shift method. Lane 1) molecular weight markers; lane 2) raw ground S-C; lane 3) S-C adjusted to pH 7.0; lane 4) precipitated proteins at pH 4 (PP4); lane 5) remaining soluble proteins at pH 4.0 (SP4).

Table 3. Functional properties of the recovered proteins (%)

Protein	Emulsion capacity	Emulsion stability	Foam forming capacity	Foam stability
PP4 [†]	23.3 ^b (± 0.0)	42.4 ^b (± 0)	105.5 ^a (± 1.9)	50.0 ^c (± 0)
SP4 [†]	20.0 ^a (± 1.1)	30.0 ^a (± 4.6)	302.2 ^b (± 20.3)	31.5 ^b (± 2.4)
3HP [†]	20.0 ^a (± 0.1)	80.0 ^c (± 0.3)	133.3 ^a (± 5.7)	0.0 ^a (± 0)
Albumin	23.3 ^b (± 1.1)	97.2 ^d (± 4.8)	286.6 ^b (± 20)	75.6 ^d (± 5.1)

Different letters in the same column indicate significant differences.

[†] PP4 = precipitated protein at pH=4; SP4 = remaining soluble protein after precipitation at pH=4; 3HP = 3 % hydrolyzed protein..

properties.

The two separate fractions may be used based on differences in properties that can be advantageous as food supplements or additives and other value-added products. For example, the highest protein and essential amino acid content of the remaining soluble protein at pH 4.0 (SP4) could be attractive for food protein-enrichment. According to the data reported in Sikorsky (2002) the protein in SP4 fraction contains more amino acids with hydrophobicity <7.0 (Table 2). The possible position of these amino acids (mainly Asp, Glu) on the surface of the folded proteins of this fraction may contributed to SP4 remained soluble at pH 4 (Trevino *et al.*, 2007). Solubility is an important quality for a protein concentrate intended for some food applications, such as beverages or gelling and emulsification. The solubility is an important feature for protein functionality and the solubility of proteins depends on the properties of the protein and solvent, pH, concentration and charge of other ions, and temperature (Sikorski, 2002). Also differences in solubility of proteins are important to separate individual proteins or groups of proteins. The differences observed in the functional properties in the distinct protein concentrates are related to the extraction methods used.

Because the pH-shift process proceeds at low temperature, it is an attractive alternative for processing fishery by-products. This process also minimizes the potential damage produced by heat to proteins and unsaturated fatty acids when heat-based extraction methods are used. From the material obtained after solubilizing at pH 7, it should be easy to separate other components from the S-C, such as oils or shells concentrated in that fraction that were not processed in the present investigation.

The moderated auto-hydrolysis process is an alternative to get protein, but increased proteolysis or the use of high temperature to stop the hydrolysis reaction, can reduce the functional properties of the protein concentrate and promote the presence of bitter peptides (Kristinsson and Rasco, 2000). If protein hydrolysates are freeze-dried, enzymes remain active; which can be advantageous, if adequately processed, for use as supplement in feeds. These enzymes if remain active in the digestive tract of the animals may enhance the digestion of feeds. Using DH% 3 on shrimp cephalothorax creates some desirable characteristics for the recovered protein. The extensive hydrolysis could yield bitter hydrolysates (Dauksas *et al.*, 2004; Kristinsson and Rasco, 2000). The pH-shift is a good choice because there is no need for heat and reaction stopping and separation of remaining components is easy.

Conclusions

Based in the amino acid profiles of the three protein concentrates, they are suitable for animal or human consumption.

Based on the statistical analysis, the pH-Shift method yielded more protein recovery from shrimp cephalothorax than the auto-hydrolysis method at the conditions tested in the present investigation. It could be considered as an alternative to recover protein from fisheries or aquaculture by-products. It is an easy and efficient method for protein recovery. The auto-hydrolysis is also a good method to recover proteins, but there are more variables to manage during the process, like temperature, time, and endogenous enzymatic activity among others. But also it is important to take into account the final use of the protein that will be recovered to select the most suitable method.

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References

- AOAC, 1995. Official methods of analysis of AOAC International. AOAC International, Arlington, VA, USA.
- Armenta-López, R., Guerrero, L.I., Huerta, S., 2002. Astaxanthin extraction from shrimp waste by lactic fermentation and enzymatic hydrolysis of the carotenoprotein complex. *J. Food Sci.*, 67: 1002-1006. doi: 10.1111/j.1365-2621.2002.tb09443.x
- CONAPESCA, 2004. Statistical Year book of fisheries and aquaculture 2004 (In Spanish). CONAPESCA, Mazatlán, Sinaloa México.
- Córdova-Murueta, J.H., García-Carreño, F.L., Navarrete-del-Toro, M.A., 2003. Digestive enzymes present in crustacean feces as a tool for biochemical, physiological, and ecological studies. *J. Exp. Mar. Biol. Ecol.*, 297: 43-56. doi: 10.1016/S0022-0981(03)00355-1
- Dauksas, E., Slizyte, R., Rustad, T., Storro, I., 2004. Bitterness in Fish Protein Hydrolysates and Methods for Removal. *Journal of Aquatic Food Product Technology*, 13: 101-114. doi: 10.1300/J030v13n02_09
- Duarte-De-Holanda, H., Netto, F.M., 2006. Recovery of components from shrimp (*Xiphopenaeus kroyeri*) processing waste by enzymatic hydrolysis. *J. Food Sci.*, 71: C298-C303. doi: 10.1111/j.1750-3841.2006.00040.x
- Fox, C.J., Blow, P., Brown, J.H., Watson, I., 1994. The effect of various processing methods on the physical and biochemical properties of shrimp head meals and their utilization by juvenile *Penaeus monodon* Fab. *Aquaculture*, 122: 209-226. doi: 10.1016/0044-8486(94)90511-8
- García-Carreño, F.L., Dimes, N., Haard, N., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitors. *Anal. Biochem.*, 214: 65-69. doi: 10.1006/abio.1993.1457
- Heu, M.S., Kim, J.S., Shahidi, F., Jeong, Y., Jeon, Y.J., 2003. Extraction, fractionation and activity

- characteristics of proteases from shrimp processing discards. *J. Food Biochem.*, 27: 221-236. doi: 10.1111/j.1745-4514.2003.tb00278.x
- Hill, S.E., 1996. Emulsions. in: Hall, G.M. (Ed.), *Methods of testing protein functionality*. Blackie Academic and Professional, London, U. K., pp. 153-185.
- Hultin, H.O., Kristinsson, H.G., Lanier, T.C., Park, J.W., 2005. Process for recovery of functional proteins by pH shifts. in: Park, J.W. (Ed.), *Surimi and Surimi Seafood*. Taylor and Francis, Boca Raton, FL, pp. 108-133.
- Ibrahim, H.M., Salama, M.F., El-Banna, H.A., 1999. Shrimp's waste: Chemical composition, nutritional value and utilization. *Food / Nahrung*, 43: 418-423. doi: 10.1002/(SICI)1521-3803(19991201)43:6<418::AID-FOOD418>3.0.CO;2-6
- Kim, Y.S., Park, J.W., Choi, Y.J., 2003. New approaches for the effective recovery of fish proteins and their physicochemical characteristics. *Fish. Sci.*, 69: 1231-1239. doi: 10.1111/j.0919-9268.2003.00750.x
- Kristinsson, H., 2001. Conformational and functional changes of hemoglobin and myosin induced by pH: functional role in fish quality. UMass, Amherst, Massachusetts, USA.
- Kristinsson, H.G., Rasco, B.A., 2000. Fish protein hydrolysates: production, biochemical, and functional properties. *Crit. Rev. Food Sci. Nutr.*, 40: 43-81. doi: 10.1080/10408690091189266
- Kristinsson, H.G., Liang, Y., 2006. Effect of pH-shift processing and surimi processing on atlantic croaker (*Micropogonias undulates*) muscle proteins. *J. Food Sci.*, 71: C304-C312. doi: 10.1111/j.1750-3841.2006.00046.x
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685. doi:10.1038/227680a0
- Li-Chang, E., 1996. Separation and purification. in: Nakai, S., Modler, H.W. (Eds.), *Food proteins: properties and characterization*. VCH, New York, N.Y., pp. 429-503.
- Limam, Z., Sadok, S., Abed, A.E., 2008. Enzymatic Hydrolysis of Shrimp Head Waste: Functional and Biochemical Properties. *Food Biotechnol.*, 22: 352 - 362. doi: 10.1080/08905430802458461
- Matringe, E., Phan Tan Luu, R., Lorient, D., 1999. Functional Properties of Milk-Egg Mixtures. *J. Food Sci.*, 64: 787-791. doi: 10.1111/j.1365-2621.1999.tb15912.x
- Mizani, M., Aminlari, M., Khodabandeh, M., 2005. An Effective Method for Producing a Nutritive Protein Extract Powder from Shrimp-head Waste. *Food Sci. Technol. Int.*, 11: 49-54. doi: 10.1177/1082013205051271
- Palafox, H., Córdova-Murueta, J.H., Navarrete-del-Toro, M.A., García-Carreño, F.L., 2009. Protein isolates from jumbo squid (*Dosidicus gigas*) by pH-shift processing. *Process Biochem.*, 44: 584-587. doi: 10.1016/j.procbio.2009.02.011
- Sikorski, Z.E., 2002. Proteins. in: Sikorski, Z.E. (Ed.), *Chemical and Functional Properties of Food Components*. CRC Press LLC, Boca Raton, pp. 133-167.
- STATGRAPHICS®, 2000. Statgraphics Plus 5.1. StatPoint Technologies, Inc. (www.statgraphics.com).
- Torten, J., Whitaker, J.R., 1964. Evaluation of the Biuret and dye-binding methods for protein determination in Meats. *J. Food Sci.*, 29: 168-174. doi: 10.1111/j.1365-2621.1964.tb01713.x
- Trevino, S.R., Scholtz, J.M., Pace, C.N., 2007. Amino Acid Contribution to Protein Solubility: Asp, Glu, and Ser Contribute more Favorably than the other Hydrophilic Amino Acids in RNase Sa. *J. Mol. Biol.*, 366: 449-460. doi.org/10.1016/j.jmb.2006.10.026
- Vázquez-Ortiz, F.A., Caire, G., Higuera-Ciapara, I., Hernández, G., 1995. High Performance Liquid Chromatographic Determination of Free Amino Acids in Shrimp. *J. Liq. Chromatogr. Relat. Technol.*, 18: 2059 - 2068. doi: 10.1080/10826079508013960
- Wenhong, C., Chaohua, Z., Pengzhi, H., Hongwu, J., 2008. Response surface methodology for autolysis parameters optimization of shrimp head and amino acids released during autolysis. *Food Chem.*, 109: 176-183. doi: 10.1016/j.foodchem.2007.11.080,
- Wenhong, C., Chaohua, Z., Pengzhi, H., Hongwu, J., Jiming, H., Jing, Z., 2009. Autolysis of shrimp head by gradual temperature and nutritional quality of the resulting hydrolysate. *LWT-Food Sci. Technol.*, 42: 244-249. doi: 10.1016/j.lwt.2008.05.026
- Wilde, P.J., Clark, D.C., 1996. Foam formation and stability. in: Hall, G.M. (Ed.), *Methods of testing protein functionality*. Blackie Academic and Professional, London, U.K.: 110-152 pp.