Physicochemical characterization of pepsin-soluble collagen extracted from the byssus of Chilean mussels (*Mytilus Chilensis*)

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Resumen. La recuperación de compuestos de alto valor agregado provenientes de desechos de la industria marina es un área de investigación prometedora. El biso del mejillón es un sub-producto de la producción de mejillones el cual es una potencial fuente de colágeno. El objetivo de este estudio fue extraer colágeno desde el biso de mejillones chilenos usando un método enzimático y caracterizar este tipo de colágeno. Se utilizó una hidrólisis enzimática mediada por pepsina con dos razones de pepsina/sustrato (1:50 ó 4:50) y dos tiempos de extracción (4 ó 24 h). La extracción se realizó a 80°C por 24 h en una solución de ácido acético. Las dispersiones de colágeno obtenidas fueron analizadas en términos de su contenido de colágeno, turbidez, viscosidad aparente y solubilidad. El tiempo de hidrólisis afectó significativamente ($p<0.05$) el contenido de colágeno, contenido de hidroxiprolina y rendimiento de la extracción. La hidrólisis enzimática a una razón pepsina/sustrato 4:50 por 24 h presentó la mejor conducta de extracción con valores de 69 mg/g proteína, 1.8 mg/g proteína y 30% para contenido de colágeno, contenido de hidroxiprolina y rendimiento de extracción, respectivamente. No se encontraron diferencias ($p>0.05$) en la viscosidad aparente y solubilidad, sugiriendo que la hidrólisis enzimática no afectó a integridad de la molécula de colágeno. Nuestros resultados indican que el biso del mejillón chileno es una buena fuente de colágeno que puede ser extraído y encontrar usos en el área de alimentos, farmacéutica y biomédica.

Palabras Claves: biso, colágeno, extracción, mejillón, pepsina.

Abstract. Recovery of high-added-value compounds from seafood waste is a promising area of research. Mussel byssus is a by-product from mussel production which is a potential source of collagen. The goal of this study was to extract collagen from the byssus of Chilean mussel using an enzymatic method and characterize this kind of collagen. For the pepsin-aided extraction, an enzymatic hydrolysis at two pepsin/substrate ratios (1:50 or 4:50) and times (4 or 24 h) was done. Then, the extraction was conducted at 80 °C for 24 h, in an acetic acid solution. All samples were analyzed for collagen content, turbidity, apparent viscosity and solubility of collagen dispersions. Hydrolysis time had significant effect ($p<0.05$) on collagen content, hydroxyproline content and extraction yield. Enzymatic hydrolysis at 4:50 pepsin/byssus ratio for 24 h gave the better extraction performance with values of 69 mg/g protein, 1.8 mg/g protein and 30%, for collagen content, hydroxyproline content and extraction yield, respectively. No differences ($p>0.05$) were found on apparent viscosity and solubility, suggesting that the enzymatic hydrolysis not affected the integrity of the collagen molecule. Our results indicate that the Chilean mussel byssus is a good source of collagen that can be extracted and find uses in the food, pharmaceutical and biomedical areas.

Keywords: byssus, collagen, extraction, mussel, pepsin.

1. Introduction

The majority of seafood waste and by-products constitute at present a serious environmental problem for their management. Although there is an attempt to decrease the waste in the world, the quantity of waste produced is increasing annually (1). Considerable amounts of protein-rich by-products from seafood processing plants are discarded without any attempt to carry out recovery. Those by-products and waste require appropriate management, especially because they are highly perishable, owing chiefly to

the action of microorganisms that find them an excellent growth medium (2, 3). Special efforts have been made to utilize the aforementioned by-products and waste by using efficient ways, including useful applications; at present, most are used as animal feed and plant fertilizers (3).

Marine by-products contain valuable protein and lipid fractions as well as vitamins and minerals. The recovery of chemical components from seafood waste materials, whichcan be used in other segments of the food industry, is a promising area of research and development for the utilization of seafood by-products (4). Extraction of those high added-value compounds, which can be profitable owing to their beneficial effect upon human health, coupled with the development of new technologies for recovery and purification, will run along with concomitant benefits towards long term sustainability of marine activities (3).

Collagen is the most abundant protein of animal origin, this material being the main constituent of animal skin, bone, and connective tissue, comprising about 30% of total animal protein (5). All collagens are extracellular proteins with specific amino acid composition. Collagens are rich in proline and hydroxyproline (20% of total amino acids), as well as glycine and alanine (over 50% of total amino acids) (5). The collagen molecule (tropocollagen) is composed of three α-chains (each containing 1000 amino acids) intertwined in the so-called collagen triple-helix, adopting a 3D structure that provides an ideal geometry for inter-chain hydrogen bonding (5, 6). Only small regions at the end of the α-chain, called telopeptides (i.e. short N- and C-terminal regions with 15-26 amino acid residues) do not form triple helical structures as they are largely made up of lysine and hydroxylysine residues, where different degrees of cross-linking have been found (3, 5, 7).

Based on the above discussion, it is clear that from a scientific, environmental and economic point of view, that research on the extraction and characterization of collagen from the mussel byssus is highly justified. In our previous paper (8) we presented a simple experimental procedure to extract collagen, the objective of this study was to improve that methodology in terms of collagen yield and partially characterize this kind of marine collagen.

2. Materials and methods

2.1 Raw materials

Mussel byssus, discarded (by-products) from mussel aquaculture for exportation, were kindly provide by Orizon S. A. Samples were stored at -80°C until used.

2.2 Characterization of mussel byssus

2.2.1 Proximate composition

Compositional measurements in terms of moisture, fat, ash and protein of byssus were conducted according to AOAC methods (9). Moisture content was determined gravimetrically by oven drying at 105 °C for 24 h. Crude fat content was evaluated by using the Soxhlet extraction method. Protein concentration was assessed by the Kjeldhal method. Total ash content was determined gravimetrically by oven heating at 550 °C. Finally, carbohydrate content was calculated by mass difference after obtaining the other component contents.

2.2.2 Amino acid analysis by high performance liquid chromatography (HPLC)
Mussel byssus was characterized in terms of its amino acid profile. HPLC measurements were developed following a methodology previously described (9). Briefly, 10 mg of sample was hydrolyzed with 300 µL of a 6 N HCl solution at 110 °C for 24 h. The hydrolyzate obtained was derivatized with 20 µL of phenylthiocyanate (10% w/v) to generate phenylthiocarbamyl amino acids, which were separated and quantified by HPLC at 254 nm. A liquid chromatograph (Waters 600 controller, MA, USA) with a diode array detector (Waters 996) and a Phenomenex (Los Angeles, CA, USA) Luna RP 18 column (150 mm×4.6 mm, particle size 5 µm) was used. Gradient separation was performed using two solvents: (A) 0.14 mol/L anhydrous sodium acetate (pH 5.9)/acetonitrile (94:6 v/v) solution and (B) HPLC-grade acetonitrile/water (60:40 v/v) solution. The injection volume was 20 µL, the column temperature was 40 °C and the analysis time was 30 min. Amino acid quantification was carried out using external standards (Sigma-Aldrich, Steinheim, Germany) of each analyzed amino acid.
In order to quantify the effectiveness of the collagen extraction method proposed in this work, amino acid profile of extracted collagen was determined using the same HPLC methodology as described above. The amount of amino acids contained in collagen dispersions, with special interest in hydroxyproline content was determined.

2.3 Isolation of collagen from mussel byssus

2.3.1 Pre-treatment of raw material
Cleaning-up of mussel byssus: Mussel byssus was washed twice with tap water (1:50 w/v) at 20 ºC for 10 min. A third wash was carried out using distilled water (1:20 w/v) at 20 °C for 10 min. The clean byssus was drained through a metal strainer and squeezed by hand. Finally, the byssus was cut into small pieces (~3 mm) with scissors.
NaOH pre-treatment: The cleaned byssus was immersed into a 0.1 N NaOH solution(1:10 w/v) for 60 min with agitation of 200 rpm at 20 ºC, in order to solubilize non-collagenous proteins and to prevent the effects of endogenous proteases on collagen. The alkaline pre-treated byssus was drained and rinsed with distilled water (1:20 w/v) at 20 ºC for 10 min, a number of times necessary to reach neutral pH.
Acid pre-treatment: In order to solubilize collagen protein, the byssus was immersed into a 0.1 N HCl solution (1:10 w/v) for 60 min with agitation of 200 rpm at 20 ºC. The acid pre-treated byssus was drained and rinsed with distilled water (1:10 w/v) at 20 ºC for 10 min, a number of times necessary to reach neutral pH.

2.3.2 Enzymatic-aided collagen extraction
Using non-specific proteases, it is possible to cut-off the non-helical ends (telopeptide region) of collagen. Pepsin solutions (pepsin from porcine gastric mucosa, EC 232-629-3, 800-2500 units/g protein, Sigma-Aldrich Co., USA) in acetic acid 0.5 N were prepared at two pepsin/byssus ratios (1:50 or 4:50 w/w), and pH was adjusted to 1.5 with 5 N HCl solution. Samples were immersed into the pepsin solution (1:6 w/v) for two different hydrolysis times (4 or 24 h) under agitation at 25 °C and 200 rpm. After the enzymatic treatment, the solution was filtered and the enzyme was inactivated by applying a thermal treatment at 98 °C for 1 min. The solution was adjusted at pH 4.0 with 5 N NaOH solution and the byssus was put back into the acetic acid solution. The samples were placed into a thermoregulated bath at 80 ºC for 24 h for collagen extraction. Then, the dispersion was filtered to eliminate the solids and the filtrate was recovered. The collagen was precipitated by adjusting the pH to 7.0 with NaOH 1N, the dispersion was centrifuged at 5000 rpm for 10 min, the supernatant was discarded and the pellet was freeze-dried at -

54 °C and 25 Pa for 24 h. The freeze-dried powder was analyzed as the pepsin-solubilized collagen (PSC). All experiments were run in triplicate.

2.4 Characterization of pepsin-solubilized collagen

For all measurements described in this section PSC was solubilized to a concentration of 6 mg/mL in acetic acid solution 0.5 N.

2.4.1 Collagen content measurements

Collagen content in dispersions was quantified using the Sircol Collagen Assay (Biocolor Life Science Assays, Carikfergus, UK). The manufacturer's protocol for the assay was followed. Briefly, 100 μL of sample was added to 1 mL of the colorimetric reagent (the dye Sirius red in picric acid) and agitated for 30 min followed by centrifugation at 12000 rpm for 10 min. The Sirius red dye was released from the pellet with the kit Acid-salt Wash Reagent and the release and recovery of the collagen bound dye was done with the Alkali Reagent. Spectrophotometric readings were taken at 550 nm on a microplate reader (Bio-Rad, model 550, Bio-Rad Laboratories Inc., Richmond, CA, USA). Absolute values were attained with a standard curve in the range of 5 - 50 μg/100 μL, composed from collagen type I standard supplied with the kit.

2.4.2 Yield of the extraction process

The extraction yield was calculated from the initial solid content of byssus and the final mass of the freeze-dried collagen as follows:

$$Collagen\ yield\ (\%) = \left(\frac{Mass\ of\ \ freeze-dried\ collagen\,(g)}{Solid\ content\ of\ byssus\,(g)} \right) 100\% \qquad \text{Equation (1)}$$

2.4.3 Turbidity of collagen dispersions

Because transparency of collagen dispersions is an important quality parameter, turbidity of dispersions was measured. Turbidity was measured by reading the absorbance of collagen dispersions at 600 nm using a spectrophotometer (Shimadzu, model UV mini 1240, Kyoto, Japan) at 25 ºC (10).

2.4.4 Apparent viscosity of collagen dispersions

Measurements of apparent viscosity of collagen dispersions were done at 100 rpm using a rotational viscosimeter (Brookfield DV-11+ viscosimeter UL adapter, Brookfield Engineering Lab Inc. Staughton, MA, USA). Temperature was kept at 25 °C by means of a thermoregulated water bath.

2.4.5 Effect of pH on collagen solubility

Collagen solubility was evaluated by methods previously published (11, 12) with slight modifications. Collagen dispersion (1 mL) was added to an Eppendorf centrifuge tube and the pH was adjusted to values ranging from 2.0 to 8.0 with either 5 N HCl or 5 N NaOH. Dispersions were made up to 1.5 mL with distilled water, previously adjusted to the same pH as the collagen dispersion. Samples were centrifuged at 5000×g at 25 °C for 30 min. Protein content in the supernatant was determined by the Bradford method, as explained in our previous paper (8). Relative solubility (%) was expressed in comparison with that obtained at the pH rendering the highest solubility.

2.5 Statistical analysis of data

Analysis of variance (ANOVA) tests were used to analyze the data at a confidence level of 95%, and treatments were compared using LSD test by means of Statgraphics Plus 5.1 software (Manugistics Inc., Statistical Graphics Corporation, Rockville, USA). Values presented are average of three replications.

3. Results and discussion

3.1 Characterization of mussel byssus

Mussel byssus was characterized in terms of its proximate composition and amino acids profile. Water is the main component of the byssus (~76%); but in dry basis mussel byssus is almost exclusively composed of proteins, with a concentration of about 82% (Figure 1), according to a previous work (13) byssal threads are 95% protein by dry weight, value slight higher than our results. The amino acid profile shows a high concentration of hydroxyproline, proline (imino acids) and glycine (Figure 2), being hydroxyproline the most abundant amino acid in the mussel byssus (~43%). A previous study (14) using two-dimensional paper chromatography detected a high concentration of glycine, proline and hydroxyproline in the byssus of the Asian green mussel. In agreement with this last study, others authors (15) demonstrated that, based on amino acid content, byssus collagenbears the hallmark of collagen types I–III: one-third of the residues are glycine, and the proline plus hydroxyproline content approaches 20%. Also, these two studies showed that the amino acid composition of mussel byssus varies along the length of the byssus thread, namely distal and proximal portions and the adhesive plaque (14, 15). This difference in composition was attributed to the high degree of specialization of the byssus. The high hydroxyproline content can be related to the high concentration of collagen among the mussel byssus proteins. It is known that the collagen content influences the toughness of muscles, but the presence of cross-links is also associated with the tough and elastic muscle of mammals (5). Probably due to their biological function, the structure of mussel byssus present high amounts of collagen, making the byssus tough enough to withstand the forces of the tide.
Following our previous work (8), in this study we improve the methodology of collagen extraction from the mussel byssus and also the collagen extracted was characterized.

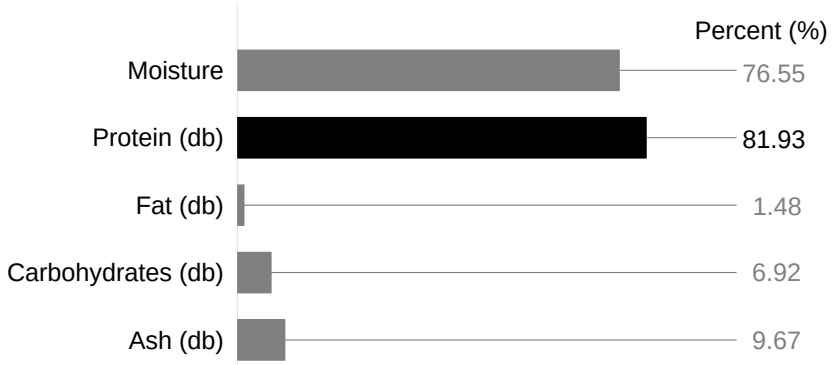

Figure 1. Proximal composition of mussel byssus. Protein, fat, carbohydrates and ash are expressed in dry basis (db).

The byssus of Chilean mussels was not completely solubilized at the extraction conditions used in this study. Soluble proteins are at best poorly extractable from mature byssal

threads (15). Traditional collagen extraction procedures, such as acid solubilization, proved ineffective with byssal threads, extensive pepsinization released several pepsin-resistant polypeptide fragments (13). Our previous results (8) suggested that the collagen molecules in mussel byssus were most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the inter-molecular cross-linking, leading to low solubility of collagen. In order to increase the collagen extraction from the mussel byssus, we applied a pepsin digestion of mechanically disrupted threads. Pepsin is typically indiscriminate in its digestion of proteins, with the notable exception of the triple helical domain of native collagen (15). With further limited pepsin digestion, the cross-linked molecules at the telopeptide region are cleaved without damaging the integrity of the triple helix (11, 12).

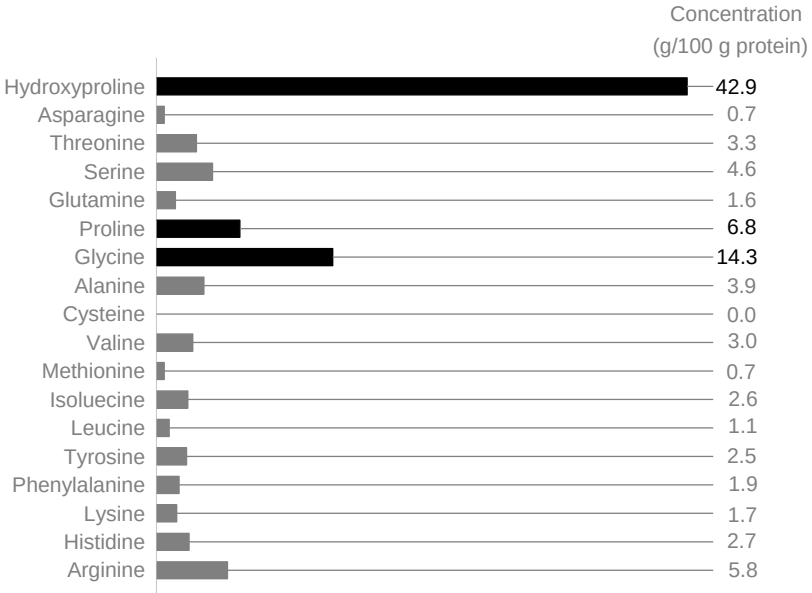

Figure 2. Amino acid profile of mussel byssus.

3.2 Pepsin-aided collagen extraction from the mussel byssus

Our results showed statistical differences ($p<0.05$) between the extractability of PSC (expressed as collagen and hydroxyproline content) for the different digestion times used in this work (Figure 3). The highest collagen (68.6 mg/g protein) and hydroxyproline contents (1.84 mg/g protein) were obtained for the hydrolysis treatment at a pepsin:byssus mass ratio of 4:50 for 24 h; whereas using an hydrolysis conditions of pepsin:byssus mass ratio of 1:50 for 4 h only 37.7 (mg/g protein) and 1.44 (mg/g protein) of collagen and hydroxyproline could be extracted. High enzyme level generally led to a greater yield of collagen extracted, additionally, a longer reaction time rendered a higher yield (8, 12). However, it was previously demonstrated (16) that enzyme concentration (0.6 to 1.4% w/v) had no significant effect on collagen yield; whereas digestion time (12 to 36 h) had a significant effect. In a previous study the yield of PSC increased with the increase of pepsin amount ranging from 20 to 40 unit/mg; however, a slight increase in the PSC yield was observed when the pepsin amount increased in the range of 40-60 unit/mg (17).

Table 1. Extraction yield, apparent viscosity and turbidity of collagen dispersions[1].

Hydrolysis conditions	Extraction yield (%)	Apparent viscosity at 100 rpm (cP)	Turbidity at 595 nm
1:50 – 4 h	19.5 ± 3.0[a]	2.35 ± 0.27[a]	0.37 ± 0.07[a]
1:50 – 24 h	29.8 ± 1.9[b]	2.31 ± 0.05[a]	0.73 ± 0.01[b]
4:50 – 4 h	22.5 ± 4.2[a]	2.16 ± 0.10[a]	0.35 ± 0.06[a]
4:50 – 24 h	28.9 ± 7.5[b]	2.24 ± 0.04[a]	0.67 ± 0.14[b]

[1]Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time. Table shows average values and standard deviation. Different letters indicate significant differences ($p<0.05$) for different hydrolysis conditions.

Also, it has been demonstrated that the effect of enzyme concentration over collagen yield depends upon the acid used as extracting medium; in general, a higher enzyme concentration increases collagen yield, but over certain critical concentration the yield becomes constant (18). Probably, an optimum level of enzyme does exit, which maximizes the yield of extracted collagen, but it could depend on the nature of the enzyme and raw material used for the extraction (8). The collagen content extracted was coincidental with hydroxyproline content measured, a linear relationship ($R^2 = 0.987$) was found between hydroxyproline and collagen content (see insert Figure 3) for different hydrolysis conditions. As well as collagen and hydroxyproline content, the yield of PSC depended on hydrolysis parameters ($p<0.05$) (Table 1), with a maximum value of about 30% in terms of mass of freeze-dried collagen. Different yields of PSC from skins of marine species had been reported, depending on fish species and process parameters. Yield of PSC (dry weight basis) were 35.0% for cuttlefish (19), 44.7% for ocellate puffer fish (20), 46.6% for grass carp (21), 27.1% for yellowfin tuna (16), 19.5% for balloon fish (22) and 60.3% for bighead carp (23). These differences in yields have been attributed to the variability in fish species, biological conditions and preparative methods for the extraction process.

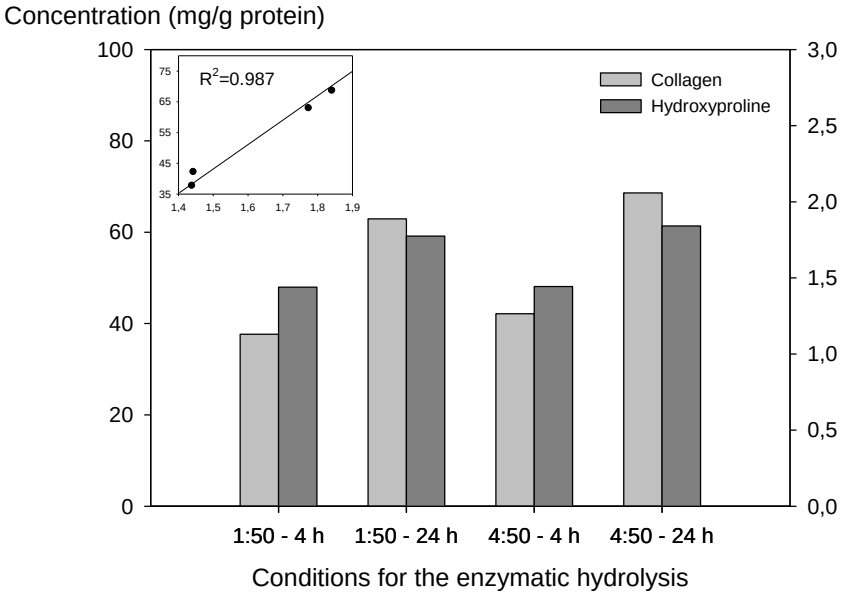

Figure 3. Collagen and hydroxyproline content for the enzyme-aided extraction procedure. Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time. Different letters indicate significant differences ($p<0.05$) between contents for the different extraction conditions. Insert figure shows a linear relationship between collagen and hydroxyproline content.

3.3 Apparent viscosity of collagen dispersions

Viscosity is one of the main factors that affect physicochemical and functional properties of collagen and gelatin. The viscosity of collagens did not presents statistical differences ($p>0.05$) (Table 1), suggesting that different hydrolysis conditions not affected the integrity of the collagen molecule, results in agreement with electrophoretic analysis (8).

3.4 Turbidity of collagen dispersions

For practical uses, pure, colorless preparations of collagen are required. Turbidity of collagen dispersion increased significantly ($p<0.05$) with enzyme concentration and hydrolysis time (Table 1). According to a previous work, turbidity values reflect concentration of colloidal material and are largely dependent on efficiency of the clarification (filtration) process (10). It is probably that the increase in the yield of the process, produced by the higher pepsin:byssus mass ratio and hydrolysis time, lead to a higher amount of non-collagenous material affecting the turbidity of the collagen dispersions.

3.5 Effect of pH on collagen solubility

Solubilities of PSC reached maximum values at pH's between 2 and 4 (Figure 4). A sharp decrease in solubility can be seen at pH's between 4 and 6 and a minimum solubility was observed at pH' between 6 and 8. No differences can be seen from solubility curves for different hydrolysis conditions (Figure 4), indicating that different conditions did not affected the collagen molecule, as discussed previously for viscosity results. The effect of pH on protein solubility could be explained by its isoelectric point (pI) value. When the pH of protein dispersion is higher or lower than the pI, the repulsive forces between charged residues of a protein molecule increases, and the protein solubility is increased by the repulsion forces between chains. In contrast, at pH near the pI total net charges of protein molecules are zero and hydrophobic interaction increases, thereby leading to the precipitation and aggregation of the protein molecule (16, 24). Isoelectric points of acid extracted collagen vary from 6 to 9 (5, 6).

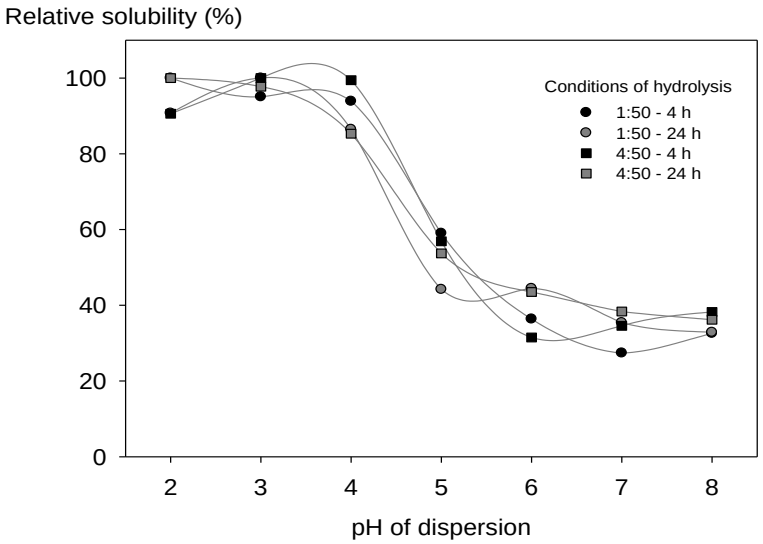

Figure 4. Relative solubility of pepsin-soluble collagen depending on the pH of dispersion. Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time.

Solubility curves were quite similar to those obtained by several studies for PSC collagens (11, 12, 16, 22, 23), where collagens are highly solubilized at pH's between 2 - 5, with relative solubilities greater than 80%. Most of these works presented maximum solubility of collagen at pH's between 3 and 4, and lowest solubility at pH 7.

4. Conclusion

An enzyme-aided extraction method was applied in order to isolate collagen from mussel byssus. Hydrolysis time had a significant effect on extraction yield, with maximum value of 30%, suggesting that yield could be increased, by increasing the time of the hydrolysis step. From rheological and solubility analysis it was concluded that condition for the pepsin hydrolysis step not affected the integrity of the collagen molecule. Also, there is clearly a need for further research to establish the functional activities of collagen from mussel byssus that impact its performance as a potential new source of collagen for food or pharmaceutical applications. All of these results could be used as a basis for crafting strategies to assist the reutilization of waste and by-products from seafood processing plants.

Acknowledgements

R.N. Zúñiga thanks CONICYT for financial support by means of the FONDECYT Project 11110156 Utilization of mussel byssus as an alternative source of collagen for the production of gelatin for food and pharmaceutical uses.

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