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ORIGINAL ARTICLE

Collagen extraction from mussel byssus: a new marine collagen source with physicochemical properties of industrial interest

F. Rodríguez[1] · L. Morán[2] · G. González[1] · E. Troncoso[3,4] · R. N. Zúñiga[1]

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Abstract Mussel byssus is a by-product of mussel production and 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 it. A pepsin-aided extraction method was employed where first an enzymatic hydrolysis at two pepsin/substrate ratios (1:50 or 4:50) and times (4 or 24 h) was done. Extraction was conducted at 80 °C for 24 h, in a 0.5 N acetic acid solution. All samples were analyzed for collagen content, amino acid profile, turbidity, viscosity, solubility, denaturation temperature and surface tension. Hydrolysis time had significant effect on collagen content, hydroxyproline content and extraction yield. Hydrolysis with a pepsin/byssus ratio of 4:50 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 were found for the viscosity and surface tension of collagen dispersions, suggesting that the enzymatic hydrolysis did not affect the integrity of the collagen molecule. Denaturation temperature of freeze-dried byssus collagen presented a high value (83–91 °C), making this kind of collagen a very interesting material for encapsulation of bioactive molecules and for biomedical applications.

Keywords Collagen · Mussel byssus · Extraction · Pepsin

✉ R. N. Zúñiga
rommy.zuniga@utem.cl

[1] Department of Biotechnology, Universidad Tecnológica Metropolitana, Las Palmeras 3360, Ñuñoa, Santiago, Chile

[2] Department of Chemical and Biochemical Engineering, Instituto Tecnológico de Zacatepec, Calzada Tecnológico 27, Zacatepec, Morelos, Mexico

[3] Department of Chemistry, Universidad Tecnológica Metropolitana, Las Palmeras 3360, Ñuñoa, Santiago, Chile

[4] Programa Institucional de Fomento a la Investigación, Desarrollo e Innovación, Universidad Tecnológica Metropolitana, Ignacio Valdivieso 2409, San Joaquín, Santiago, Chile

Introduction

Considerable amounts of by-products from seafood processing plants are discarded, increasing the wastes worldwide. Those by-products and wastes require appropriate management, especially because they are highly perishable, owing chiefly to the action of microorganisms (Ferraro et al. 2010; Wasswa et al. 2007). Special efforts have been made to utilize these by-products and wastes by efficient ways (e.g. animal feed and plant fertilizers) (Ferraro et al. 2010). However, valuable nutrients and bioactive compounds in by-products are lost without any attempt to carry out recovery.

Marine by-products contain valuable protein and lipid fractions as well as vitamins and minerals (Ferraro et al. 2010). Recovery of chemical components from seafood waste, which can be used in other segments of the industry, is a promising area of research and development (Arvanitoyannis and Kassaveti 2008). Extraction of those high added-value compounds, coupled with the development of new technologies for recovery and purification, will run along with concomitant benefits towards long term sustainability of marine activities (Ferraro et al. 2010). Thus, the rising interest in the valorization of industrial by-products is one of the main reasons why exploring different marine species as new sources of collagen (Gómez-Guillén et al. 2011; Silva et al. 2014). In addition, after the outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease crisis, restrictions on collagen trade have

been established and alternative safe sources of collagen are needed. Another problem of bovine collagen application is the risk of autoimmune and allergenic reactions (Zhao and Chi 2009). The huge number of marine species having very different intrinsic characteristics has aroused the scientific and commercial interest in optimizing the extracting conditions as well as characterizing the yields, and physicochemical and functional properties of marine collagen (Gómez-Guillén et al. 2011; Subhan et al. 2015).

Collagen is the most abundant high-molecular-weight protein in both invertebrate and vertebrate organisms, this material being the main constituent of animal skin, bone, and connective tissue, comprising about 25–30% of total proteins (Foegeding et al. 1996; Silva et al. 2014). All collagens are extracellular proteins with specific amino acid composition. Generally, collagens are formed by polypeptide chains constituted by repeating triplets Gly-X-Y of glycine and two other amino acids, wherein X is generally proline and Y is generally hydroxyproline (Foegeding et al. 1996; Silva et al. 2014; Yang et al. 2014). 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 (Foegeding et al. 1996; Stevens 2010). 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 (Ferraro et al. 2010; Foegeding et al. 1996; Ledward 2000).

Collagens have been elected as one of the key biological materials in biomedical applications due to their high biocompatibility (Silva et al. 2014). Because of the aforementioned problems presented by mammalian collagens, marine collagen sources with potential application in tissue engineering are being intensively studied as novel candidates to be used for biomedical applications (Silva et al. 2014; Subhan et al. 2015; Yang et al. 2014). Collagen from fish wastes (skin, bones and scales) is the most studied source for scaffold design; but its low denaturation temperature and variable composition limits its application in this field (Silva et al. 2014; Subhan et al. 2015). Hence, invertebrate marine collagen sources are now under investigation. Scaffolds from jellyfish and marine sponges collagen have been developed, obtaining materials with high porosity, interconnected porous structure, high cell viability and no cytotoxicity (Song et al. 2006; Lee et al. 2007; Pallela et al. 2012; Hoyer et al. 2014). These promising results lead to a further increase in the research in this area.

Mussels (*Mytilus sp.*) are found in a variety of habitats, including rocky intertidal, salt marsh, subtidal, and hydrothermal vents. Mussels adhere to surfaces by the byssus, a structure composed of individual threads representing a collagen fiber reinforced composite that can withstand the wave forces, which can reach local velocities up to 25 m/s (Harrington and Waite 2008; Lucas et al. 2002; Suhre et al. 2014). Byssus comprises three distinct collagen types, surrounded by matrix proteins, and further adhesive and cohesive proteins in the thread cuticle and the adhesive plaque (Suhre et al. 2014). Byssal threads are composed of over 50% collagen (Harrington and Waite 2008), although differences in concentration had been shown between species (Lucas et al. 2002; Qin and Waite 1995; Suhre et al. 2014). In addition, it has been highlighted that "*byssal collagens have no match with any known collagen sequence*" (Qin and Waite 1995). Extraordinary properties include the high melting temperature, lack of banding patterns and certain resistance to denaturants, acids and proteases (Qin and Waite 1995). Processing of mussels leads to the generation of a large biomass of mussel waste (i.e. byssus and shells), which is generally discarded. If substantial amounts of collagen could be obtained from the wastes, they would provide alternatives to mammalian collagen in foods, pharmaceutical and biomedical materials using the same approaches investigated for collagen extracted from invertebrate marine sources.

It is clear that from a scientific, environmental and economic point of view, research on the extraction and characterization of collagen from the mussel byssus is highly justified. In our previous work (Vallejos et al. 2014) we presented a simple experimental procedure to extract collagen. This work shares our approach's motivation and complements it, since its goal was to improve the extraction methodology, evaluated in terms of collagen yield, and complementing the partial characterization of this kind of marine collagen. To our knowledge this is the first study in characterizing this kind of marine collagen searching possible industrial applications.

Materials and methods

Raw materials

Mussel byssus, by-product discarded from mussel aquaculture for exportation, was kindly provided by Orizon S. A. Samples were stored at −80 °C until used. All experiments were done in a period of 4 months.

Characterization of mussel byssus

Proximate composition

Compositional measurements in terms of moisture, fat, ash and protein of byssus were conducted according to AOAC

methods (AOAC 1995). Moisture content was determined gravimetrically by oven drying at 105 °C for 24 h. Crude fat content was evaluated by the Soxhlet extraction method. Protein concentration was assessed by the Kjeldhal method. Total ash content was determined gravimetrically by oven heating at 550 °C. Carbohydrate content was calculated by mass difference after obtaining the other component contents.

Amino acid analysis by high performance liquid chromatography (HPLC)

Amino acid profile from Chilean mussel byssus was obtained by using HPLC, following the methodology of previous studies (Janssen et al. 1986; Vallejos et al. 2014). Briefly, 10 mg of sample was hydrolyzed with 300 µL of 6 N HCl solution at 110 °C for 24 h. The hydrolysate 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) provided 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: 0.14 mol/L anhydrous sodium acetate (pH 5.9)/acetonitrile (94:6 v/v) solution, and 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, USA) of each analyzed amino acid.

Isolation of collagen from mussel byssus

The method for collagen extraction from the mussel byssus applied in this work (see Fig. 1) is a modification of our previous study (Vallejos et al. 2014). The pre-treatment of the raw material was done in the same way as previously described, but for the extraction step we used acetic acid instead of hydrochloric acid to increase the yield of the process, as it was demonstrate in previous experiments (data not shown).

Pre-treatment of raw material

Cleaning-up of mussel byssus: mussel byssus was washed twice with tap water (1:50 w/v) at 20 °C 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. After this, the byssus was cut into small pieces (~3 mm) with sterile scissors.

NaOH pre-treatment: in order to solubilize non-collagenous proteins and to prevent the effects of endogenous proteases on collagen, the cleaned byssus was immersed into a 0.1 N NaOH solution (1:10 w/v) for 60 min at 200 rpm at 20 °C. The alkaline pre-treated byssus was drained and rinsed with distilled water (1:20 w/v) at 20 °C for 10 min, a number of times necessary to reach neutral pH.

Acid pre-treatment: to solubilize collagen protein and demineralize the samples, the byssus was immersed into a 0.1 N HCl solution (1:10 w/v) for 60 min at 200 rpm at 20 °C. The acid pre-treated byssus was drained and rinsed with distilled water (1:10 w/v) at 20 °C for 10 min, a number of times necessary to reach neutral pH.

Enzymatic-aided collagen extraction

Pepsin solutions (pepsin from porcine gastric mucosa, EC 232-629-3, 800–2500 units/g protein, Sigma-Aldrich) in 0.5 N acetic acid 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 in order to cut off the non-helical ends (telopeptide region) of collagen. Pre-treated samples were immersed into the pepsin solution (1:6 w/v) for two different hydrolysis times (4 or 24 h) at 200 rpm and 25 °C. After the enzymatic treatment, the solution was filtered and put into test tubes to inactivate the enzyme 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. Samples were heated at 80 °C for 24 h for collagen extraction. Then, the dispersion was filtered under vacuum using filter paper (Whatman N°1) to eliminate the remaining solids (i.e. material not solubilized by acid and/or pepsin) and the filtrate was recovered. The solubilized collagen was precipitated by adjusting the pH to 7.0 with 1 N NaOH solution, the dispersion was centrifuged at 5000 rpm for 10 min, the supernatant was discarded and the pellet was freeze-dried (Virtis BenchTop Pro 9L ES-55, SP Scientific, PA, USA) at −54 °C and 25 Pa for 24 h. The freeze-dried powder was analyzed as the pepsin-solubilized collagen (PSC). All extraction experiments were run in triplicate.

Characterization of pepsin-solubilized collagen

For all measurements, PSC was solubilized to a concentration of 6 mg/mL in 0.5 N acetic acid solutions, except for DSC analyses where the freeze-dried powder was used. All measurements were done in triplicate.

Amino acid profile by HPLC

In order to quantify the effectiveness of the collagen extraction method here proposed, amino acid profiles were determined using the HPLC methodology previously

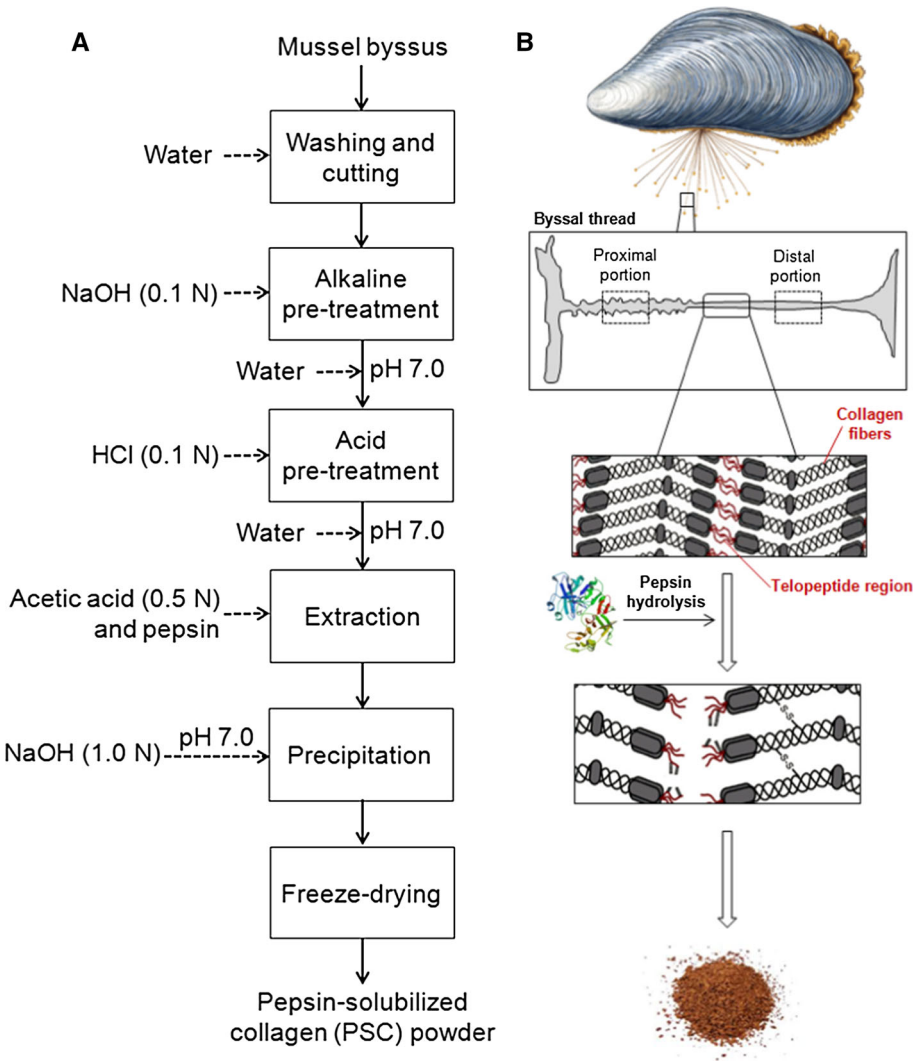

Fig. 1 **a** Flow chart describing the extraction method of collagen from mussel byssus by using the enzymatic extraction procedure, **b** scheme outlining critical physiological steps in the extraction process of collagen from byssal thread (adapted from Hagenau et al. 2009)

described (Section "Amino acid analysis by high performance liquid chromatography (HPLC)"). The amount of amino acids contained in collagen dispersions was determined, with special interest in the imino acid content (proline and hydroxyproline content).

Percentage of proline hydroxylation was calculated on the basis of the amino acid composition (Ahmad et al. 2010):

$$\text{Hydroxylation}\,(\%) = \frac{\text{Hyp}}{(\text{Pro} + \text{Hyp})} \times 100\% \qquad (1)$$

where Hyp and Pro are the amount of hydroxyproline and proline in the collagen (g/100 g protein), respectively.

Collagen content measurements

Collagen content in dispersions was quantified using the Sircol Collagen Assay (Biocolor Life Science Assays, Carikfergus, UK), following the manufacturer's assay protocol. Briefly, 100 µL of sample were added to 1 mL of the colorimetric reagent (Sirius red dye in picric acid) and agitated for 30 min followed by centrifugation at 12,000 rpm for 10 min. The 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., CA, USA). Absolute values were obtained with a standard curve in the range of 5–50 µg/100 µL, composed of collagen type I standard supplied with the kit.

Yield of the extraction process

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

$$\text{Collagen yield}\,(\%) = \frac{\text{Mass of freeze} - \text{dried collagen}\,(\text{g})}{\text{Mass of solids of byssus}\,(\text{g})} \times 100\% \qquad (2)$$

Turbidity of collagen dispersions

Because transparency of collagen dispersions is an important quality parameter, turbidity of dispersions was measured by reading the absorbance of collagen dispersions at 600 nm using a spectrophotometer (Shimadzu, model UV mini 1240, Kyoto, Japan) at 25 °C and using 0.5 N acetic acid solution as blank (Shon et al. 2011).

Apparent viscosity of collagen dispersions

Measurements of apparent viscosity of collagen dispersions were done at 25 °C and 100 rpm using a rotational viscometer (Brookfield DV-11 + viscometer UL adapter, Brookfield Engineering Lab Inc. Staughton, MA, USA). Readings were taken after 2 min of rotation.

Effect of pH on collagen solubility

Collagen solubility was evaluated following the method previously reported (Jongjareonrak et al. 2005; Nalinanon et al. 2007), with only slight modifications. Briefly, collagen dispersion (1 mL) was added to an Eppendorf centrifuge tube and 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 rpm at room temperature for 30 min. Protein content in the supernatant was determined by the Bradford method (Vallejos et al. 2014). Relative solubility (%) was normalized against that obtained at the pH providing the highest solubility.

Denaturation temperature determination

Denaturation temperature of freeze-dried collagen was determined using a differential scanning calorimetry (DSC) equipment (Mettler-Toledo, model DSCF822e, Switzerland). Temperature calibration was adjusted with Indium thermogram. Samples (10 mg) were weighed and sealed in aluminum pans and scanned at 5 °C/min over the range of 0–140 °C with a nitrogen gas flow rate of 50 mL/min. An empty pan was used as reference. The onset, peak and endset of the transition were reported. Total denaturation enthalpy (ΔH) was calculated as the area of the transition in the DSC thermogram divided by the sample mass. A commercial sample of bovine collagen (collagen from bovine Achilles tendon C9879, Sigma-Aldrich) was used as control.

Surface tension measurements

Collagen dispersions were used for surface tension measurements by means of the shape analysis of pendant drops, using an automated contact angle goniometer (Ramé-Hart Instruments CO., NJ, USA) in combination with the DROP image software. The surface tension of a 9-μL capillary pendant drop was record for 120 s at 25 °C. Prior to measurements it was corroborated experimentally that the surface tension of the ultrapure water/air system was 72 mN/m (Zúñiga et al. 2012).

Statistical analysis

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

Results and discussion

Characterization of mussel byssus

Mussel byssus was characterized in terms of its proximate composition (Fig. 2) and amino acids profile (Table 1). Water is the main component of the byssus (~76%) but dry basis mussel byssus is almost exclusively composed of proteins (~82%) (Fig. 2). According to Harrington and Waite (2008) byssal threads are 95% protein by dry weight, value higher than our results, which can due to differences in the species studied. The amino acid profile shows a high concentration of hydroxyproline, proline (imino acids) and glycine (Table 1), being hydroxyproline the most abundant amino acid in the mussel byssus (~43%). Bharathi and Ramalingam (1989) used two-dimensional paper chromatography and detected a high concentration of glycine, proline and hydroxyproline in the byssus of the Asian green mussel. Also, Qin and Waite (1995) demonstrated that, based on amino acid content, byssus collagen bears the hallmark of collagen types I–III: one-third of the residues are glycine, and the proline plus hydroxyproline content approaches 20%. These two studies showed that the amino acid composition of mussel byssus varies along

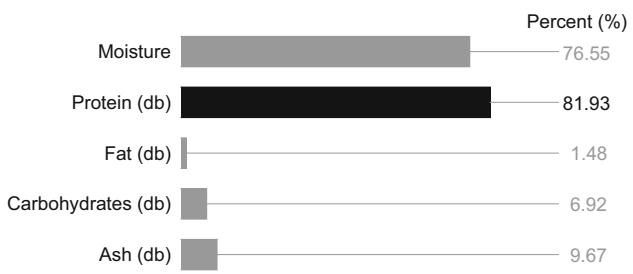

Fig. 2 Proximate composition of mussel byssus. Protein, fat, carbohydrates and ash are expressed in dry basis (db)

Table 1 Amino acid composition (g/100 g protein) of mussel byssus and pepsin-soluble collagen (PSC) extracted from mussel byssus

	Mussel byssus	Enzyme-aided extraction conditions[a]			
		1:50—4 h	1:50—24 h	4:50—4 h	4:50—24 h
Hyp	**42.9**	**2.2**	**3.0**	**2.2**	**2.8**
Asp	0.7	11.2	10.6	12.3	10.9
Thr	3.3	3.9	3.8	3.7	3.5
Ser	4.6	5.3	5.4	5.3	5.3
Glu	1.6	9.4	7.2	9.0	9.5
Pro	**6.8**	**6.0**	**6.3**	**5.9**	**5.9**
Gly	**14.3**	**9.0**	**10.2**	**8.9**	**10.0**
Ala	3.9	4.4	4.8	4.5	4.8
Cys	0.0	7.5	6.0	7.5	5.4
Val	3.0	4.5	4.4	4.6	4.2
Met	0.7	1.5	5.4	1.5	5.2
Ile	2.6	3.9	3.8	3.8	3.6
Leu	1.1	5.7	5.6	5.7	5.5
Tyr	2.5	3.6	3.2	3.6	3.0
Phe	1.9	3.0	3.0	2.9	2.9
Lys	1.7	7.1	6.2	6.9	6.4
His	2.7	2.0	1.9	2.1	2.1
Arg	5.8	9.8	9.2	9.7	9.0
Imino acids	**49.7**	**8.2**	**9.3**	**8.1**	**8.7**

Bold values correspond to imino acids

[a] Conditions of hydrolysis: pepsin:byssus mass ratio and hydrolysis time

the length of the byssus thread, namely distal and proximal portions and the adhesive plaque (Bharathi and Ramalingam 1989; Qin and Waite 1995), and this difference in composition was attributed to the high degree of specialization of the byssus. A high hydroxyproline content can be related to the high concentration of collagen among the mussel byssus proteins (Qin and Waite 1995). Tendons are another biological structure with high collagen content (more than 50% collagen) and at low strain values ($\sim 10\%$) byssal threads behave mechanically like tendons, reflecting their similar composition (Harrington and Waite 2008). Probably due to their biological function, the structure of mussel byssus presents high amounts of collagen, making the byssus tough enough to withstand the forces of the tide.

Pepsin-aided collagen extraction from the mussel byssus

The byssus of Chilean mussels was not completely solubilized at the extraction conditions here established. Soluble proteins are at best poorly extractable from mature byssal threads. Traditional collagen extraction procedures (e.g. acid solubilization) were ineffective with byssal threads (Qin and Waite 1995; Harrington and Waite 2008). We previously 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 (Vallejos et al. 2014). To increase the collagen extraction from the mussel byssus, we performed 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 (Qin and Waite 1995). With further limited pepsin digestion, the cross-linked molecules at the telopeptide region are cleaved without damaging the integrity of the triple helix (Jongjareonrak et al. 2005; Nalinanon et al. 2007).

Extractability of PSC (expressed as collagen and hydroxyproline content) showed statistical differences ($P < 0.05$) for the different digestion times used (Fig. 3), but not for the pepsin to byssus mass ratio. The highest collagen (68.6 mg/g protein) and hydroxyproline content (1.84 mg/g protein) were obtained for the hydrolysis treatment at a pepsin:byssus mass ratio of 4:50 for 24 h, although no significant differences ($P < 0.05$) were found in collagen and hydroxyproline content between this treatment and that performed at 1:50 pepsin:byssus mass ratio; whereas using an hydrolysis condition 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 respectively were extracted. It has been found that high enzyme level generally led to a greater yield of collagen extracted, additionally, a longer reaction time increased the

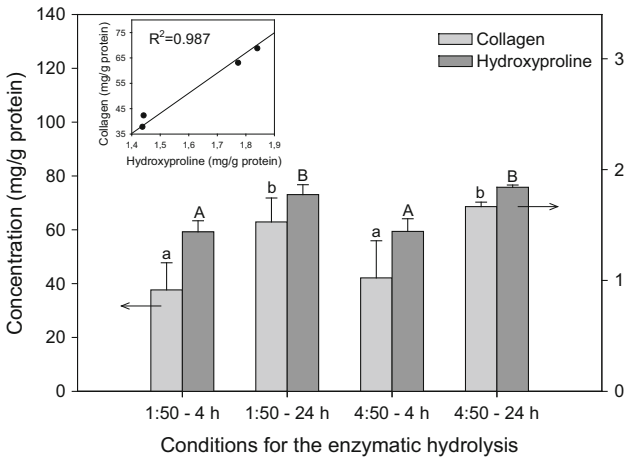

Fig. 3 Collagen and hydroxyproline content for the enzyme-aided extraction procedure. Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time. *Insert* figure shows a linear relationship between collagen and hydroxyproline content. Different *letters* indicate significant differences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

Table 2 Extraction yield, apparent viscosity and turbidity of collagen dispersions

Hydrolysis conditions	Extraction yield (%)	Apparent viscosity (cP) at 100 rpm	Turbidity at 600 nm
1:50—4 h	19.5 ± 3.0[a]	2.35 ± 0.27[a]	0.27 ± 0.07[a]
1:50—24 h	29.8 ± 1.9[b]	2.31 ± 0.05[a]	0.64 ± 0.01[b]
4:50—4 h	22.5 ± 4.2[a]	2.16 ± 0.10[a]	0.25 ± 0.06[a]
4:50—24 h	28.9 ± 7.5[b]	2.24 ± 0.04[a]	0.58 ± 0.14[b]

Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time. Table shows average values (n = 3) and standard deviations. Different letters indicate significant differences ($P < 0.05$) for different hydrolysis conditions

yield (Nalinanon et al. 2007; Vallejos et al. 2014). However, Woo et al. (2008) demonstrated that enzyme concentration (0.6–1.4% w/v) had no significant effect on collagen yield, whereas digestion time (12–36 h) had a significant effect. According to Wang et al. (2008), the yield of PSC increased with the increase of pepsin amount (20–40 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. Skierka and Sadowska (2007) showed that the effect of enzyme concentration on 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. An optimum level of enzyme which maximizes the yield of extracted collagen probably does exist but it could depend on the nature of the enzyme and raw material used for the extraction (Vallejos et al. 2014), probably in our work the optimum level is below the lower content of pepsin used here. The collagen content extracted was coincident with hydroxyproline content determined by HPLC, where a linear relationship ($R^2 = 0.987$) was found between hydroxyproline and collagen content (insert Fig. 3) for different hydrolysis conditions. As well as collagen and hydroxyproline content, the yield of PSC depended on hydrolysis parameters ($P < 0.05$) (Table 2), 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 species and process parameters. Yield of PSC (dry weight basis) were 35.0% for cuttlefish (Nagai et al. 2001), 44.7% for ocellate puffer fish (Nagai et al. 2002), 46.6% for grass carp (Zhang et al. 2007), 27.1% for yellowfin tuna (Woo et al. 2008), 19.5% for balloon fish (Huang et al. 2011), and 60.3% for bighead carp (Liu et al. 2012). Differences in yields have been attributed to the variability in species, biological conditions and preparative methods for the extraction process.

Amino acid profile of collagen from mussel byssus

Amino acid composition of PSC obtained was similar regardless hydrolysis conditions (Table 1), and no effect of enzyme/substrate ratio or hydrolysis time was observed. As shown in Table 1, the imino acid content had an average value of ~8.6% for the four treatments employed. This value was lower than those obtained by Woo et al. (2008) for PSC from yellowfin tuna skin (~20%). It is known that imino acids play a key role in the thermal stability of collagen triple helix (Ledward 2000; Stevens 2010). When Gly–X–Y is Gly–Pro–Hyp, the hydroxyproline residues increase the heat stability of the collagen triple helix because of bonding with the hydroxyl group of the pyrrolidine ring (Stevens 2010). Therefore, the amounts of proline and hydroxyproline are important for the physicochemical properties of collagen.

The total degree of proline hydroxylation for mussel byssus collagen was in the range of 26–32%. Degrees of hydroxylation were 47.9% for cuttlefish (Nagai et al. 2001) and 42.6% for unicorn leatherjacket (Ahmad et al. 2010). The degree of proline hydroxylation influences the thermal stability of collagen, as well as the imino acid content. A higher degree of hydroxylation is associated with higher denaturation temperature for collagens with similar amino acid profiles.

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 collagen dispersions did not present statistical differences ($P > 0.05$) (Table 2), suggesting that different hydrolysis conditions did not affect the integrity of the collagen molecules, this being in agreement with electrophoretic (Vallejos et al. 2014), solubility and surface analysis.

Turbidity of collagen dispersions

For practical uses, pure, and colorless preparations of collagen are required. Turbidity of collagen dispersion was significantly increased ($P < 0.05$) with hydrolysis time (Table 2). Turbidity values reflect concentration of solubilized colloidal material and are largely dependent on efficiency of the clarification (filtration) process (Shon

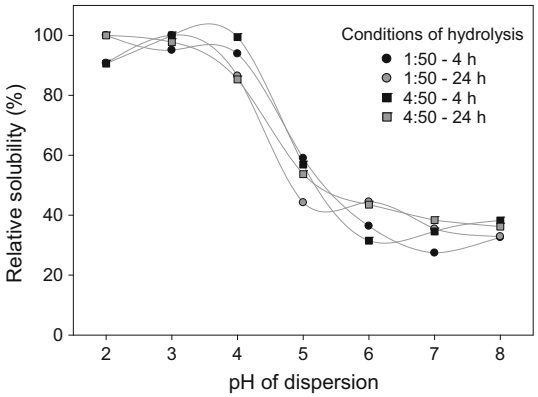

Fig. 4 Relative solubility of pepsin-soluble collagen depending on the pH of dispersion. Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time. *Error bars* are not shown for sake of clarity. Standard deviation between 2 and 8%

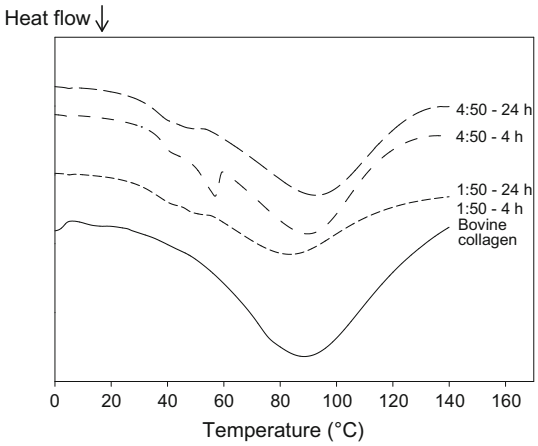

Fig. 5 DSC thermograms of pepsin-soluble collagen dispersions extracted from mussel byssus. Conditions of hydrolysis are pepsin:-byssus mass ratio and hydrolysis time

et al. 2011); however, in this study the collagen extracted was not clarified. Shon et al. (2011) obtained a turbidity value of 0.28 for skate collagen, which is a very similar value as those obtained in this study for the low hydrolysis time (0.27 and 0.25); but for the higher hydrolysis time the turbidity values were at least two-fold higher (0.64 and 0.58) than those obtained for low hydrolysis time. During enzymatic treatment of collagen, it is likely that non-collagen proteins were hydrolyzed. Therefore, the increase in the yield of process, induced by the higher pepsin:byssus mass ratio and hydrolysis time, leads to a higher amount of collagenous and non-collagenous material extracted, hence affecting the turbidity of collagen dispersions.

Effect of pH on collagen solubility

PSC solubility reached maximum values at pH's between 2 and 4 (Fig. 4). A sharp decrease in solubility could be seen at pH's between 4 and 6, and a minimum solubility was observed at pH's between 6 and 8. No significant differences ($P > 0.05$) were found from solubility curves for different hydrolysis conditions (Fig. 4), indicating that different conditions did not affected the collagen molecule, as previously discussed for viscosity results. The effect of pH on protein solubility could be explained by its isoelectric point (pI). When the pH of protein dispersion is higher or lower than the pI, the repulsive forces between charged residues of a protein molecule increase, 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 (Cheftel et al.1996).

Solubility curves were quite similar to those obtained by several studies for PSC collagens (Huang et al. 2011; Jongjareonrak et al. 2005; Liu et al. 2012; Nalinanon et al. 2007; Woo et al. 2008), where collagens are highly soluble at pH's between 2 and 5, with relative solubility 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.

Thermal stability of collagen dispersions

High temperatures induce structural unfolding of protein molecules. For collagen, thermal denaturation is interpreted as unfolding of the triple helix into random coils. For the four extraction conditions freeze-dried byssus collagen showed a single denaturation peak between 83 and 91 °C (Fig. 5). The single peak suggests a unique fraction of collagen molecules in terms of their thermal stability. Significant differences ($P < 0.05$) were found for the hydrolysis conditions employed in this study, the higher the pepsin:byssus ratio the higher the denaturation temperatures (Table 3); however, no statistical differences ($P > 0.05$) were found between denaturation temperatures of byssus and lyophilized bovine collagen used as control. The denaturation enthalpy (ΔH) had values between 116 and 187 J/g, values similar to bovine collagen (130 J/g); however, enthalpies for collagen extracted at lower extraction times were statistically different ($P < 0.05$) from bovine collagen. These observations are attributed to the high imino acid content (Table 1) and the degree of proline hydroxylation of byssus collagen.

Thermal properties of marine collagen are often measured as collagen dispersions. Thermograms for dispersions of PSC from marine sources presented denaturation

Table 3 Thermal transition temperatures and denaturation enthalpy of freeze-dried collagen

Hydrolysis conditions	Onset (°C)	Maximum (°C)	Endset (°C)	Enthalpy (J/g)
1:50—4 h	43.7 ± 0.0^{b}	84.4 ± 0.8^{a}	114.2 ± 3.8^{a}	173.2 ± 16.3^{b}
1:50—24 h	39.0 ± 0.7^{a}	83.2 ± 2.7^{a}	116.7 ± 1.7^{a}	115.9 ± 18.6^{a}
4:50—4 h	46.5 ± 1.8^{c}	90.2 ± 0.9^{b}	118.0 ± 2.6^{a}	186.8 ± 8.9^{b}
4:50—24 h	42.1 ± 0.9^{b}	90.7 ± 1.5^{b}	125.2 ± 0.9^{b}	142.8 ± 6.1^{a}
Bovine collagen	59.3 ± 1.9^{d}	89.0 ± 2.2^{b}	124.1 ± 1.4^{b}	130.2 ± 20.9^{a}

Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time. Table shows average values ($n = 3$) and standard deviations. Different letters in the same column indicate significant differences ($P < 0.05$) for different samples

temperatures in the range of 24.6–31.5 °C for fish species (Jongjareonrak et al. 2005; Nalinanon et al. 2007; Zhang et al. 2007; Ahmad et al. 2010; Benjakul et al. 2010; Huang et al. 2011), 32.2–34.1 °C for ray species (Bae et al. 2008), 34.4 °C for shark (Kittiphattanabawon et al. 2010), 34.8 °C for squid (Veeruraj et al. 2013) and 35 °C for eel (Veeruraj et al. 2013). These values were always lower than calf skin collagen (41–44 °C) or porcine skin collagen dispersions (37 °C) (Zeng et al. 2009; Subhan et al. 2015).

Thermal stability of collagen is associated with the restriction of the secondary structure of the polypeptide chain governed by the pyrrolidine rings of proline and hydroxyproline and, partially, by the hydrogen bonding through the hydroxyl group of hydroxyproline (Benjakul et al. 2010). Hydroxyproline is important in maintaining the stabilization of the trimers in collagen, also iminoacid content (proline and hydroxyproline) has showed a direct positive correlation with the thermal stability of protein via hydrogen bonds, which stabilize the secondary structure of collagen (Ahmad et al. 2010; Nalinanon et al. 2007; Zhang et al. 2007). Differences in hydroxyproline content might determine the denaturation temperature and enthalpy of collagens from different fish species (Bae et al. 2008; Nalinanon et al. 2007). Calf skin collagen (215 residues per 1000 residues) and pig skin collagen (220 residues per 1000 residues) have higher imino acid content (Zhang et al. 2007) than PSC from skin of balloon fish (174 residues per 1000 residues) (Huang et al. 2011); bighead carp (165 residues per 1000 residues) (Liu et al. 2012) and grass carp (186 residues per 1000 residues) (Zhang et al. 2007), and denature at higher temperature. In general, collagen containing a low content of imino acids undergoes denaturation at lower temperatures than those with higher contents. Qin and Waite (1995) reported an imino acid content of 194 residues per 1000 residues for mussel byssus collagen, value closer to that of calf skin collagen, suggesting that thermal properties of byssus collagen might be similar to that of mammals. Although several studies linked collagen thermal stability with environmental and body temperature, this is not the case. As mentioned before, it is most likely that due to the biological function of mussel byssus collagen, the need of a highly extensive and stiff byssus (Harrington and Waite 2008) results in these unique thermal properties.

The denaturation temperature of the extracted collagen is a key property that determines its use for biomedical applications (Silva et al. 2014). Mammalian collagen has been extensively used as scaffolding material in regenerative medicine; but this protein is considered a great pathologic risk for transmitted diseases. On the other hand, the safety of marine collagen as potential material for biomedical applications has been confirmed by in vitro and in vivo analysis, but its application is hindered by its low denaturing temperature (Subhan et al. 2015). Hence, the high denaturation temperature of mussel byssus collagen, equals to bovine collagen, opens an opportunity to study this molecule for biomedical applications.

Surface tension of collagen dispersions

Interfacial processes play a major role in the self-assemblies of collagen molecules. Adsorption processes of amphiphilic molecules diffusing at interfaces are most often characterized using surface tension as a parameter (Fathima et al. 2011). Surface tension of collagen dispersions decreased from ~59 to 52 mN/m in 120 s (Fig. 6), with no statistical differences ($P > 0.05$) between different hydrolysis conditions. A sharp linear decrease was found in the first 15 s indicating that collagen molecules diffused quickly and covered the water/air interface, then a slower decrease in surface tension was seen towards the equilibrium values. Fathima et al. (2011) reported a surface tension value of 55.7 mN/m for rat tail tendon collagen, whereas Li et al. (2013) found a value of 70.9 mN/m but the concentration of the collagen dispersions used were below 8×10^{-3} mg/mL. Kezwon and Wojciechowski (2014) using a bovine calf skin collagen dispersion of 4 mg/mL at 21 °C, found a value of ~57 mN/m. These authors demonstrated that at concentrations below 0.3 mg/mL surface tension did not practically decrease.

Collagen surface properties are based on the presence of charged groups in the protein side chains, and on certain parts of the collagen sequence containing either hydrophilic or hydrophobic amino acids. The weak surface

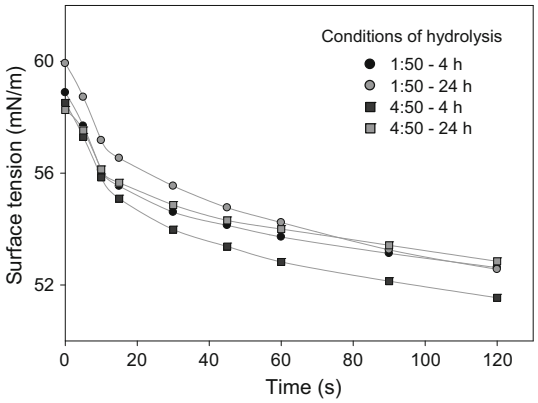

Fig. 6 Surface tension kinetics of pepsin-soluble collagen dispersions. Conditions of hydrolysis are pepsin:byssus mass ratio and hydrolysis time. *Error bars* are not shown for sake of clarity. Standard deviation between 0.06 and 2.32 mN/m

activity of native collagen is a consequence of the specific amino acid composition and spatial arrangements (Kezwon and Wojciechowski 2014). Collagen in its native form is too hydrophilic (hence not sufficiently amphiphilic) to display significant affinity to the water/air surface. However, these surface properties allow collagen to be used as encapsulating material for essential oils (Ocak 2012), probiotic bacteria (Su et al. 2011) or drugs (Lee et al. 2009) for controlled release.

Conclusion

An enzyme-aided extraction method was employed to isolate collagen from mussel byssus. Following our previous results, in this study we improve the methodology of collagen extraction from the mussel byssus and then the collagen extracted was characterized. Hydrolysis time had a significant effect on extraction yield, with a maximum value of 30%, indicating that the yield of the process can be increased by increasing hydrolysis time. From rheological, solubility, surface tension and thermal analysis it was concluded that pepsin hydrolysis step did not affect the integrity of the collagen molecule. The high value of denaturation temperature of byssus collagen opens an opportunity 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, pharmaceutical and biomedical applications. All of these results could be used as a basis for crafting strategies to assist the reutilization of waste and by-products from mussel processing plants.

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