Process Biochemistry 47 (2012) 570–577

Process Biochemistry

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

Recovery of protein, chitin, carotenoids and glycosaminoglycans from Pacific white shrimp (*Litopenaeus vannamei*) processing waste

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ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form
18 November 2011
Accepted 16 December 2011
Available online 30 December 2011

Keywords:
Autolysis
Bioactive molecules
Litopenaeus vannamei
Polysaccharides
Processing waste

ABSTRACT

Shrimp head waste is a major byproduct of crustacean processing in North-eastern Brazil and represents an interesting source of bioactive molecules. Additionally, its use increases the sustainability of processing fishery products. The present study reports a process developed for recovering bioactive molecules from shrimp heads through autolysis. A protein hydrolysate (120 ± 0.4 g) formed by a 9% (w/v) solution was recovered and lyophilized from 1 kg of shrimp heads. Approximately 195 ± 0.5 mg of carotenoids was recovered as an ethanolic extract. The recovery of chitin and chitosan were 25 ± 2 g kg^{-1} and 17 ± 4 g kg^{-1} wet processing waste, respectively. Chitosans were characterized by ^{13}C NMR, and FT-IR analysis and exhibited a variable degree of deacetylation (60–80%). Sulfated glycosaminoglycans that exhibited electrophoretic migration similar to mammalian standards were also recovered (79 ± 2 mg kg^{-1} wet processing waste), and their degradation products suggested the presence of C6-sulfated heparan sulfate. These data point to the feasibility of an integrated process for isolating highly bioactive molecules, such as sulfated- and amino-polysaccharides, with a broad spectrum of applications from shrimp processing waste.

1. Introduction

Shrimp production reached 3,275,726 T in 2007 and continues to be the second most important commodity traded monetarily, accounting for 16.5% of international fishery revenues [1]. The production of cultured crustaceans in North-eastern Brazil reached 63,750 T in 2006, accounting for approximately 98% of the amount produced in the country. The marine Pacific white shrimp, *Litopenaeus vannamei*, is the most commonly farmed shrimp species in Brazil and one of the three most commonly farmed species in the world, along with *Penaeus monodon* and *Penaeus chinensis*.

Increased farmed shrimp production has lead to a higher amount of processing waste, which poses novel environmental problems. In fact, shrimp processing waste is one of the main byproducts of fishery industries. Hydrolysis is a common method used to treat fish and shrimp waste and can be used to recover bioactive molecules. Hydrolysis is easily adaptable to industrial conditions, where it has been employed to produce highly nutritive protein hydrolysate [2,3]. Additionally, it is one of the steps in the extraction of other bioactive molecules, such as chitin (for chitosan production) and carotenoids [4,5].

The use of commercial proteases has been proposed for the recovery of biomolecules from shrimp processing waste [2–4,6]. However, the high cost of commercial enzymes represents an economic obstacle. The shrimp midgut gland and fish viscera are sources of digestive enzymes, such as peptidases [7,8]. Moreover, shrimp heads are a source of high-quality protein hydrolysate, chitin and chitosan [3,4,6], carotenoids [9,10] and sulfated glycosaminoglycans [11,12].

Shrimp processing waste is composed mainly of the cephalothorax, carapace and tail, which together represent 50% of the whole shrimp [10]. The remaining tissues can be recovered by hydrolysis

Abbreviations: ΔU,2S-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid 2-sulfate)-(1→4)-2-sulfamino-D-glucose 6-sulfate; ΔU-GlcNS,6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-sulfamino-D-glucose 6-sulfate; ΔUGlcNS, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-sulfamino-D-glucose; ΔU-GlcNAc, 6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-glucose 6-sulfate; ΔU-GlcNAc, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-glucose; ΔU-GalNAc, 4S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-glalactose 4-sulfate; ΔU-GalNAc, 6S, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-galactose 6-sulfate; ΔU-GalNAc, O-(4-deoxy-hex-4-enopyranosyluronic acid)-(1→4)-2-acetamido-D-galactose.

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doi:10.1016/j.procbio.2011.12.012

as soluble protein for the formulation of feed diets and food flavor [14]. Lyophilized *L. vannamei* head hydrolysate is composed of 44% protein, with a high content of essential amino acids (41% amino acid profile) [14]. Shrimp head waste is rich in protein (50–65% dry weight) and provides essential amino acids when used as a protein source in aquatic animal feeds as well as livestock and poultry diets [3].

In addition to using hydrolysis to recover protein, solvents or vegetable oils can be used to recover shrimp pigments in the form of a lipid–carotenoid extract. Carotenoids are the most widespread class of pigments in nature, present in virtually all organisms. In crustaceans, carotenoids, such as astaxanthin, are found complexed to proteins and are responsible for the animals' color and various biological events [9]. These pigments are suitable additives in animal feed and have been used as diet and health supplements [10,19,33].

Another approach is the extraction of polysaccharides, which come mainly from the chitin carapace and sulfated glycosaminoglycans. Chitin is an abundant natural structural amino-polysaccharide and is formed by 2-acetamido-2-deoxy-β-D-glucose through β (1→4) linkages. This white, hard and inelastic polysaccharide has low solubility, immunogenicity and chemical reactivity, much like cellulose [5]. The structure of chitosan is somewhat different: it is made up of poly(2-amino-2-deoxy-β-D-glucose) connected by β (1→4) linkages.

The sulfated glycosaminoglycan (GAG) profiles of various invertebrates and crustaceans have been studied to discover whether they have the potential to affect the circulatory system, as do the mammalian analogs [11,12,24].

The present study describes an integrated method to recover protein, chitin, carotenoids and GAGs from shrimp processing waste using proteolytic endogenous enzymes and characterizes the polysaccharides obtained.

2. Materials and methods

2.1. Raw material

Shrimp head processing waste, comprising the cephalothorax without the body carapace, from *L. vannamei* juveniles (total body weight about 10–12 g) were provided by a local fishery processing plant (EMPAF Ltd.). The samples were collected on different dates (04/02/2008, 10/04/2008 and 11/01/2009). Fresh heads were immediately stored on ice (0 °C) and transported to the laboratory where they were packed in plastic bags (1 kg per bag) and stored at −20 °C until use. Only analytical-grade reagents were used.

2.2. Proteolytic activity

Shrimp heads were mixed with distilled water at a ratio of 1 kg of wet processing waste to 1 L of water and ground in an industrial food processor (Engefrio, Recife, Brazil). Protease activity of the shrimp head homogenate was determined based on a previously described method [13]. One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing azocasein to produce a 0.001-unit change in absorbance per minute.

2.3. Shrimp head autolysis

Hydrolysis was performed based on a previously described method [14] for *L. vannamei*, without the addition of any commercial proteases. The heads (1.0 kg) were minced in 1.0 L of distilled water (1:1 ratio; particle size approximately 5 mm), and autolysis occurred in a vessel placed in a water bath at 40 °C for 2 h with constant stirring (700 rpm). The preparation was then heated for 10 min at 100 °C and filtered through gauze (1-mm^2 mesh) to retain the head carapace (solid phase) used for chitin extraction and chitosan production. The liquid phase was centrifuged at 10,000 × *g* (Sorvall RC 6 Plus Centrifuge, Thermo Scientific, USA) at 4 °C for 10 min, generating a precipitate from which carotenoids and glycosaminoglycans were extracted. The supernatant was a solution containing the protein hydrolysate. The schematic procedure is summarized in Fig. 1.

2.4. Chitin extraction and chitosan preparation

The solid phase (chitinous residue) was dried at 70 °C and treated with 1 M HCl and 1 M NaOH to remove $CaCO_3$ and proteins, respectively. The depigmentation was performed with 0.5% (w/v) $KMnO_4$ and 1% (w/v) $Na_2S_2O_4$. The chitin flakes (25 g) were washed with abundant distilled water and dried at 70 °C. Chitin was added to 500 mL of 50% (w/v) NaOH and incubated in a water bath at 65 °C for 24 h with constant stirring. N-deacetylation was performed a second time under the same conditions. The chitosan was washed with distilled water until a neutral pH was reached, then dried at 70 °C and pulverized in an electric mill (IKA® A11 Basic, IKA®-Works Inc., China) to reduce the particle size to below 250 μm. Finally, the chitosan (1%, w/v) was solubilized in 3% (v/v) acetic acid, filtered through paper filter (14 μm), precipitated with 1 M NaOH until a pH of 11.0 was reached, neutralized to pH 7.0 and centrifuged (10,000 × *g* for 15 min at 25 °C). This purified chitosan was lyophilized and collected as a fine powder.

2.5. Residual solution treatment

Calcium and protein were obtained by mixing alkaline and acid washes from the chitin purification to obtain an insoluble phase. The mixture was centrifuged at room temperature for 10 min at 10,000 × *g*, and the precipitate was dried at 70 °C.

2.6. Nuclear magnetic ressonance and Fourier transform-infrared spectroscopic analysis of chitin and chitosan

Decoupled ^{13}C NMR spectra were obtained using a Bruker Avance DRX-400 spectrometer with a 5-mm inverse probe. ^{13}C NMR acquisitions were performed using the WALTZ-16 pulse sequence with the following parameters: FIDRES: 0.8466 Hz; AQ: 0.5906 s; DW: 15.75 s; DE: 5.5 μs; D1: 110 ms; D2: 3.4 ms; PL12: 17 dB (decoupler ^{1}H). FT-IR spectra were measured in KBr pellets in transmission mode within a range of 4000–500 cm^{-1} using an FT-IR Bomem MB100 spectrophotometer. The degree of deacetylation (DD%) was calculated based on the ratio A_{1320}/A_{1420} reported by [15]: $DD\% = 100 - [(A_{1320}/A_{1420} - 0.3822)/0.03133]$.

2.7. Extraction of carotenoids and HPLC analysis

To extract fat and carotenoids, 1500 mL of 90% (v/v) ethanol were added to the precipitate in three 500-mL portions. The remaining sediment was lyophilized. This lyophilized sediment, hereafter referred to as "dried sediment," was used for the further extraction of sulfated glycosaminoglycans (GAGs). The pigmented extract was concentrated (100 rpm, 40 °C) in a flash evaporator (IKA® HB 05.06CN Rotary Evaporator, IKA Works Inc., China), and the evaporated ethanol from this concentration step was recovered for further extractions. HPLC separations were carried out using a 300 mm × 3.9 mm (length × I.D.) water-wet C18 reverse-phase column (15–20 μm). Chromatographic analysis was conducted in a ÄKTA Purifier (GE Healthcare, Buckinghamshire, United Kingdom) equipped with an auto-sampler injector and controlled by the UNICORN 4.11 software. Chromatography was performed isocratically at 23 °C. The mobile phase consisted of acetonitrile, methanol, chloroform and water at a proportion of 60:25:10:5 (v/v/v/v) and had a flow rate of 1.0 mL min^{-1}. The injection volume was 50 μL. Detection by absorbance was performed at 480, 455 and 474 nm. Calibration curves were established by injecting pure astaxanthin (Sigma–Aldrich, Germany) at ten different concentrations as a standard in the HPLC mobile phase. The quantity of the carotenoids obtained could thus be directly calculated from the areas of the peaks.

2.8. Extraction and partial purification of glycosaminoglycans

Approximately 85 g of the dried sediment obtained following carotenoid and lipid ethanol extraction was submitted to another proteolysis in a solution (final volume, 0.85 L) of 4.0 mg mL^{-1} Maxatase (Biocon Laboratories, São Paulo, Brazil) in 50 mM Tris–HCl, pH 8.0, with 0.15 M NaCl (1 g of the sediment per 10 mL of the enzyme solution) for 24 h at 60 °C. The proteins were then precipitated with trichloroacetic acid (TCA, final concentration 10%, w v^{-1}) at 4 °C and centrifuged at 10,000 × *g* for 20 min. The glycosaminoglycans present in the supernatant after TCA precipitation were precipitated by adding two volumes of methanol, stored at −20 °C for 18 h, centrifuged (10,000 × *g* at 4 °C for 10 min), and dried at room temperature. The dried precipitate was resuspended in water to form a 6% (w v^{-1}) solution, centrifuged (10,000 × *g* at 4 °C for 10 min), dialyzed and lyophilized. The fraction obtained after precipitation with methanol was analyzed by electrophoresis and fractionated with increasing volumes of acetone. For this analysis, the sample was dried and resuspended in 0.5 M NaCl to form a 5% (w v^{-1}) solution, to which acetone was added at a final concentration of 30%, and stored at 4 °C for 18 h. The precipitate was collected by centrifugation at 10,000 × *g* for 20 min. Acetone aliquots were added to the supernatant to obtain different volume fractions (F0.3–F2.0). The highest acetone volume fraction was 200% (F2.0).

2.9. Electrophoresis of glycosaminoglycans

Three electrophoretic systems on agarose gels with different buffers were used: a PDA (1,3-diaminepropane acetate) buffer (0.5 M, pH 9.0), a Tris-acetate buffer (0.5 M, pH 8.0) and a discontinuous barium acetate (0.5 M, pH 8.0)/PDA system, as previously described [11,12]. Samples were spotted on 0.6% agarose gel slides in each buffer and underwent electrophoresis at 5 °C in a chamber. GAGs were quantified by densitometry at 525 nm.

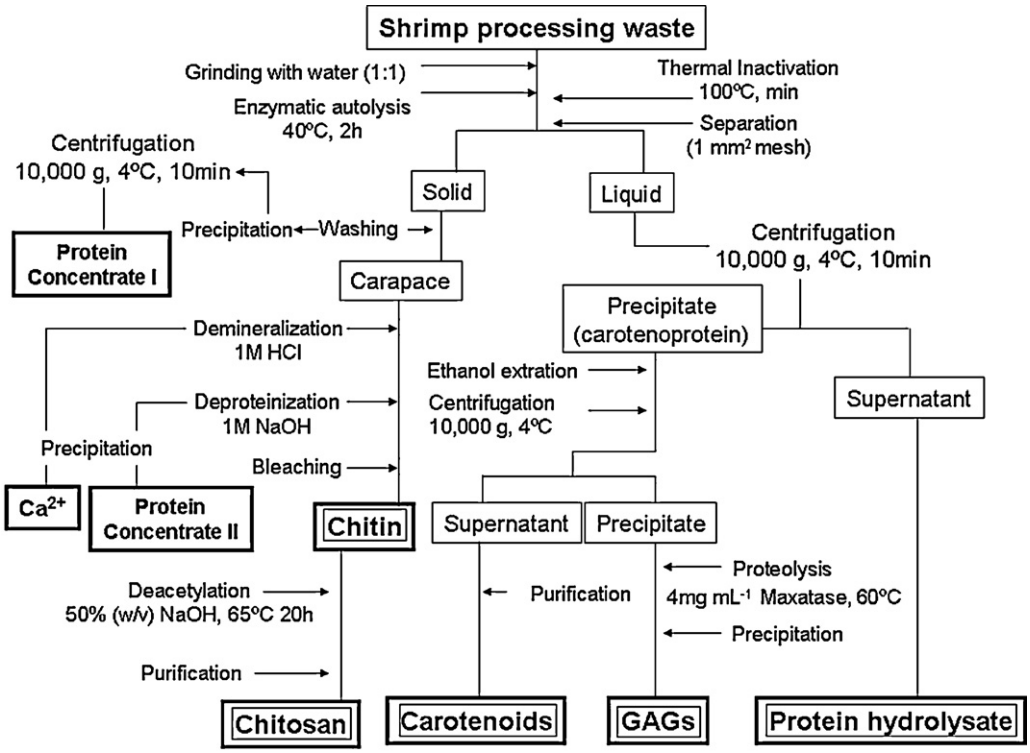

Fig. 1. Scheme for the use of *L. vannamei* shrimp head waste for the recovery of protein, chitin, carotenoids and glycosaminoglycans and production of chitosan.

2.10. Enzymatic degradation with glycosaminoglycan lyases

The F0.8 and F1.0 fractions were selected to undergo enzyme degradation with glycosaminoglycan lyases, according to methods previously described [11,12,25] and summarized as follows: 100 μg of GAGs were incubated with 0.1 U of enzymes in 0.05 M ethylenediamine-acetate buffer pH 7.0 at 30 °C for 8 h in a final volume of 40 μL. The degradation products underwent paper descendent chromatography on Whatman No. 1 filter paper, using isobutyric acid/1 M NH_3 5:3 ($v\,v^{-1}$) as mobile phase for 24 h. The products were detected by short-wave UV shadowing and silver nitrate staining. The incubation mixtures were also analyzed by HPLC in a Zorbax SAX 4.6 mm × 150 mm ion exchange column (Äkta Purifier, GE Helathcare, Buckinghamshire, United Kingdom) and the dissacharides formed were eluted using a NaCl gradient (0–1.5 M) with a flow of 1 mL min^{-1} and monitored at 232 nm.

2.11. Protein hydrolysate production

The liquid supernatant obtained following hydrolysis, solid separation and centrifugation (10,000 × *g* for 10 min at 4 °C), hereafter referred to as "protein hydrolysate," was stored in polyethylene flasks at −20 °C until analysis. One-milliliter aliquots were lyophilized to determine their dry matter content. Analyses were carried out as described previously [14] to determine the hydrolysate composition and the amino acid profile.

3. Results and discussion

The present study reports the development of an optimized process for recovering bioactive compounds with a large array of applications from shrimp processing waste (Table 1). This process economic feasibility lies in the fact that no expensive exogenous enzymes were employed. Trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of penaeids [16,17]. Indeed, the proteolytic activity of *L. vannamei* head homogenates using azocasein as a substrate was 6.76 ± 0.8 mU min^{-1} mg^{-1}. The proteolytic activity in midgut gland extracts from the penaeids *Farfantepenaeus paulensis* and *Farfantepenaeus subtilis* using the same substrate were 6.49 ± 0.2 mU min^{-1} mg^{-1} and 11.97 ± 0.74 mU min^{-1} mg^{-1}, respectively [7,18]. This information demonstrates the presence of endogenous shrimp peptidases that may potentially hydrolyze the high protein content in shrimp heads, releasing peptides and amino acids.

Despite these data, it is generally thought that the content and composition of the enzymes in shrimp heads are not constant. Shrimp physiology may be influenced by seasonal changes and other physico-chemical factors such as salinity, temperature or food availability. In fact, the shrimps used in this study were obtained from commercial farms, in which the animals are cultured in outdoor tanks in North-eastern Brazil, a tropical zone where temperatures generally range between 24 and 28 °C throughout the year. Moreover, the tanks where the shrimp are farmed are subject to rigidly controlled salinity and feed conditions. Matrices and the post-larvae production are under highly standardized genetic control. These conditions are designed to minimize the effect of seasonal changes that could affect shrimp physiology, body composition and digestive enzymes.

Several methods are available for the recovery of chemical components from crustacean processing waste. Such methods are used to obtain protein hydrolysates, protein concentrates, carotenoids, chitin and calcium compounds from shrimp, crabs, krill and lobster heads and carapaces [19,20]. However, these well-established processes use crustacean waste for recovering some compounds by homogenizing and cooking the materials. A process was developed [4] in which shrimp heads are hydrolyzed using a commercial protease to obtain protein hydrolysate, chitin and carotenoids, with improved efficacy. Tissues that undergo hydrolysis instead of homogenization and cooking yield products with different properties that facilitate the extraction of compounds with more desirable physico-chemical characteristics and lower amounts of chemical contaminants [3,6].

Fig. 1 contains a flow chart representing the process for obtaining the protein hydrolysate and recovering chitin, carotenoids and glycosaminoglycans (GAGs) from shrimp processing waste as well as calcium and the protein concentrate from chitin purification. The carapace washings yielded a protein-rich precipitate (concentrate I). Chitin was obtained after the washed carapace was

Table 1
Yield of bioactive molecules isolated from 0.236 ± 0.028 kg (dwb[a,b], $n = 3$) of shrimp head waste as well as some properties and technological applications.

Products	Yield (dwb[a])	Properties	Application
Hydrolysate[c]	120 ± 0.4 g	High protein quality[g] and sugar content [14], source of essential amino acids [29].	Animal feed supplement [14], replacement for fish protein and feed flavor [3,31].
Carotenoids[d]	195 ± 0.5 mg	Natural pigments, pro-vitamin A activity [9,10,21].	Fish skin and flesh pigmentation and feed supplementation [21,36].
Astaxanthin[d]	83 ± 0.2 mg	Powerful antioxidant, anticancer, photoprotective health promotion [32,33,35].	Human health enhancer, nutraceutical [34].
Chitin[e]	25 ± 2 g	N-acetylglucosamine, insoluble, biodegradable, nontoxic, physiologically inert, high protein affinity [5,23].	Lectin affinity chromatography, biosensor, enzyme and cell immobilization, films, wound dressing material [5,23].
Chitosan[e]	17 ± 4 g	Cationic, acid soluble, nontoxic, biodegradable, biocompatible, renewable, film forming, hydrating agent, nontoxic, antimicrobial, wound healing properties, hydrolyzable by lysozyme, complexes with metals [5,23,24].	Food coating, removal of metal ions, anti-tumor, hemostatic, anticoagulant, bacteriostatic, surgical sutures, artificial skin, rebuilding of bone, controlled drug delivery, encapsulating material [23,24].
Sulfated glycosaminoglycans[f]	79 ± 2 mg	Heparan sulfate-like and heparin-like, anticoagulant, antithrombotic, cofactor II activity [12,28].	New anticoagulant, anti-inflammatory, anti-hemorrhagic drugs [11,27].

[a] Dry weight basis.
[b] From 1.0 kg shrimp head wet weight.
[c] Dry matter from 9% lyophilized solution.
[d] Obtained from 53 g of crude carapace.
[e] Estimated from astaxanthin standard chromatogram peak area.
[f] Obtained from 85 g of dried sediment after extraction of carotenoids and lipids with ethanol, estimated by densitometry.
[g] Total of 44% crude protein, of which 41% is essential amino acids.

demineralized, deproteinized and bleached, while chitosan was produced by alkaline deacetylation of chitin. The liquid phase was centrifuged, yielding a supernatant with a high protein content (the protein hydrolysate) and a precipitate formed by a complex mixture of proteins, lipids and liposoluble compounds mostly carotenoids. From this precipitate, carotenoids and lipids were extracted with ethanol. After this step, the residual insoluble mass underwent further proteolysis for the recovery of GAGs. The main products obtained are summarized in Table 1. At the end of the process, the following products were obtained from 1 kg of wet shrimp head processing waste and were used for further extraction and purification: a protein and calcium concentrate (48.1 g), an ethanolic extract (lipid concentrate) (44 g), a precipitate after ethanol extraction (85.0 g), carapace content (53 g) and a protein hydrolysate (1.5 L) (9% dry matter). This method proved to be suitable for the recovery of multiple biomolecules from shrimp head waste. Also, the proportion of bioactive molecules extracted was reproducible even though the raw materials (shrimp heads) were collected on different dates (04/02/2008, 10/04/2008 and 11/01/2009).

After autolysis, the solubilized tissues form the liquid hydrolysate (supernatant), and the pigmented sediment is formed mainly by the carotenoid–protein complex [9]. Organic solvent extraction is traditionally used for carotenoid extraction from natural sources, mainly with organic solvents [10,33]. The total carotenoid content in the concentrated ethanolic extract was 194.52 mg kg^{-1} of heads, of which 111.96 mg was unidentified carotenoids; astaxanthin accounted for 82.56 mg (Table 1). A

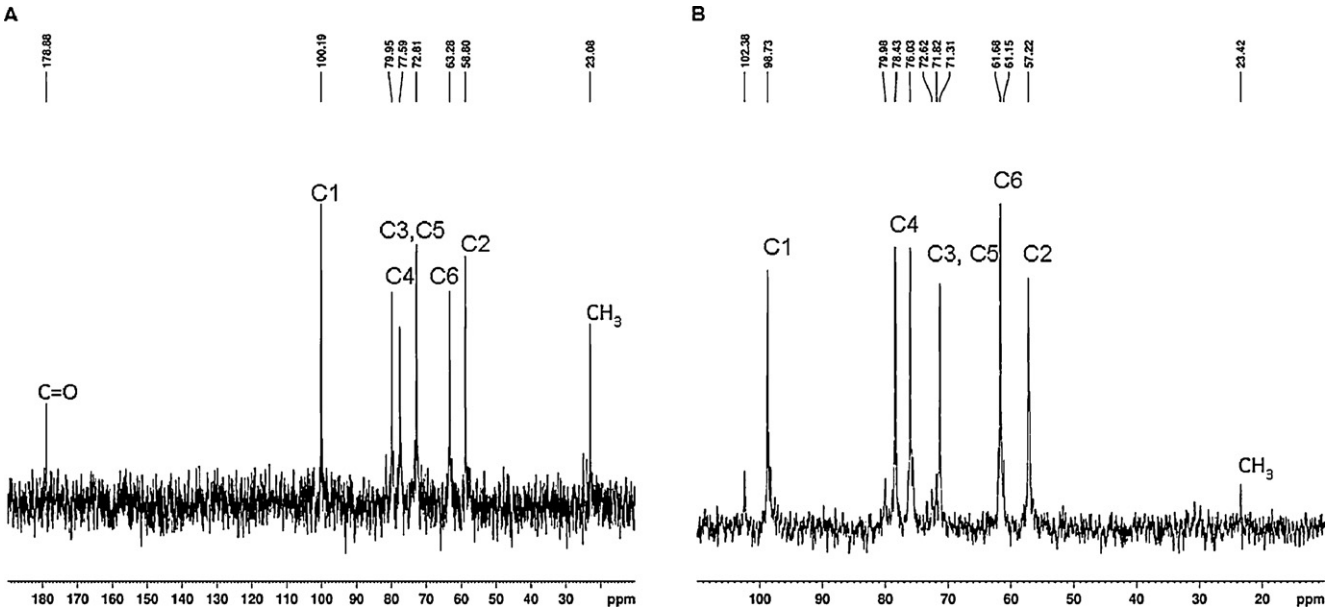

Fig. 2. ^{13}C NMR spectra of chitosan that has been deacetylated once (A) and twice (B), using 50% (w v^{-1}) NaOH at 65 °C for 20 h; carbon peaks represent atoms in the N-(acetyl)-glucosamine structure; the disappearance of the peak at 178.8 ppm and the slight decrease in the peak at 23.3 ppm, which represent the C═O and CH_3 of the acetyl group, respectively, indicate that deacetylation has occurred.

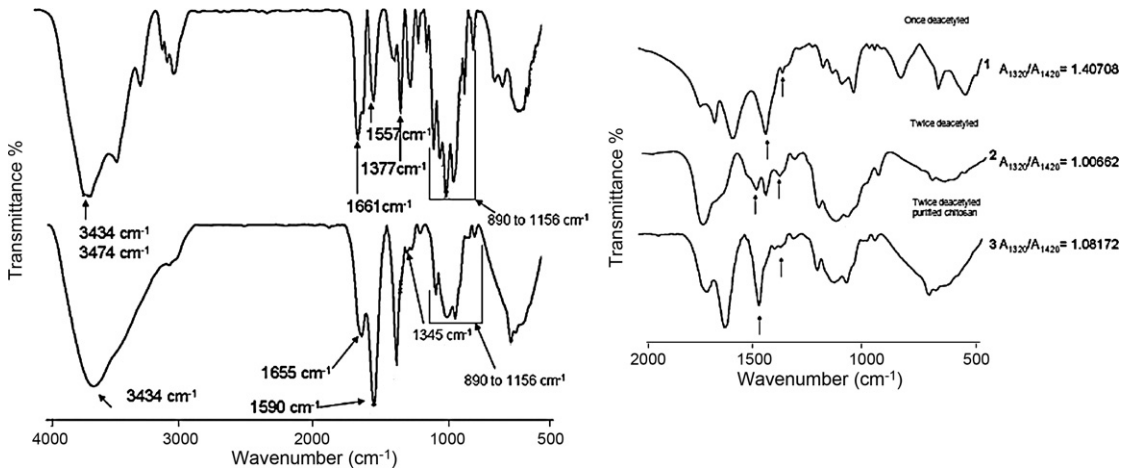

Fig. 3. (A) FT-IR comparison of chitin and chitosan from *L. vannamei* and (B) FT-IR comparison of chitosan (1) deacetylated once, (2) deacetylated twice and (3) purified and deacetylated twice.

method was previously optimized for the extraction of carotenoids from non-hydrolyzed shrimp waste using different organic solvents [10]. The maximal yield of carotenoids from *Penaeus indicus* using this method was 43.9 mg kg^{-1} of waste using a mixture of isopropyl alcohol and hexane and only 31.9 mg kg^{-1} of wet waste using ethanol [10]. In a previous study, the biological material originating from krill, shrimp or crawfish waste underwent proteolysis and was shown to promote the more efficient release of astaxanthin [21].

Crude chitin represented 53.3 g kg^{-1} of waste (average dry weight), and chitin and chitosan accounted for approximately 25 g and 17 g, respectively (Table 1). In a previous study, 88 g kg^{-1} of chitin was obtained from 1 kg of processing waste from the shrimp *Penaeus semisulcatus* [3]. The production of chitin and chitosan is based on the treatment of the crustacean carapace. In the exoskeleton of shrimp, chitin is closely associated with proteins, so the deproteinization step is very important in the extraction process. During shell demineralization and deproteinization, the solubilized protein and calcium carbonate can be recovered as a protein concentrate and Ca^{2+}, respectively (Fig. 1). This recovery is possible because solid head waste contains 30–35% protein and calcium carbonate, whereas chitin accounts for the other major fractions [17,22].

The ^{13}C NMR spectra of *L. vannamei* chitosan for samples that underwent one or two deacetylation cycles are displayed in Fig. 2. The deacetylation is evident, as the peak at 178.8 ppm disappears, while the one at 23.3 ppm slightly decreases, representing C=O and CH_3 of the acetyl group, respectively. The other peaks correspond to C1 (δ 100.1 and 100.5); C2 (δ 58.8 and 57.2); C3 and C5, which appear as a single signal (δ 72.8 and 71.6); C4 (δ 78.7 and 78.4); and C6 (δ 63.2 and 61.1). The absence of other peaks suggests that the samples are free of impurities [25].

The FT-IR graphics of chitin and chitosan from *L. vannamei* are displayed in Fig. 3A and are in accordance with the characteristic profiles previously described [25]. The bands at 1661–1671 cm^{-1} are attributed to the vibrations of the amide I bond and correspond to the amide I stretching of C=O. The band at 1345 cm^{-1} corresponds to a CO—NH deformation of the CH_2 group (amide III). The sharp band at 1377 cm^{-1} corresponds to a symmetrical deformation of the CH_3 group, the band at 1557 cm^{-1} corresponds to the stretching or N—H deformation of amine II and the bands between 890 and 1156 cm^{-1} represent polysaccharide structures. For chitosan, the band at 1590 cm^{-1} was more intense than that at 1655 cm^{-1}, which suggests effective deacetylation (Fig. 3B). When chitin deacetylation occurs, the band observed at 1655 cm^{-1}

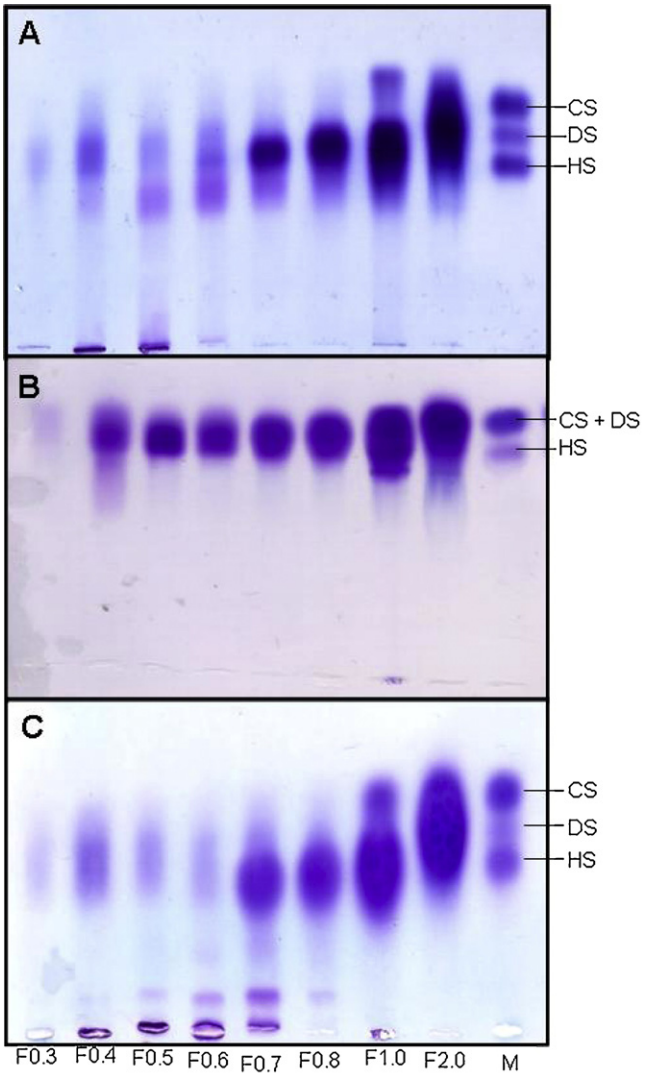

Fig. 4. Electrophoresis of glycosaminoglycans extracted from *L. vannamei* head waste using several different buffers: (A) 0.05 M propylenediamine-acetate (PDA) buffer, pH 9.0; (B) 0.05 M Tris-acetate buffer, pH 8.0; (C) discontinuous 0.04 M barium acetate, pH 4.0/PDA buffer. "M" is a mixture of chondroitin, dermatam and heparam sulfate (1.0 mg mL^{-1}).

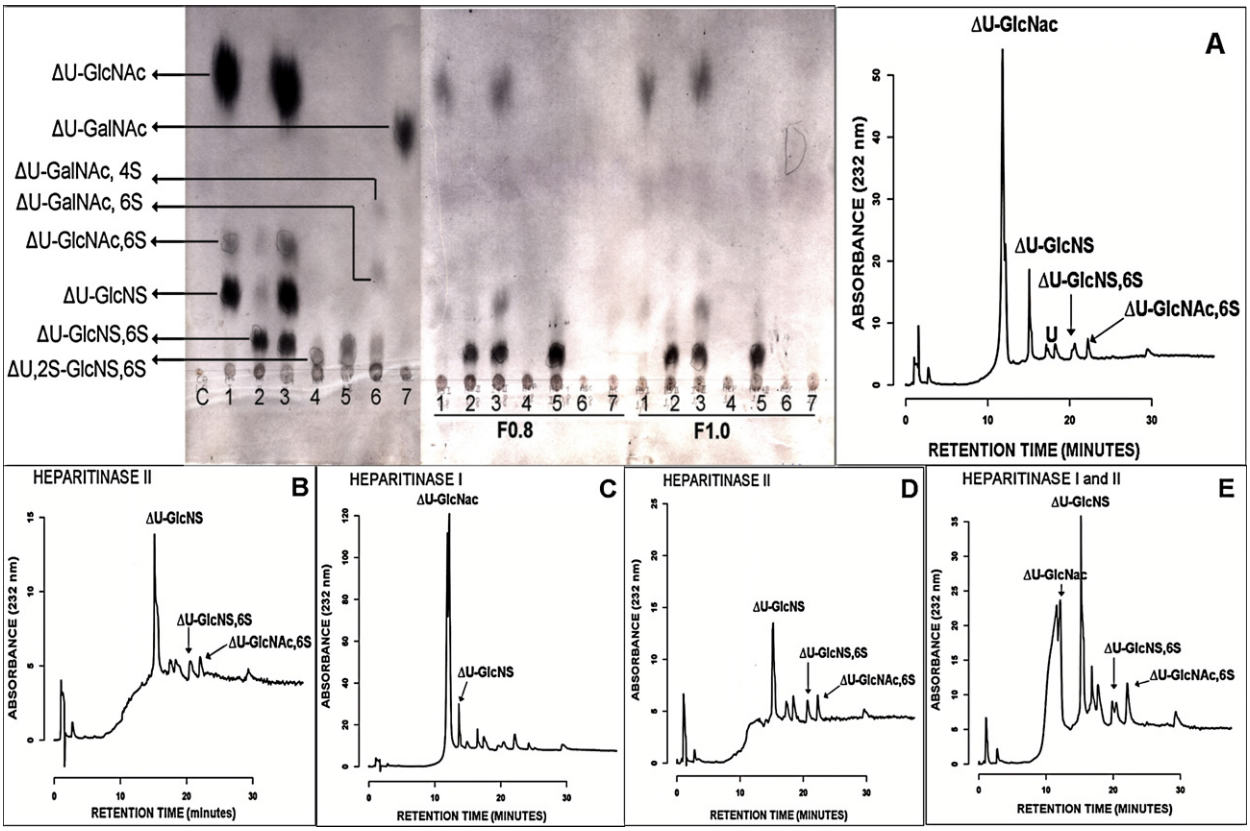

Fig. 5. Paper chromatography analysis of products formed through action of heparitinases I (1) and II (2), a mixture of these two enzymes (3), heparinase (4), mixture of heparinase and heparitinase II (5) and chondroitinases AC and ABC (6 and 7, respectively) and SAX-HPLC profile of products formed by heparitinases degradation of standard heparam sulfate (A), F0.8 (B) and F1.0 (C–E).

decreases and then increases again at 1590 cm^{-1}, indicating the presence of NH_2 groups. The band from 1500 to 1700 cm^{-1} demonstrates an intensification of the peak at 1590 and a decrease at 1655 cm^{-1}.

A method was developed for measuring the degree of deacetylation in chitin and chitosan using FT-IR analysis in a selected ratio of absorbance bands, calibrated using ^{1}H liquid and ^{13}C CP MAS solid state NMR as absolute techniques [15]. The absorption ratio A_{1320}/A_{1420} gives the smallest experimental error, regardless of the technique and state of the material. Fig. 4 displays the difference between chitosans analyzed by FT-IR and by the corresponding A_{1320}/A_{1420} ratio. The FT-IR-calculated degree of deacetylation for *L. vannamei* chitosan that was deacetylated once, deacetylated twice or purified deacetylated twice were 62% ± 5, 79% ± 2 and 77%, respectively. The degree of deacetylation and the molecular mass are the most important properties of chitosan, as both factors influence solubility and reactivity and, as such, are important in differentiating chitosan from chitin and cellulose [15].

The glycosaminoglycan content in the sediment after centrifugation of the hydrolysate and extraction with ethanol was measured by densitometry in a dried agarose gel with PDA buffer and accounted for 79 ± 2 mg kg^{-1} of *L. vannamei* processing waste (Table 1). Fractions purified from the glycosaminoglycan extract by acetone precipitation underwent electrophoresis in an agarose gel with various buffer systems (Fig. 4).

Propylenediamine acetate at 0.05 M and pH 9.0 buffer distinguishes glycosaminoglycans based on the positions of sulfate groups relative to diamine (chondroitin and dermatan sulfate have identical charge densities but different structures and, consequently, different migration lengths). This buffer separates chondroitin sulfate from dermatan sulfate, heparan sulfate and heparin. The discontinuous buffer used (0.04 M barium acetate pH 4.0/0.05 M diaminopropane acetate pH 9.0) separates heparin from heparan sulfate, dermatan sulfate and chondroitin sulfate. The Tris-acetate buffer (0.06 M Tris, pH 8.0) separates glycosaminoglycans based on charge; thus, heparin has the highest migration rate in this system [26].

The electrophoretic migration of glycosaminoglycans from *L. vannamei* was similar to that of the mammalian glycosaminoglycan standards (chondroitin sulfate, dermatan sulfate and heparan sulfate) and stained metachromatically in toluidine blue. In the barium-diaminopropane discontinuous system, three fractions displayed a glycosaminoglycan that migrates like the slow-moving component of heparin (Fig. 4C). The presence of sulfated glycosaminoglycans has been reported in some invertebrates [28]. These studies report that heparan sulfate-like and heparin-like compounds are present in many species. The presence of heparan sulfate is ubiquitous in tissue-organized life forms, and its structural variability is known to be tissue dependent [27,28]. This observation has lead to speculation that these compounds may be involved in cell–cell recognition and growth control [26].

The precipitation fractions F0.8 and F1.0 were chosen for degradation by glycosaminoglycan lyases because these fractions had the highest extraction yields (data not shown) and because a mixture of other compounds was found in F0.4–F0.7 and F2.0, as illustrated in Fig. 4. The degradation of glycosaminoglycan extracts from *L. vannamei* processing waste is displayed in Fig. 5. These fractions were extensively degraded by heparitinase II and, to a lesser extent, heparitinase I. Heparitinase II is a glycosaminoglycan lyase from *Flavobacterium heparinum* that cleaves glycosidic linkage between 2- or 6-O-sulfated glucosaminide-glucuronic acid on heparin sulfate and heparin, leaving mainly ΔU-GlcNAc, 6S

ΔU-GlcNS, ΔU-GlcNS, 6S and traces of ΔU-GlcNAc heparitinase I cleaves exclusively N-acetyl or N-sulfo-glucosaminide-glucuronic acid linkage on heparan sulfate, leaving ΔU-GlcNS and ΔU-GlcNAc. Sulfation of the glucosamine residue at C-6 is impeditive for enzyme activity of heparitinase I. These two heparitinases degrade heparan sulfate on its constituent disaccharides [29]. The main products formed by action of heparitinases I and II on fractions F0.8 and F1.0 were ΔU-GlcNS, 6S, ΔU-GlcNAc, ΔU-GlcNS as seen in Fig. 5(B–E). These data suggests that one of the major glycosaminoglycans extracted from the dried shrimp sediment is a heparan sulfate with a high degree of C6 sulfation.

A sulfated glycosaminoglycan isolated from *Penaeus brasiliensis* head processing waste was previously described [12]. Its anticoagulant activity and susceptibility to heparinase and heparitinase II were similar to those of mammalian heparins. A heparan sulfate from the brine shrimp *Artemia franciscana* with structural features similar to heparan sulfate and heparin has been isolated and characterized [28]. In another study that employed a different process, a heparin-like glycosaminoglycan that exhibited anti-inflammatory activity and reduced anti-coagulant properties was isolated from *L. vannamei* [11]. At that time, the absence of hemorrhagic events due to this compound demonstrated that it was better than mammalian heparin as a possible anti-inflammatory drug. In that study, the authors also suggested that, unlike crustacean heparin analogs, mammalian products are at risk for contamination by pathogens such as prions. The use of these biomaterials represents a new approach for applications in the fields of biotechnology, biomedicine and pharmaceutics.

At the end of the process, the content of dry matter remaining in the protein hydrolysate was 120 ± 0.4 g (Table 1) in a 9% ($w\,v^{-1}$) solution. Protein hydrolysates, which can be used as a functional food ingredient or a flavor enhancer, were prepared from *L. vannamei* heads through autolysis [30]. Endogenous enzymes from shrimp heads have a strong autolytic capacity for releasing threonine, serine, valine, isoleucine, tyrosine, histidine and tryptophan. Feed formulations for the Nile tilapia (*Oreochromis niloticus*) have been designed using shrimp head silage powder [31] and autolysate [14] as a replacement for fish meal, with no adverse effects on growth or nutrient uptake.

This process – carried out on a pilot scale – can be used for larger amounts of materials, such as those in industry reactor vessels, which can have volumes of up to 1000 L. The requirements for the extraction procedures can be met using an industrial centrifuge. The process should be performed as close to the processing plant as possible to avoid transportation and refrigeration costs. Moreover, the entire process can be carried out to obtain all of the available biomolecules or may be adjusted to meet the demand for a particular biomaterial using simple steps.

Acknowledgments

This study was financially supported by the Brazilian fostering agencies MPA, CNPq, FINEP, EMBRAPA, UFPE and FACEPE. The authors would like to thank EMPAF Ltd. for providing the shrimp heads.

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