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Antioxidant and Antimicrobial Enhancement by Reaction of Protein Hydrolysates Derived from Shrimp By-Products with Glucosamine

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Abstract

Shrimp cooking juice and shrimp carapaces are interesting sources of protein which are habitually disposed of at large quantities by crustacean industries. In this work, protein obtained from the cooking juice and carapaces of Pacific white shrimp (*Litopenaeus vannamei*) was hydrolyzed by an enzyme extract of *Enterococcus faecalis* DM19 isolated from raw camel milk. The hydrolysates were afterwards heated separately with glucosamine (GlcN) at 100 °C for 0, 40, 60, 120 and 180 min. The reaction was followed by measuring the change in absorbance at 294 and 420 nm and also by fluorescence ($\lambda_{exc} = 340$ nm; $\lambda_{em} = 440$ nm). Browning and intermediate products increased with heating time showing a concomitant decrease in free amino groups, while the pH decreased as well. Amino acid analysis showed that the amounts of lysine, arginine, and histidine decreased significantly after heating for 180 min. The infrared spectra suggested the appearance of new molecules after heating, presumably Schiff bases and/or aromatic compounds. The antioxidant and antimicrobial activities of the heated hydrolysates were compared to those of the native hydrolysates. Interestingly, the reducing ferric ion activity was improved 22-fold after conjugation. Meanwhile, both scavenging radical and metal chelating ion activities were increased sevenfold. Besides, the shrimp carapace hydrolysate heated in presence of GlcN exhibited a good antibacterial activity against gram positive and gram negative bacteria. To conclude, protein hydrolysates derived from the shrimp by-products studied in this work could be heated in presence of GlcN at 100 °C and be potentially used as antioxidant and/or antibacterial ingredients for different purposes.

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Keywords

Maillard reaction
Shrimp by-products
Enzymatic hydrolysis
Glucosamine
Antioxidant activity
Antimicrobial activity

Statement of Novelty

Crustacean industries produce a large amount of protein rich residues that are hardly valorized. In this work, protein obtained from the cooking juice and carapaces of Pacific white shrimp was hydrolyzed by microbial proteases. The hydrolysates were further incubated at 100 °C in the presence of glucosamine. New biomolecules were formed during the incubation. The antioxidant activity increased significantly, and potent antimicrobial activity against gram negative and gram positive bacteria was also observed after incubation. This work demonstrates that the incubation of glucosamine with protein hydrolysates from crustacean disposals could yield molecules with interesting applications with interest for industries. The valorization of these by-products would also contribute to the protection of the environment.

Introduction

Shrimp and prawn are the most valued products in the global seafood market, including fisheries and aquaculture, with a total capture production of 3.5 million tons and an export value of more than 23,000 million (American) dollars in 2016 [1]. These products are usually processed at sea or on shore and can be bought peeled, with the vein removed or intact, pre-cooked, frozen, fresh, canned, etc. The shrimp processing has led to a higher amount of solid by-products, mainly carapaces, heads and tails, which can account up to 20–50% of total weight [2]. It means that more than one million tons of solid by-products could be produced by shrimp processing industries each year around the world. In addition, the shrimp pre-cooking process produces highly pollutant wastewaters that can account around 1 L per kg of shrimp cooked [3]. Whatever the kind, unfortunately, these wastes are not usually processed and are directly discharged into the environment causing contamination problems, notwithstanding the fact that they contain several compounds which deserve to be valued [4]. Among these products, scientific interest has focused on proteins since they represent a potential source of biologically active peptides and essential amino acids necessary for the proper

functioning of the organism.

Protein-rich by-products can be utilized in the production of bioactive peptides using specific protease producing microorganisms [5]. Among the microorganisms employed in the bioconversion of wastes, lactic acid bacteria (LAB) has garnered great attention owing to its potential to effectively convert inexpensive agri-food industrial residues into valuable bioactive protein hydrolysates [6]. Nonetheless, this depends on the protease-producing ability of the starter culture during the fermentation process [5]. For example, lactic acid fermentation of freshwater fish heads by *Pediococcus acidilactici* NCIM5368, *Enterococcus faecium* NCIM5335 and *P. acidilactici* FD3 has resulted in the recovery of bioactive protein hydrolysates [7]. Likewise, in another work, shrimp head fermentation by *Streptococcus thermophilus*, produced protein hydrolysates, which exhibited a high antioxidant activity [8]. However, protease activity of the *Enterococcus* genus has never been exploited on shrimp wastes.

Maillard reaction is a type of non-enzymatic browning which involves the reaction of carbonyl compounds, especially of reducing sugars, with compounds that possess primary amino groups, such as amino acids, proteins (mainly the ϵ -amino group of Lys and α -amino groups at N-terminal position) or any nitrogenous compound [9]. This reaction produces a large number of the so-called Maillard reaction products (MRP) which can contribute to the quality and acceptability of stored or processed foods. During the last decade, investigations have been focused on fish protein hydrolysates to enhance, via conjugation with sugars, functional and biological properties such as antimicrobial or antioxidant [10]. In this regard and by conjugation of proteins, glucosamine (GlcN) has been reported to be an interesting sugar so as to obtain molecules with promising applications in the food industry [11]. GlcN is an amino sugar obtained by the hydrolysis of chitin, the second most abundant polymer in nature and a major component of insect exoskeletons, fungal cell walls and crustacean shells [12]. GlcN could then be obtained from shrimp waste usually discarded by seafood processing industries. Hrynets et al. [13] reported that GlcN showed higher reactivity than fructose, galactose, glucose or xylose in the Maillard reaction at moderate temperatures (25 and 37 °C) as well as at high temperatures (125 °C) respectively. GlcN could be a potential candidate for peptide glycation to obtain antioxidant and/or antimicrobial molecules from shrimp waste.

The main objective of this work was to improve the antioxidant and

antibacterial activities of shrimp waste hydrolysates by the conjugation with GlcN. The accomplishment of this objective may lead to valorize important by-products derived from shrimp processing, such as cooking juice and carapaces.

Materials and Methods

Materials and Chemicals

Enterococcus faecalis DM19, isolated from raw camel milk, was supplied by the Laboratory for Biology of Microorganisms and Biotechnology, University of Oran 1 Ahmed Benbella, Algeria. It was selected due to its proteolytic activity detected in plate count agar with skimmed milk added, where it exhibited a large clear halo (unpublished data). D-glucosamine (2-amino-2-deoxy-D-glucose, GlcN) hydrochloride, o-phthaldialdehyde (OPA), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), ethylenediaminetetraacetic acid (EDTA), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate (Ferrozine) and L-serine were from Sigma-Aldrich Co. (St. Louis, MO). Potassium persulfate, L(+)-ascorbic acid, iron(II) heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), iron(III) chloride (FeCl_3), iron(II) (FeCl_2) and 2-mercaptoethanol were obtained from Panreac (Barcelona, Spain) while 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) was from Fluka (Buchs, Switzerland). All other chemicals were of analytical reagent grade.

Sample Preparation

Cultured Pacific white shrimp (*Litopenaeus vannamei*) were kindly provided by Angulas Aguinaga (Burgos, Spain). They were transported frozen at $-20\text{ }^\circ\text{C}$ to Madrid (Spain), where they were divided into two batches after arrival. Shrimp of the first batch (500 g) were thawed at low temperature and peeled manually. Carotenoids were then extracted from the shells, as described by Gómez-Estaca et al. [14]. Carotenoid-free shells were then carefully rinsed with distilled water and frozen at $-20\text{ }^\circ\text{C}$ until use.

Shrimp (500 g) of the second batch were cooked in 1000 mL of distilled water for 5 min at $100\text{ }^\circ\text{C}$. The shrimp were removed and the protein containing cooking water was further freeze-dried and kept at $-20\text{ }^\circ\text{C}$ until use.

Preparation of the Enzyme Extract

One liter of MRS broth buffered with 0.1 M sodium phosphate buffer (pH 7.0)

was added with a 2% of skimmed milk, inoculated with *E. faecalis* DM19 and then left to incubate for 24 h at 30 °C. The culture was then centrifuged at 10000×g for 20 min at 4 °C (Beckman J2-MC, Palo Alto, California, USA). The collected supernatant was filtered through a 0.22 µm filter and then freeze dried. The resulting powder was stored at – 20 °C and used as crude enzyme extract for shrimp protein hydrolysis.

Determination of Enzymatic Activity

The protease activity in the supernatant (crude enzyme extract) was determined according to Park et al. [15] with slight modifications, using azocasein as substrate. One volume of supernatant was mixed with two volumes of azocasein solution (0.5%, w/v) prepared in 0.2M sodium phosphate buffer (pH 7.0). After incubation at 40 °C for 24 h, the reaction was stopped by adding trichloroacetic acid (TCA) at 12%, (w/v). The mixture was homogenized with vortex and then centrifuged at 5000×g for 30 min at 4 °C. Then, 750 µL of supernatant were mixed with 250 µL of NaOH (0.5 M) and the absorbance of the resulting mixture at 440 nm was against a blank prepared as the sample, with the exception that TCA was added before the supernatant.

One unit (AU) of proteolytic activity corresponded to the amount of enzyme releasing 1 µg of azocasein per min under the assay conditions. The extinction coefficient of azocasein ($\epsilon'_{1\%/440\text{nm}} = 37 \text{ L cm}^{-1} \text{ g}^{-1}$) was used for calculating proteolytic activity. The specific activity was measured by the number of protease activity units per mg of protein in 1 mL of extract according to the following formula:

$$\text{Specific activity (U/mg)} = \frac{\text{Enzymatic activity (U/mL)}}{\text{total protein content (mg/mL)}}.$$

Sample Hydrolysis and Degree of Hydrolysis

The proteins from the carapaces or from the cooking juice were hydrolyzed in controlled conditions of pH and temperature. The pH of the reaction was 7, and was controlled with 0.1M NaOH by using a pH-Stat (TIM 856, Radiometer Analytical, Villeurbanne Cedex, France). For each reaction, 50 g of raw material were homogenized with 150 mL of sodium phosphate buffer (0.2 M, pH 7). The system was stirred and brought to the desired temperature

(40 °C). Then, 2 g of enzymatic extract (111 Units) were added (enzyme:substrate ratio of 1:25, w:w). After 2 h, the samples were heated at 90 °C for 10 min to inactivate the enzymes, and the hydrolysates of proteins from the cooking juice (SCJH) or carapaces (SCH) were cooled down at room temperature. Protein hydrolysates were then centrifuged at 5000×g (Sorvall centrifuge RT 6000B, DuPont Co., Delaware, USA) for 20 min at 4 °C to separate insoluble and soluble phases. Finally, the supernatants (hydrolysates) were freeze dried, ground in a mortar and stored at – 20 °C before analysis.

The degree of hydrolysis (DH), defined as the percent ratio of peptide bonds cleaved to the total number of peptide bonds in the protein substrate, was determined by the method described by Adler-Nissen [16] as follows:

$$DH = \frac{V \cdot N}{\alpha \cdot M_p \cdot h_{tot}} \times 100,$$

where V is the amount of NaOH consumed to keep the pH constant during the reaction, N is the normality of NaOH, M_p is the mass of the protein substrate in the reaction (determined as $N \times 6.25$), α is the average degree of dissociation of the α NH_2 groups released during hydrolysis ($\alpha = 10^{(\text{pH}-\text{pKa})}/1 + 10^{(\text{pH}-\text{pKa})}$) and h_{tot} is the total number of peptide bonds per unit weight of shrimp, calculated from the amino acid composition. The α value was 0.90. The protein content of shrimp carapace was 24.85%, whereas that of shrimp cooking juice was 18.86%. The h_{tot} values were calculated from the amino acid composition of the raw material assuming complete recovery of all the amino acids. The h_{tot} values were 9.46 and 9.43 meq/g of protein for shrimp cooking juice and shrimp carapace, respectively.

Reaction of Protein Hydrolysates with Glucosamine

Non-enzymatic glycation of shrimp protein hydrolysates was performed according to the method described by Sumaya-Martinez et al. [17] with some modifications. One hundred mg of freeze-dried hydrolysate were dissolved in 40 mL of 0.2 M sodium phosphate buffer (pH 8.0) followed by the addition of 800 mg of GlcN. Control samples (hydrolysates without GlcN) were also prepared. The mixtures were transferred to Pyrex tubes tightly capped and heated at 100 °C in a temperature-controlled water bath equipped with a continuous stirring system (120 rpm). Aliquots of each sample (SCJH-GlcN and SCH-GlcN) were removed after incubation for 40, 60, 120 and 180 min, cooled immediately in ice water, freeze dried and stored at – 20 °C.

Measurement of pH

The pH of the samples was monitored during heating using a pH-meter (Metrohm 827 pH lab, Swiss).

Absorbance Measurements

The UV-absorbance and browning were measured at 294 nm and 420 nm, respectively by using a spectrophotometer UV-1601 (Shimadzu, Japan), as described by Ajandouz et al. [18]. Before analysis, the samples were diluted tenfold times with Milli-Q[®] water (Millipore, Molsheim, France). The absorbance at 294 nm was measured as an indication of the formation of intermediate products of non-enzymatic browning, while that at 420 nm was measured as an index of the brown polymers formed in more advanced stages.

Fluorescence Spectroscopy

Fluorescence of the samples was determined over heating time as described by Morales and Jiménez-Pérez [19] with a slight modification using a Synergy MX Microplate Reader (BioTek, Winooski, USA). Samples were previously diluted 30-fold times with Milli-Q[®] water. The fluorescence intensity was then measured at an excitation wavelength of 340 nm and emission wavelength of 440 nm. The scan rate was 120 nm/min.

Determination of Free Amino Group Content

The amount of available amino groups in the samples before and after heating was determined by the *o*-phthaldialdehyde (OPA) method, according to Nielsen et al. [20]. Three hundred microliters (300 μ L) of freshly prepared OPA reagent was added to 10 μ L of sample prepared at 0.6 mg/mL. The OPA reagent consisted of mixing OPA with 2-mercaptoethanol at a ratio of 1:200 (v/v). After vortexing and a minimal 2 min delay in the dark at room temperature, the absorbance at 340 nm was recorded against OPA reagent. Absorbance readings were converted into free amino contents (in mg serine equivalent/mL) using a calibration curve obtained with L-serine (0–0.2 mg/mL) as standard.

Amino Acid Composition

The freeze-dried samples were diluted with ultrapure water and hydrolysed with continuously boiling 6 N HCl containing 0.1% phenol with norleucine as internal standard, at 110 °C for 24 h. After hydrolysis, HCl was removed

under vacuum. The dried samples were reconstituted in the application buffer, and injected onto a Biochrom 20 amino acid analyzer (Pharmacia, Barcelona, Spain). The amino acid content was expressed as milligrams of residues per gram of sample.

Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectra of freeze-dried samples were recorded between 4000 and 650 cm^{-1} using a Perkin–Elmer Spectrum 400 Infrared Spectrometer (Perkin–Elmer Inc., Waltham, MA, USA) equipped with an ATR prism crystal accessory. The spectral resolution was 4 cm^{-1} . Measurements were performed at room temperature using approximately 1 mg of dried sample, which was placed on the surface of the ATR crystal, and pressed with a flat-tip plunger until spectra with suitable peaks were obtained. Background interference was eliminated using the Spectrum software version 6.3.2 (Perkin–Elmer Inc.).

ABTS Radical Scavenging Activity

The ABTS radical [2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] scavenging capacity of the samples was determined according to Alemán et al. [21]. The stock solution of ABTS radical consisted of 7 mM ABTS in potassium persulfate 2.45 mM, kept in the dark at room temperature for 16 h. An aliquot of stock solution was diluted in Milli-Q water to obtain a working solution of ABTS radical with an absorbance of 0.70 ± 0.02 at 734 nm. Twenty microliters (20 μL) aliquot sample (25 mg/mL) was mixed with 980 μL of ABTS reagent and then left to stand in the dark at 30 °C for 10 min. Absorbance values were read at 734 nm using a UV-1601 spectrophotometer (Model CPS-240, Shimadzu, Japan). Results were expressed as mg ascorbic acid Equivalent Antioxidant Capacity (AAEAC)/g of sample based on a standard curve of ascorbic acid.

Ferric Reducing Antioxidant Power (FRAP)

A measure of the reducing power of the samples was performed according to Alemán et al. [21] with some modifications. Thirty microliters (30 μL) of sample (25 mg/mL) were incubated with 90 μL of Milli-Q water and 900 μL of FRAP reagent, containing 10 mM of TPTZ and 20 mM of FeCl_3 at 37 °C for 30 min. Absorbance values were read at 595 nm. Results were expressed as mmol Fe^{2+} equivalents/g of sample based on a standard curve of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

Iron Binding Ability

The Fe²⁺ chelating activity of the samples was measured according to Dinis et al. [22]. Briefly, 800 µL of sample (25 mg/mL) were mixed with 10 µL of 2 mM FeCl₂ and 20 µL of 5 mM Ferrozine. The mixture was kept at room temperature for 10 min prior to measuring the absorbance at 562 nm.

Chelating ability was expressed as µmol EDTA equivalents/g of sample based on a standard curve of EDTA (5 mM).

Molecular Weight Profile

The molecular weight (MW) distribution of the hydrolysates before and after conjugation with GlcN was obtained by size-exclusion chromatography (HPLC model SPE-MA10AVP, Shimadzu). The column used was a Superdex peptide PC 3.2/30 column (GE Healthcare Bio Sciences, Barcelona, Spain). First, 10 mg of powdered samples were dissolved in 1 mL of Milli-Q water, centrifuged at 9000×g for 5 min, and filtered at 0.45 µm before injection. The injection volume was 10 µL and the flow rate was 0.1 mL/min through the column. Acetonitrile:Milli-Q water (30%, v/v) with 0.01% (v/v) trifluoroacetic acid was used as isocratic mobile phase. Absorbance was monitored at 214 nm. Molecular weight standards including bovine serum albumin (6.64 kDa), aprotinin (6.511 kDa), vitamin B12 (1.355 kDa), angiotensin II (1.046 kDa), hippuryl-L-histidyl-L-leucine (0.429 kDa) and glycine (0.075 kDa) were run through the column at the same conditions and used for MW calculation. Plots of retention time for molecular weight standards were used to construct calibration curve, from which hydrolysate molecular distributions were computed.

Antimicrobial Properties of MRP

The antimicrobial activity of the samples was tested against gram positive and gram negative bacteria from the Spanish Tye Culture Collection (CECT). The activity was determined using the disk diffusion method [23]. Cell suspension (10⁶ cfu/mL, 100 µL) was added onto agar plates before paper disks (diameter of 5.0 mm, Whatman No. 1) containing test solutions were placed on plates. An antibiotic solution (chloramphenicol, 0.3 g/100 mL) was used as control. After incubation, the diameter of the inhibition zone (considered as the antimicrobial activity) was measured with Corel Draw X6 software. Results were expressed as mm of inhibition growth (magnification ratio 1:10).

Statistical Analyses

All experiments were carried out in triplicate. The results were expressed as mean $\pm$ standard deviation. Mean separation and significance were analyzed using the IBM SPSS[®] software (SPSS Statistical Software, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was carried out to discriminate among the means at different heating times. Tukey's HSD procedure was used as post-hoc test. Differences between pairs of means were determined using Fisher LSD (Least Significant Difference). A statistical difference at $P < 0.05$ was considered significant.

Results and Discussion

Measurement of pH

The pH of each sample incubated with GlcN decreased markedly ($P < 0.05$) within the first 40 min (Table 1). Thereafter, the pH decreased slightly during the remaining heating time. In parallel, the pH of the hydrolysates heated without GlcN showed minor changes. These results were in accordance with those reported by Morales and Jiménez-Pérez [19] and Jiang et al. [24].

Brands, van Boekel [25] showed that the pH drop resulted in part from the formation of organic acids, such as acetic acid and formic acid during the Maillard reaction process. It has been demonstrated, as well, that due to its degradation at high temperature, GlcN could give rise to acetic acid [26], glyoxal and methylglyoxal [11] which affect the pH of the system.

Furthermore, significant amounts of amino groups were released when heating the hydrolysates in the absence of GlcN (Table 2) and this fact could have a significant effect in the decrease of the pH in these samples. In this field, Eric et al. [27] reported that the decrease in pH, and therefore the acidification of the medium during the heating process, is mainly due to the involvement of amine groups during the chemical reaction to form MRP. Lan et al. [28] indicated that the incubation of peptides causes the release of amino acids, which could contribute to a slight decrease in the pH of the system. As well, Van Chuyen et al. [29] observed that the incubation of peptides with carbonyl compounds may induce peptide bond breaking and hence, a decrease in pH.

Table 1

pH values (mean $\pm$ standard deviation, $n = 3$) of the hydrolysates heated alone or in presence of GlcN as a function of heating time

Heating time (min)	SCH	SCH-GlcN	SCJH	SCJH-GlcN
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Heating time (min)	SCH	SCH-GlcN	SCJH	SCJH-GlcN
0	8.00 ± 0.00 ^a	8.00 ± 0.00 ^a	8.00 ± 0.00 ^a	8.00 ± 0.00 ^a
40	8.00 ± 0.00 ^a	6.98 ± 0.03 ^b	7.99 ± 0.01 ^{ab}	6.98 ± 0.02 ^b
60	8.00 ± 0.00 ^a	6.87 ± 0.02 ^c	7.99 ± 0.01 ^{ab}	6.91 ± 0.01 ^c
120	8.00 ± 0.00 ^a	6.61 ± 0.02 ^d	7.98 ± 0.01 ^{ab}	6.64 ± 0.01 ^d
180	8.00 ± 0.00 ^a	6.50 ± 0.01 ^e	7.97 ± 0.02 ^b	6.55 ± 0.01 ^e
Different letters (a, b...) in the same column indicate significant differences at different heating times ($P < 0.05$)				

Table 2

Changes in free amino group content (mean ± standard deviation, n = 3) of the hydrolysates heated alone or in the presence of GlcN

Heating time (min)	SCH	SCH-GlcN	SCJH	SCJH-GlcN
0	2.97 ± 0.04 ^c	7.30 ± 0.18 ^a	2.49 ± 0.06 ^c	4.96 ± 0.18 ^a
40	8.95 ± 0.13 ^a	1.61 ± 0.03 ^b	6.13 ± 0.43 ^a	0.40 ± 0.13 ^b
60	8.33 ± 0.27 ^b	1.29 ± 0.05 ^b	6.15 ± 0.03 ^a	0.30 ± 0.01 ^b
120	8.63 ± 0.04 ^{ab}	0.60 ± 0.50 ^c	5.69 ± 0.13 ^b	0.32 ± 0.02 ^b
180	8.50 ± 0.10 ^b	0.62 ± 0.08 ^c	5.48 ± 0.35 ^b	0.32 ± 0.02 ^b
Results are expressed in mg of Ser equivalents/ml. Different letters (a, b, c) in the same column indicate significant differences at different heating times. Values with different letters are significantly different ($P < 0.05$)				

UV Absorbance and Browning Intensity

Browning is the easiest measurable parameter of the Maillard reaction and is often used as an indicator of the reaction extent [18], while absorbance at 294 nm has been generally used as an indicator of the presence of colorless intermediate compounds [30]. Figures 1 and 2 illustrate, respectively, the absorbance evolution of the samples at 420 nm and 294 nm during heating time. The browning intensity of the samples incubated with GlcN significantly increased with heating during the first 40 min (Fig. 1). From then on, browning intensity of these samples reached a plateau phase for 20 min and later increased significantly, to finally remain stable until the end of the heat

treatment. In contrast, no changes were observed in the hydrolysates incubated alone for 3 h at 100 °C. This means that colored molecules were formed during heating in the presence of GlcN, mainly low molecular weight (LMW) brownish compounds instead of higher colored molecules, as proposed by Poulsen et al. [31]. These LMW molecules mainly derive from the reaction of sugar and intermediate products with free amino acids and peptides, which are abundant in shrimp hydrolysates. Similar results were found by Cai et al. [32]. Accordingly, polymerized brown pigments were formed proportionally with the generation of intermediate products. In addition, Renn and Sathe [33] observed a faster browning when the model systems showed an excess of amino acids. Kim and Lee [34] suggested that the degree of browning was related to the degree of hydrolysis of peptides or proteins upon heating, which could be the case in our study. According to Jiang et al. [24], the browning rate was remarkably influenced by the initial pH and the browning products were favorably formed in alkaline conditions.

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Fig. 1

Browning intensity of the samples as a function of heating time. Error bars represent the standard deviation of the mean of triplicate experiments. The absorbance at 420 nm of each sample over heating time was compared, and values with different letters indicate results significantly different ($P < 0.05$)

Fig. 2

Development of absorbance at 294 nm in the samples as a function of heating time. Error bars represent the standard deviation of the mean of triplicate experiments. The absorbance at 294 nm of each sample over heating time was compared, and values with different letters indicate results significantly different ($P < 0.05$)

As depicted in Fig. 2, a sharp increase in $A_{294\text{nm}}$ of the hydrolysates-GlcN model systems was observed after heating for 40 min ($P < 0.05$). As previously observed in the formation of browning compounds, the $A_{294\text{nm}}$ remained stable or showed a slight decrease during the following 20 min of heating, to then significantly increase and remain stable up to the end of the reaction time. The results suggested that intermediate products were produced to a great extent within the first 40 min and during the last 1–2 h of reaction. Moreover, heating should have increased the $A_{294\text{nm}}$ of the hydrolysates in the absence of sugar. However, the absorbance remained constant or even decreased. The reason could be the complete exposure of aromatic residues in the peptide molecules before and during heating.

Therefore, the results depicted in Figs. 1 and 2 suggest that the UV absorbing compounds produced during the intermediate stage could be further transformed into brown pigments. The increments in UV absorbance and browning of SCJH-GlcN model system were much higher than those of SCH-GlcN, thus indicating that the browning degree could be dependent on the

peptide composition of the hydrolysates. Moreover, it coincides with a higher loss of Lys, Arg and mainly His in SCJH after glycation (Table 3).

Table 3

Amino acid content (mg/g sample) in shrimp hydrolysates before and after heating in the presence of glucosamine

Amino acids	SCH	SCH-GlcN	SCJH	SCJH-GlcN
Aspartic acid + asparagine (Asx)	16.59 ± 0.30	13.53 ± 0.14*	4.47 ± 0.18	3.24 ± 0.16*
Threonine (Thr)	5.72 ± 0.04	5.27 ± 0.23	1.52 ± 0.14	1.15 ± 0.13
Serine (Ser)	5.21 ± 0.11	6.02 ± 0.03*	1.38 ± 0.01	1.63 ± 0.01*
Glutamic acid + glutamine (Glx)	24.03 ± 0.08	19.53 ± 0.27*	10.09 ± 0.27	7.36 ± 0.25*
Glycine (Gly)	11.72 ± 0.01	9.90 ± 0.11*	9.09 ± 0.06	5.95 ± 0.04*
Alanine (Ala)	9.86 ± 0.20	10.10 ± 0.18	3.89 ± 0.07	4.25 ± 0.96
Cysteine (Cys)	1.23 ± 0.17	1.54 ± 0.25	1.27 ± 0.16	0.94 ± 0.01
Valine (Val)	7.67 ± 0.24	6.85 ± 0.30*	3.02 ± 0.10	1.66 ± 0.08*
Methionine (Met)	2.63 ± 0.01	1.59 ± 0.21*	0.46 ± 0.35	0.26 ± 0.18
Isoleucine (Ile)	6.32 ± 0.10	4.49 ± 0.06*	1.75 ± 0.24	1.11 ± 0.04*
Leucine (Leu)	11.89 ± 0.38	9.41 ± 0.52*	3.60 ± 0.48	2.52 ± 0.44
Tyrosine (Tyr)	2.92 ± 0.11	3.39 ± 0.23	0.07 ± 0.02	0.25 ± 0.24
Hydroxylysine (Hyl)	0.05 ± 0.03	0.38 ± 0.30	0.13 ± 0.06	0.00 ± 0.00*
Histidine (His)	4.80 ± 0.02	3.58 ± 0.04*	7.99 ± 0.20	0.48 ± 0.65*

The data are expressed as mean ± standard deviation (n = 3)

*Represents significant differences between pair of samples before and after heating

Amino acids	SCH	SCH-GlcN	SCJH	SCJH-GlcN
Lysine (Lys)	14.83 ± 0.11	6.83 ± 0.58*	10.92 ± 0.52	2.05 ± 0.13*
Arginine (Arg)	9.41 ± 0.06	4.67 ± 0.47*	8.00 ± 0.76	2.68 ± 0.30*
Hydroxyproline (Hyp)	0.94 ± 0.08	0.99 ± 0.03	0.36 ± 0.14	0.00 ± 0.00*
Proline (Pro)	12.01 ± 0.13	10.39 ± 0.01*	10.55 ± 0.06	8.51 ± 0.08*
TOTAL	147.83 ± 2.52	118.46 ± 3.96*	78.56 ± 4.52	44.04 ± 3.7*
The data are expressed as mean ± standard deviation (n = 3)				
*Represents significant differences between pair of samples before and after heating				

Fluorescence Intensity

Previous studies have reported that fluorescent compounds in MRP show maximum excitation at wavelengths between 340 and 370 nm, and maximum emission between 420 and 450 nm [35]. In our work, the maximum excitation was observed at 330 nm, while the maximum emission was found at 440 nm. As shown in Fig. 3, a continuous marked increase in fluorescence over time was noticeable in samples incubated with GlcN, unlike the fluorescence of the hydrolysates heated alone. The increase in fluorescence was in fact significant when heating for longer periods of time. This result indicates the production of fluorescent chemical structures during heating of the hydrolysates in the presence of GlcN. This was probably due to the increase in precursors, which was associated with the development of browning. Obviously, according to Cai et al. [32], a longer heating time forces fluorescence compounds to accumulate gradually and reach a maximum before being converted into visible brown pigments. Similarly, the two chemical processes are dependent on each other with the exception that they function concomitantly in our case. Furthermore, in contrast to the non-enzymatic browning reaction, the production of the main fluorophore that seems responsible for the overall measured fluorescence is favored at neutral or at a slightly acid pH [24], which could be the case in our present study.

Fig. 3

Changes in fluorescence intensity of the samples as a function of heating time. Error bars represent the standard deviation of the mean of triplicate experiments. The fluorescence intensity of each sample over heating time was compared, and values with different letters indicate results significantly different ($P < 0.05$)

Determination of Free Amino Group Content

It is known that α -amino groups of peptides and ϵ -NH₂ groups of Lys are able to react with carbonyl groups of reducing sugars. After 40 min of incubation of the hydrolysates with GlcN, an important decrease of the α -amino group content was observed (Table 2). From then on, the amounts of free amino groups in the system were very scarce (SCJH-GlcN). This means that an extended heating of the samples catalyzed covalently the interaction between the terminal α -amino (or ϵ -amino) groups of practically all peptides or free amino acids and the carbonyl groups of GlcN. Thus, it seems that numerous and different MRP intermediates were formed. Our result was consistent with that of Liu et al. [36]. From the results, the decrease observed in free amino groups complied with the increase in browning, fluorescence and colorless intermediate compound generation.

The amount of amino groups in protein hydrolysates significantly increased after heating for 40 min in the absence of GlcN (Table 2). It clearly suggests that peptide degradation into smaller peptides or free amino acids occurred in that period of time, as observed by Lan et al. [28]. The release of amino groups during heating was much higher in SCH, being this attributable to the higher amount of protein of this sample (Table 3). From then on, the amount of free amino groups remained constant or even decreased slightly, suggesting that some peptides could cyclize spontaneously at prolonged heating times.

Amino Acid Composition

Table 3 shows the amino acid composition of the protein hydrolysates before and after reaction with GlcN. The SCH showed a relevant amount of Glx (24 mg/g) followed by Asx (16.59 mg/g), Lys (14.83 mg/g) and Pro (12.01 mg/g), while the most abundant amino acid in SCJH was Lys (10.92 mg/g), followed by Pro (10.55 mg/g), Glx (10.09 mg/g), Gly (9.09 mg/g), Arg (8 mg/g) and His (7.99 mg/g). The total amount of amino acids in SCH was around two fold times higher than that in SCJH. In both samples, the reaction with GlcN decreased dramatically the amount of amino acids, reaching this decrease values around 20% (SCH) and 56% (SCJH). It suggests a higher reactivity of GlcN with free amino groups in SCJH, mainly because of the abundance of amino acids with amino groups in the side chain, such as Lys, Arg or His [31], either in peptide chains or in their free form.

The reaction with GlcN affected the relative amount of each amino acid in the samples differently. Among the most abundant amino acids, Lys and Arg were the most reactive and their content decreased dramatically after incubation of the hydrolysates with GlcN. It is important to note that the relative amount of His also decreased differently on each glycated sample. Thus, the His content in SCJH decreased almost completely after the reaction with GlcN, but this reduction was slight in SCH. This suggests that almost all His residues in SCJH were located in the N-terminal position of peptides, or remained as free amino acids in this hydrolysate. In SCH, the decrease in the amounts of Asx and Glx was higher after the reaction with GlcN, when compared to SCJH. It indicates a higher reaction of the sugar with Asn and Gln in this sample. The decrease in the relative amount of certain amino acids after heating could explain the increment in the relative proportion of Ser or Ala. Presumably these amino acids were scarcely located at the N-terminal position in the peptides or in their free form in the system.

Molecular Weight Distribution

The molecular weight (MW) distribution of the hydrolysates showed a relevant peak that corresponded to peptides around 197 Da (SCH) and 186 Da (SCJH), as depicted in Fig. 4. Therefore, the protein hydrolysates were mainly composed of short peptides, presumably dipeptides. As well, a low amount of high molecular weight peptides with MW higher than 1 kDa were found in the hydrolysates. These profiles are consistent with the high DH values calculated for each hydrolysate (12.60% and 9.89% for SCH and SCJH respectively).

Fig. 4

Changes in the molecular weight profile (range 100–7000 Da) of SCH (**a**) and SCJH (**b**) when heated for 180 min in the presence of GlcN. Identification: Red (non-glycated), blue (glycated for 40 min), green (glycated for 60 min), brown (glycated for 120 min), pink (glycated for 180 min). There were not significant differences in the molecular weight of the hydrolysates when heated without glucosamine (data not shown)

The molecular weight profile of each hydrolysate, measured at 214 nm with the Superdex peptide column, changed during heating with GlcN. The highest MW molecules (> 1 kDa) disappeared during the first 40 min of heating, suggesting that they reacted with GlcN giving place to new molecules that were not retained by the column or could not be read at 214 nm. At the same time, the browning observed in the samples SCJH-GlcN and SCH-GlcN (Fig. 1) indicated that melanoidins could be formed from these higher MW peptides. Moreover, the low MW peptides present in the hydrolysates (186 and 197 Da) disappeared during the first 40 min of reaction while larger molecules (MW of 267 Da) appeared (Fig. 4). It indicates that molecules with similar molecular weight were formed in SCJH-GlcN and SCH-GlcN during

heating in presence of GlcN. These molecules seemed to remain stable until the end of the heating incubation and could be conjugates sugar-peptide. Interestingly, the presence of new LMW molecules coincided with a diminution in the amount of certain amino acids such as His, Lys and Arg (Table 3). These results are consistent with the study of Liu et al. [37]. Nonetheless, the MW of glycated peptides can be calculated by a mass shift of 161 Da between the native and glycated peptides [38], and in our work this mass shift was around 70–80 Da, which indicates the formation of advanced glycated end products (AGE's) during heating, as reported by Schmidt et al. [39]. As well, numerous MRP intermediates with different molecular weight and composition could be formed during heating, due to the presence of a large variety of reactive intermediates and other molecules such as free amino acids and peptides in the system.

Fourier Transforms Infrared Spectroscopy (FTIR)

Shrimp protein hydrolysates and their MRP had nearly similar infrared absorption, suggesting that the reaction with GlcN did not lead to important appearance of functional groups associated with Maillard products such as Amadori compounds (C=O), Schiff base (C=N) or pyrazines (C–N) (Fig. 5). It is worth mentioning that the profiles of the samples glycated did not show the bands associated with GlcN reported by Benavente et al. [40] (3370–3300, 1615 and 1094 cm^{-1}), suggesting that all molecules of GlcN reacted with peptides during the heating reaction.

Fig. 5

FTIR Spectra of the hydrolysates before and after heating in the presence of GlcN

Several infrared (IR) absorption bands characteristics of proteins [41] were observed in the spectra of the hydrolysates. However, not all these bands were found because of the abundance of peptides. They cannot adopt complex conformations, in contrast with proteins. An absorption band ascribed fundamentally to N–H stretching vibrations (Amide A band) could be seen at 3252 cm⁻¹, which was mainly attributed to the abundance of peptide bonds. A similar band with the same intensity was also identified in SCJH-GlcN. However, the lower concentration of amino acids in this sample suggests a lower content of peptide bonds; this band could also be attributed to potential presence of melanoidins in this sample. In SCH-GlcN the band shifted to 3238 cm⁻¹ and showed a lower intensity than in the non-glycated sample. It

suggests that the reaction of SCH with GlcN could affect in some way the strength of hydrogen bonds in this sample [42], as well as the presence of melanoidins. The band at 2927–2930 cm^{-1} observed in all samples could be attributed to C–H stretching vibrations, and showed a lower intensity in the SCH-GlcN. The reason could be the potential lower concentration of peptide bonds. The amide I band is the most intense absorption band in proteins, but its intensity in the samples studied (1594–1626 cm^{-1}) was not strong when compared with the bands around 3252 and 1032 cm^{-1} . This band is mainly due to the C=O stretching vibrations of the peptide bonds, and was identified for the hydrolysates at 1615–1626 cm^{-1} . In addition, the contribution of the ϵ -amino group of Lys, with maximal bending vibration at 1629 cm^{-1} [43], to the intensity of these bands cannot be ruled out. After reaction with GlcN, the absorption bands at 1615–1626 cm^{-1} shifted to 1594–1599 cm^{-1} , suggesting the conjugation of GlcN to peptides and the formation of Schiff bases and/or aromatic rings (C=N stretching and C=C bending vibrations with characteristic absorption around 1600 cm^{-1}). The lower amount of Lys after reaction with GlcN, and hence the loss of free ϵ -amino groups could also explain the low intensity at 1615–1626 cm^{-1} . Moreover, this fact indicates that Amadori compounds (with C=O linkages) were not predominant in the samples after reaction with GlcN.

The amide II band, mainly associated with in-plane N–H bending vibrations and also with C–N and C–C stretching vibrations, was identified in all samples as a weak band at 1480–1500 cm^{-1} . A broad absorption band at 1032 cm^{-1} observed in all profiles reflected the stretching vibrations of C–O, C–C and/or C–OH, and could be attributed to the presence of glycosaminoglycans in the samples [4]. The presence/absence of pyrazines in the glycated samples could not be confirmed, as C–N vibrations present characteristic absorption around 1000–1350 cm^{-1} , which coincided with the strongest absorption peak observed in the IR spectra of all samples. The small band at 865 cm^{-1} could correspond to C–H bending vibrations of aromatic rings. Moreover, a weak band was observed in all samples at 779 cm^{-1} . This band could be associated with aromatic C–H bending vibrations.

Antioxidant Properties

The antioxidant activity of the hydrolysates did not change when heated in the absence of sugar (Fig. 6). However, the Maillard reaction strongly enhanced the antioxidant activity, mainly after the first 40 min of reaction.

Fig. 6

Reducing power activity (**a**), ABTS radical scavenging capacity (**b**) and metal ion chelating activity (**c**) of the samples before and during heating for 180 min. Bars represent the standard deviation from triplicate determinations. Different letters (a, b, c...) indicate significant differences ($P < 0.05$) as a function of heating

The reducing ferric ion ability of the hydrolysates was improved 22-fold after heating for 40 min in the presence of GlcN (Fig. 6a). Then, it remained stable for 20 min and finally rose significantly up to 180 min of reaction. The

highest reducing power ability at any time of heating was that of SCJH-GlcN. In accordance with our results, Vhangani and Van Wyk [44] also reported higher antioxidant activity of fructose-lysine and ribose-lysine mixtures principally when heating time increased. The increment in the reducing power after glycation suggests the presence in glycated samples of electron donors that could reduce the oxidized intermediates of lipid peroxidation processes [45]. During the Maillard reaction, several pyrroles with hydroxyalkyl side-chains could be formed and act as reducing agents, as suggested by Yanagimoto et al. [46]. In this regard, Hwang et al. [47] reported that hydroxyl groups of MRP may play a role in the reducing activity through their redox potential of transferring electrons. Amarowicz [48] suggested that reductones, which are formed at alkaline pH values, exhibit high reducing power activity due to their ability to donate hydrogen atoms.

The best ABTS radical-scavenging activity was that of SCJH-GlcN (Fig. 6b). The antioxidant activity increased slight but significantly in both glycated samples after 40 min of glycation. The radical-scavenging activity could be produced by donating hydrogen atoms or by combination with radicals. In this regard, Eichner [49] observed that MRP intermediates may scavenge oxygen. Morales and Jiménez-Pérez [19] also found free radical scavenging activity of MRP, but they observed different activity of different model systems including alanine, glycine or lysine with the same sugar (glucose). It aims to think that the radical-scavenging activity of MRP is produced by a wide variety of molecules that are formed in the course of the reaction.

The highest iron-chelating activity was, as previously described, that of SCJH-GlcN (Fig. 6c). In this case, the antioxidant activity did not increase after 40 min of reaction. In fact, it dropped slowly or become stable until the end of reaction. This might be attributed to the formation of acids at higher time-temperature combinations, which can affect the antioxidant activity of the products formed, as observed by Vhangani and Van Wyk [44]. The high chelating activity could be attributed to the formation of molecules with hydroxyl group [50] and/or melanoidins [51].

These results suggest that different molecules with different antioxidative mechanisms were formed mainly during the first 40 min of heating reaction with GlcN. Probably, these molecules were not degraded and remained stable until the end of reaction. As well, the obtained results reflect the multiple antioxidative functions exhibited by MRP, probably with different modes of action. Thereby, further investigations need to be conducted to elucidate this

phenomenon. In addition, as it was already reported in a previous work [52], we can deduct from the results of the present study that there was a direct relationship between antioxidant activity and both color intensity of the Maillard reaction solutions and intermediate product formation.

Antimicrobial Properties of MRP

From the results obtained (Table 4), no inhibitory activity was observed for native hydrolysates. Chloramphenicol was used for control purposes, giving halos around 9 mm (data not shown). However, after the reaction with GlcN, the SCH exhibited antibacterial activity against all strains tested ($p \leq 0.05$). This means that the glycation at the temperature used (100 °C) led to the formation of molecules responsible for the antibacterial activity described in Table 4. In contrast, the glycation of SCJH did not yield antibacterial compounds, thus confirming that glycation of SCH or SCJH gave place to the formation of different molecules possessing different bioactivities. Hong et al. [38] and Martínez [53] also reported antimicrobial activity of peptides from a fish gelatin hydrolysate after conjugation with GlcN. Martínez [53] found in the conjugates different glycopeptides as well as α -dicarbonyl compounds such as diacetyl, glyoxal and methylglyoxal that could be involved in the antimicrobial activity against *Escherichia coli*. These last compounds are intermediate compounds formed during the Maillard reaction with ability to inhibit the growth of *Salmonella*, *Staphylococcus aureus* and *Streptococcus mutans* [54]. As well, more advanced products as melanoidins and different pyrazines have been identified as antibacterial compounds. Thus, melanoidins inhibit the growth of *E. coli*, *S. aureus* and *Listeria innocua*, while pyrazines also show activity against different bacterial and fungi species [53]. The antimicrobial effect of melanoidins seems to be related to their metal chelating properties. Some of these compounds could be present in SCH-GlcN and be responsible for the antimicrobial activity observed against different bacterial species.

Table 4

Antibacterial activity of hydrolysates against Gram-positive and Gram-negative bacteria before and after glycation with GlcN

Microorganisms	Samples tested			
	SCJH	SCJH-GlcN	SCH	SCH-GlcN

Microorganisms	Samples tested			
	SCJH	SCJH-GlcN	SCH	SCH-GlcN
Gram ⁺				
<i>Lactobacillus helveticus</i> DSM 20075	na	na	na	8.40 ± 0.50 ^c
<i>Listeria innocua</i> CECT 910	na	na	na	7.56 ± 0.48 ^{bc}
<i>Staphylococcus aureus</i> CECT 240	na	na	na	7.25 ± 0.10 ^{ab}
Gram ⁻				
<i>Citrobacter freundii</i> ECT 401	na	na	na	6.60 ± 0.28 ^a
<i>Escherichia coli</i> CECT 515	na	na	na	6.96 ± 0.11 ^a
<i>Pseudomonas fluorescens</i> CECT 4898	na	na	na	8.66 ± 1.60 ^c
Results are expressed as the mean of the diameter of the inhibition zone in mm ± standard deviation (n = 3)				
na no antimicrobial activity				

Song et al. [55] reported antibacterial activity of MRP derived from the glycation of enzymatic hydrolysates of anchovies with glucose, against both gram negative and gram positive bacteria (*E. coli*, *Pseudomonas fluorescens*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *S. aureus*, *Bacillus subtilis*, *Bacillus megaterium*, and *Sarcina lutea*). Similarly, it has been observed that melanoidins formed from casein and lactose inhibited both in vitro and in vivo *Helicobacter pylori* [56]. A similar remark was made in gluten-glucose and β -lactoglobulin-GlcN model systems, where the antibacterial effect against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 of MRP was more pronounced when heating time was increased [57].

The antimicrobial effect of MRP is not yet understood, but it is likely that they exert different inhibitory mechanisms. Lanciotti et al. [58] reported that MRP may interfere with the absorption of serine, glucose and oxygen and thereby inhibit the enzymes of the microorganism sugar catabolism by causing irreversible changes in the internal and external cell membranes, and therefore the inhibition of nutrient transport in gram negative bacteria. However, other authors suggest that melanoidins may develop antimicrobial activity by

binding essential metals, such as iron, copper, and zinc, key elements for the growth and survival of pathogenic bacteria [59]. In the present work the enhancement of the antimicrobial effect was very evident in carapace protein hydrolysate after the reaction with GlcN, but not in the case of that obtained from cooking juice. It suggests that the composition in MRP differed in both glycated samples.

Conclusion

The antioxidant activity of hydrolysates derived from shrimp cooking juice or carapace protein was remarkably improved by their conjugation with GlcN at high temperature. The improvement in antioxidant activity was correlated with the increase in browning and intermediate products and was consistent with the decrease in free amino groups and pH. The highest antioxidant activity was obtained by heating the shrimp cooking juice protein hydrolysate with GlcN, while heating shrimp carapace protein hydrolysate with GlcN led to the production of compounds with relevant antimicrobial activity against diverse gram negative and gram positive bacteria. These interesting results lead to think that shrimp waste (carapaces and cooking juice) could be used for the production of molecules with a promising interest as antioxidants and antimicrobials. In fact, shrimp waste could also be the source of GlcN, as this sugar can be obtained by deacetylation and chitin hydrolysis. Additional research must be performed in order to understand the composition and potential presence of undesirable and unhealthy AGE's in the final products.

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