Received: 22 July 2020 | Revised: 26 March 2021 | Accepted: 30 March 2021

DOI: 10.1111/jfpp.15526

ORIGINAL ARTICLE

Journal of Food Processing and Preservation | Institute of Food Science + Technology | WILEY

Characterization of liquid protein hydrolysates shrimp industry waste: Analysis of antioxidant and microbiological activity, and shelf life of final product

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Funding information
National University of Mar del Plata, EXA 874/18 and EXA 875/18

Abstract

Proteases from shrimp wastes were characterized and protein hydrolysates were obtained. Shrimp protein hydrolysates (SPH) were produced by autolysis (H0) and added 1% (H1) and 2% vol/vol (H2) enzyme extract of shrimp. The hydrolysis degree was determined using a colorimetric method; the capability of hydrolysates to scavenge free radicals was measured with DPPH, and the antimicrobial activity of the SPH was evaluated by the microdilution test. The degree of protein hydrolysis ranged between 43% (H0) and 71.5% (H2) after 90 min, and it functioned as a source of lysine, leucine, aspartic acid, glutamic acid, and glycine. After 10 min of reaction, all hydrolysates reached 50% of scavenging effect. In addition, the SPH prepared with food additives showed acceptable microbiological quality and pH during 40 days at room temperature. This study aims at introducing a low-cost process which produces SPH with commercial applications in the food industry.

Practical applications

Currently, shrimp processing wastes represents an environmental and economic problem, since such seafood industry must pay for the landfarming service. This waste treatment is not environmentally efficient, and it affects the value of the final product. However, these wastes have a large number of compounds which hold biological activities of interest that can be used to obtain a high added value product such as protein hydrolysates. This by-product has several potential applications for the food and pharmaceutical industries. In this study, a protein hydrolysate solution was obtained utilizing processed shrimp waste as an enzymatic resource and a protein substrate as well. This research demonstrated the feasibility of obtaining a good protein source that also holds useful antioxidant properties. It is important to highlight that the by-product was obtained without resorting to freeze-drying technology, which makes the industrial process more expensive. To accomplish that, we included low-cost food preservatives and assessed the shelf life of this product of high nutritional quality.

1 | INTRODUCTION

The shrimp *Pleoticus muelleri* (Bate, 1888) is an important fishing resource of the Argentine Sea, which has a large distribution area in south-west Atlantic waters from 20°S, Espíritu Santo, Brazil, to 50°S, Santa Cruz, Argentina (Gavio & Boschi, 2004). During last year, 126,480 tons of *P. muelleri* were landed and processed (MAGyP, 2021). This produced a volume of waste (heads, broken specimens, and noncommercial sizes) equivalent to 30% of the total processed capture, generating a problematic situation in terms of waste disposal, contamination, and management (Pereira et al., 2020).

In an attempt to recycle these wastes, private investors have tried to produce shrimp meals and oils, but for commercial and economic reasons, these projects proved unviable. This situation has led to the investigation of other sustainable alternatives to avoid the disposal of wastes in municipal garbage dumps which imply high economic and environmental costs. Consequently, it is necessary to provide an added value to these wastes composed by viscera, and following this objective, several studies have shown the interest of extracting enzymes from these organs (Caruso, 2015; Ideia et al., 2020). In this sense, proteolytic enzymes from marine organisms have great potential applications in the industry. For example, these molecules have the property to maintain their activity at low temperatures, which would be useful in food processing to avoid bacterial contamination and unwanted chemical reactions (Gudmundsdóttir & Pálsdóttir, 2005). Thus, the use of marine enzymes in food technology is becoming a promising application for the development of new processes and products (Rossano et al., 2011).

Shrimp wastes represent a valuable source of protein that can be transformed by enzymatic hydrolysis into highly nutritional protein hydrolysates due to the release of bioactive substances (Gunasekaran et al., 2015). Most researchers used commercial enzymes to produce protein hydrolysates and verified that the type of enzyme affects the bioactivity of the peptides (Cheung & Li-Chan, 2010). In fact, many endogenous hydrolytic enzymes (such as proteases, lipases, etc.) which are found in fishery waste can hydrolyze the tissues spontaneously under certain conditions. This phenomenon is called "autolysis" (Cao et al., 2019). However, the use of endogenous enzymes for hydrolysates could have many advantages and could improve the properties of resulting peptides, since it allows the control of the hydrolysis and thus minimizes undesirable reactions. In this way, the viscera inside shrimp head contain most of the endogenous enzymes which can participate in hydrolysis process. Shrimp protein hydrolysates (SPH) have been applied to diverse fields like biotechnology, medicine, and nutrition, including animal nutrition (Ambigaipalan & Shahidi, 2017). In cultured fishes, they have been included in feeds both as a new protein source and, in small amounts, as flavoring to enhance the attractiveness of foods (Hou et al., 2017). However, just a few works utilized endogenous proteases as this study. Previous studies on *P. muelleri* have characterized the digestive proteases from midgut gland concerning the molting stages (Fernández Gimenez et al., 2001) and their dietary effect (Velurtas et al., 2011). Also, recent studies have analyzed the properties of proteases enzymes of this shrimp species and their use as milk clotting for cheese manufacture (Pereira et al., 2017) and as a dietary supplement for *Oreochromis niloticus* cultured fish (Rodriguez et al., 2017). Most of the studies lyophilize the hydrolysate obtained maintaining the structure and original appearance of the food for a long period of time, but this process is most feasible on a laboratory scale. To enlarge production to an industrial scale, a high investment in equipment is necessary, in addition to considering an increase in process times and energy costs (Ramírez-Navas, 2007). Moreover, microorganisms such as aerobic mesophilic bacteria, fecal coliforms, *Escherichia coli* and *Staphylococcus aureus* have been found in samples of shrimp (Abd-El-Aziz & Moharram, 2016) and could remain in protein hydrolysates. Therefore, to develop liquid shrimp hydrolysates, information about the effect of storage conditions (time and temperature) on microbial changes and shelf life is required. The principal aim of this work was to make different liquid protein hydrolysates and evaluate their shelf life. For this, shrimp wastes were employed as the source of proteins and enzymes. Thus, three versions of shrimp hydrolysates were produced by autolysis of raw material without addition of shrimp enzyme extract (H0), 1% of shrimp enzyme extract addition (H1), and 2% of shrimp enzyme extract addition (H2). To evaluate the feasibility of using the enzymes present in the shrimp residues, its proteases were characterized through electrophoresis SDS-PAGE, exposition to inhibitors (SBTI and TLCK), and the quantification of its activity and stability. Then, we elaborated the protein hydrolysates and determined their amino acid profiles, antioxidants, and antimicrobial activities, as well as the effect of food preservatives on the durability of the final product. Finally, this work would contribute to the sustainable management of marine wastes, increasing its commercial value and reducing the negative environmental impact that it currently generates.

2 | MATERIALS AND METHODS

2.1 | Biological materials

Five batches of *P. muelleri* shrimp waste were selected randomly from the company PESCO (Mar del Plata, Argentina), containing mainly heads (heads with midgut gland) and broken tails (abdomen), and were stored at −20°C during approximately one month to perform all the analysis.

2.2 | Characterization of enzymes of shrimp wastes

One batch of five individuals was randomly taken for proteolytic enzymes assays. Each shrimp was weighed, and cephalothorax or head (H), abdomen (A), and midgut gland (MG) were excised. Every one of the H, A, and MG samples was separately homogenized in chilled distilled water (1:4 wt/vol) at low speed using a blender. Subsequently, the homogenates were centrifuged (Presvac EPF-12R,

Argentina) for 30 min at 4°C and 14,000 ×*g*. Lipids were eliminated, and then, soluble protein was evaluated in the supernatant portion (Bradford, 1976) with bovine serum albumin (Sigma A 9647) as the standard protein. The supernatant was used as enzyme extract for the following assays.

Alkaline protease activity of H, A, and MG was evaluated according to García-Carreño (1992), using 0.5% wt/vol azocasein in 50-mM TRISHCl (pH 8.0) as substrate. An enzymatic preparation of 10 μl was mixed with 500 μl of the substrate solution in a 1.7-ml microcentrifuge tube and then incubated for 10 min at 25°C. The reaction was stopped by the addition of 500 μl 20% (wt/vol) of trichloroacetic acid (TCA) and cooled in ice for 10 min. The undigested substrate precipitate was separated by centrifugation (5 min, 10,000 ×*g*). Afterwards, the absorbance of the supernatant was measured at 366 nm against distilled water (Shimadzu UV-2102 PC, UV-visible Scanning Spectrophotometer). For blanks, TCA was added before the substrate. The specific activity was expressed as activity units per mg protein (U/mg protein). One unit of enzyme activity was defined as the change in absorbance per min. Assays were run in triplicate.

All enzymes extracts (H, A, and MG) were incubated with two specific protease inhibitors. Trypsin inhibitor from Soybean (SBTI, Fluka 93,618) was used as a specific inhibitor of proteases belonging to the serine class, and N-α-tosyl-L-lysyl-chloromethyl-ketone (TLCK, Fluka 90,182) was used as a specific inhibitor of trypsin. Solutions (10 μl) of 250-μM SBTI in distilled water and 10-mM TLCK in 1-mM HCl pH 3 were separately mixed with the enzyme extracts (10 μl) and incubated for 60 min at 25°C. Then, the activity was evaluated as previously described. Activity in inhibition assays was reported as percentages, considering the activity measured in the absence of inhibitor as 100%. Assays were done in triplicate.

Separation of proteins from the enzyme extracts (H, A, and MG) was done by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Aliquots of enzyme extracts containing 20 μg of protein diluted in loading buffers 1:1 vol/vol and 5 μl of low molecular mass protein standards (Sigma SDS7) were all loaded into individual wells of the gel. The electrophoresis was run at 30 mA per gel in an electrophoresis unit (BIO RAD model Mini PROTEAN Tetra System) refrigerated by a circulating bath at 4°C. Once the front dye reached the bottom of the 0 gel, electrophoresis was stopped. Gel was stained with an aqueous solution containing 0.5% wt/vol Coomassie Blue R-250 in 40% vol/vol methanol and 7% vol/vol acetic acid. The excess dye in the gel was washed with an aqueous solution containing 40% vol/vol methanol and 7% acetic acid.

Protease composition was studied after separation of proteins by substrate-SDS-PAGE (García-Carreño & Haard, 1993). Aliquots of enzyme extracts containing 10 mU of activity were run as described in the previous section. Once electrophoresis was stopped, the gel was immersed in 3% casein in 50-mM Tris·HCl pH 8 for 30 min to allow the substrate to diffuse into the gel. Next, the gels were allowed to hydrolyze the casein in its vicinity for 60 min at 25°C. Then, the gels were washed with distilled water and stained as above. Clear bands over blue background showed proteolytic activity.

The enzyme preparations containing 10 mU of activity were incubated with protease inhibitors to identify trypsin and serine protease activities. SBTI and TLCK solutions were separately added to the extracts at a ratio of 1:1 (inhibitor/extract) and incubated later at 25°C for 60 min. Distilled water replaced inhibitors in trials without inhibition. Then, the treated enzyme preparations were subjected to substrate SDS-PAGE. After electrophoresis separation, the molecular weight lane was cut apart and immediately stained. For protein substrate digestion by the active fractions on the SDS-PAGE, gels were soaked in casein as described above. Gels were washed in water and immediately fixed and stained in Coomassie Blue solution. Bands with and without protease inhibitors were then compared. A significant reduction of intensity on the band with activity was recorded as inhibition, to identify the type of enzyme involved. Since TLCK is a specific inhibitor for trypsin and SBTI for serine protease, inhibited bands on substrate SDS-PAGE could be attributed to the presence of such enzymes.

Several trials were run to assess the effect of pH and temperature on enzyme activity. Since the maximum values of proteolytic activity were registered in midgut gland, the samples utilized were protease extracts of this organ. The effect of different pH values ranging from 6 to 9 was evaluated using a universal buffer at room temperature (25°C), while the temperature effect between 20°C and 80°C was assessed using buffer 50-mM TrisHCl pH 8. The pH stability of alkaline protease was also checked by pre-incubating the extracts in different buffers (pH 7. 8. 9. 10, and 11) for 60 min at 25 C. To test the thermal stability of enzyme extracts, these were kept in a water bath at 20°C, 40°C, 50°C, 60°C, and 80°C for up to 60 min. In both assays, samples were taken every 20 min, and the residual activity was measured as previously described.

2.3 | Collection and characterization of SPH

SPH was produced by autolysis, based on the method described by Leal et al. (2010). The raw material (1 kg of shrimp heads) was washed and ground in distilled water (1:1), and different proportions of *P. muelleri's* midgut gland enzyme extracts were added: 0% (H0), 1% (H1), and 2% vol/vol (H2) of total volume. The mixture (shrimp heads and enzyme extracts) was vigorously stirred, and the blend was submitted in a water bath (45 ± 2°C). The degree of hydrolysis (DH) for each hydrolysate was determined according to Baek and Cadwallader (1995). For this, three aliquots (500 μl) were taken at 0, 10, 20, 30, 40, 50, 60, and 90 min, with a subsequent rise in temperature (100°C, 10 min) for enzyme deactivation. Each aliquot was immediately transferred into test tubes containing 1,000 μl of 0.3-M TCA solution. These solutions were stored at room temperature for 20 min and filtered through Whatman filter paper; 25 μl of the resultant filtrate was mixed with 225 μl of distilled water, 1,250 μl of 0.5-N NaOH solution and 0.25 ml of 1.0-N Folin and Ciocalteu's phenol reagent (Sigma F9252). The resulting solution was immediately mixed,

incubated at 30°C for 15 min, and then centrifuged at 2,000 ×*g* for 10 min to remove turbid material. Supernatant absorbance was measured at 578 nm. The blank (amount of 0.3-M TCA soluble peptides of un-hydrolyzed shrimp protein substrate expressed as tyrosine) was prepared by adding 1,000 μl of 0.3-M TCA solution to un-hydrolyzed shrimp protein substrate (zero time). Final hydrolysis (maximum amount of 0.3-M TCA soluble peptides as tyrosine) was determined after the hydrolysis of 0.1-g shrimp substrate with 4-ml 6-N HCl at 110°C for 24 hr. The DH was defined as follows:

$$\mathrm{DH} = \frac{\text{Hydrolysis at time } t - \text{blank}}{\text{Hydrolysis final - blank}} \times 100$$

The proximate composition, moisture, lipid, protein, and ash contents of protein liquid hydrolysates material were determined using Standard Methods (AOAC, 1953, 2008, 2013). The amino acid composition was determined on samples of hydrolysates H0–90 min. The analysis was done by Fares Taie Laboratories, Mar del Plata, Argentina. Assays were done in triplicate.

2.4 | Bioactive properties of the SPH

Scavenger capability of hydrolysates on free radical 2,2-diphenyl-1-picrilidrazil (DPPH Calbiochem® 300267) was performed by the method described by Shimada et al. (1992). A volume of 1.5 ml of each liquid hydrolysate (H0, H1, and H2) was mixed with 1.5 ml of 0.1-mM DPPH solution in 95% ethanol. After 30 min, the samples were analyzed in a spectrophotometer Epoch BioTek Microplate Spectrophotometer (Gen5™ Software) at 517 nm. The blank was the absorbance of the control reaction (containing all reagents except the sample). The lower absorbance means was the greater scavenger capability of the hydrolysate by DPPH radical. Results were determined using following equation:

$$\%\,\mathrm{SE} = 1 - \frac{\mathrm{ABS\,sample}}{\mathrm{ABS\,blank}} \times 100$$

The scavenger capability (%SE) is connected with the absorbance of the blank sample (ABS blank) and the absorbance of the sample (ABS sample).

Based on the results obtained for the antioxidant capacity of the hydrolysates evaluated, SPH0 was selected to develop the analyses of antimicrobial activity and durability of the final product since it has the least complexity of processing.

The antimicrobial activity of the SPH was evaluated by the micro dilution test according to the Clinical and Laboratory Standards Institute (CLSI, 2015). SPH0 was treated with activated carbon and centrifuged three times at 10,000 rpm during 1 hr. The supernatant was collected and filtered with membrane 0.22 μm (Titan syringe filters; Sri Scientific Resources Inc., USA). The resulting extract was added to the culture medium in order to achieve different final concentrations. A bacterial suspension of the strains *E. coli* ATCC 25,922 and *S. aureus* ATCC 25,923 (10^5–10^6 CFU/ml) was added to the wells with Mueller–Hinton Broth, while a suspension of *Paenibacillus larvae* (ERIC I genotype) was added to Mueller–Hinton Broth with yeast extract, glucose and sodium pyruvate (MYPGP) prepared following the composition reported by Dingmann and Stahly (1983). All microtiter plates (with positive and negative controls) were incubated at 35 ± 0.5°C for 24–48 hr according to each strain.

2.5 | Evaluation of the shelf life of the SPH with preservants addition

To evaluate the shelf life of the SPH, the SPH0 was prepared with 0.5% citric acid, 0.4% tartaric acid, and 0.2% potassium sorbate as preservative and acidulant (Pardio et al., 2011). SPH samples were kept in 50-ml centrifuge tubes with caps. The different tubes were stored at room temperature (25°C), fridge (4°C), and freezer (−20°C). Microbiological counts and pH determinations of the samples were performed at different time intervals (0, 20, and 40 days).

From each sample, 10 g were homogenized in 90 ml of sterile physiological saline solution (0.85% sodium chloride). Serial dilutions were prepared by adding 1- to 9-ml sterile physiological saline solution. An aliquot of each dilution was plated on Plate Count Agar (Britania, Buenos Aires) to determine the growth of aerobic mesophilic bacteria (35 ± 0.5°C, 48 hr); Violet Red Bile Agar (Britania, Buenos Aires) to determine the presence of *Enterobacteriacea* (35 ± 0.5°C, 48 hr); Molds and Yeast Medium (Britania, Buenos Aires) to determine the counts of molds and yeasts (25°C, 5–7); Baird Parker Agar (Britania, Buenos Aires) to detect *S. aureus* (35 ± 0.5°C for 24–48 hr); Violet Red Bile Lactose Agar (Britania, Buenos Aires) (44 ± 1°C for 24 hr) for the presumptive isolation of *E. coli*; and Clostridium Media (Britania, Buenos Aires) (44 ± 1°C for 24 hr in anaerobiosis) to detect *Clostridium perfringens* (Fangio & Fritz, 2014). For the investigation of *Salmonella* spp., 25 g of the SPH were taken aseptically and homogenized with 225 ml of Peptone Water (Britania, Buenos Aires) and incubated at 35°C for 24 hr for pre-enrichment. Five ml of pre-enriched sample were transferred to 25 ml of Selenite Cystine Broth and Tetrathionate Broth and incubated at 37°C for 24 hr for selective enrichment. A loopful of enriched sample was streaked onto a plate with Hektoen Agar (Britania, Buenos Aires) and incubated at 35°C for 24–48 hr for the examination of *Salmonella*.

On Days 0, 20, and 40, all samples were subjected to determine their pH. Five grams of sample were homogenized with 50 ml of boiled distilled water cooled at 25°C. The mixture was stirred for 30 min and decanted. The pH value was measured in the supernatant, using a pHmeter HI 2,211 (HANNA, Buenos Aires) (Fangio & Fritz, 2014).

2.6 | Statistical analysis

Enzymatic activity was analyzed with analysis of variance (ANOVA) after testing normality and homogeneity of variances, while

MANOVA and then ANOVA were used to examine the scavenger capability of hydrolysates in the first step. ANOVA with post hoc Tukey was used to compare the values of microorganism counts and pH. For all analyses, significant differences were considered at $p \leq .05$. ANOVA was used to test differences among the regression lines between DH. Arcsin transformation was applied to percentages. The analysis was made using NCSS and Past Software.

3 | RESULTS

The protein content and protease activity of different body regions of shrimp are shown in Table 1. Protein content in midgut gland and abdomen was significantly higher than in the head. Specific enzyme activity was found in midgut gland and head extracts, when these were evaluated with azocasein at pH 8 (0.4 and 0.3 U/mg protein, respectively). However, the alkaline proteolytic activity in the abdomen was null.

The results indicate that protease activities, in H and MG extracts, were inhibited in a magnitude of 73.2% and 69.1% by SBTI, whereas the TLCK reduced the enzymatic activity in a 31.6% and 30.0%, respectively (Table 1).

The protein fractions and their molecular weight detected by SDS-PAGE are shown in Figure 1. Several protein fractions have been observed with molecular weights of 14, 17.4, 21.2, 29, 36, 39.5, and 52.6 kDa, for midgut gland and head. Zymograms of midgut gland extracts and head showed a similar pattern of proteolytic enzymes and are indicated in Figure 1. Active bands inhibited with TLCK were 14 and 17.4 kDa, while SBTI inhibited 14-, 17.4-, and 21.2-kDa bands.

The proteolytic alkaline activity of *P. muelleri* was weakly affected by pH changes. The alkaline proteases were mostly stable in the pH range 7–9, with an optimum at pH 8 (Figure 2a). Moreover, the optimum temperature was registered between 60°C and 70°C (Figure 2b).

When enzyme stability was evaluated over a period of 1 hr, the results demonstrated that proteolytic enzymes were stable at all pHs analyzed (Figure 2c). Furthermore, the thermal stability of proteases was registered from 20°C to 80°C, while the enzyme activity was measured every 20 min under standard conditions. The activity remained mostly stable during one hour at temperatures ranging from 20°C to 50°C. On the other hand, at 60°C and 80°C, a total loss of proteolytic activity was found at 40 and 20 min, respectively (Figure 2d).

The proximal content of the liquid protein SPH0 was: 7.3% of protein (87.95% on a dry weight basis); 91.7% of moisture, 0.38% of ether extract, and 0.99% of ash. The total amino acid content in SPH (H0–90 min) was 40.9% essential and 59.1% non-essential, mainly glutamic acid, lysine and glycine (Table 2).

Figure 3 shows the hydrolysis curves for shrimp *P. muelleri* with the addition of 1 (H1) and 2 (H2) and without shrimp enzymes added (H0). All curves presented a high rate of hydrolysis, but this rate decreased throughout the reaction until reaching a steady phase where no apparent hydrolysis took place. The DH reached after 30 min was statistically higher in H2 (71.5%), while the maximum hydrolysis degree for H0 and H1 was 50% and 43%, respectively.

The free radical scavenger capability of hydrolysates made with different doses of *P. muelleri*'s enzyme related to the different reaction times is shown in Table 3. H0 and H1 hydrolysates showed the highest values of DPPH scavenging capability at 10 min, while H2 present the maximum value of scavenger capability at 20 min. At 30 min, all hydrolysates did not show statistical differences.

The antimicrobial activity of hydrolysates was evaluated against *E. coli*, *S. aureus*, and *P. larvae*. Hydrolysate did not show antimicrobial activity against any of the Gram-positive or Gram-negative strains.

To evaluate the shelf life of the SPH, the microbiological quality of SPH treated with additives was analyzed at different conditions and times of storage. Aerobic mesophilic bacteria showed that the initial microbiological quality of hydrolysates was 1.4×10^2 CFU/g, and at the end of storage time of 40 days, samples stored at room temperature showed acceptable values of 1.5×10^4 CFU/g (Figure 4). In addition, samples stored at 4°C and −20°C showed no significant differences ($p > .05$) throughout storage time. The initial counts of molds and yeasts was 0.7×10^1 CFU/g, reaching a level of 3×10^3 CFU/g at room temperature and 2.4 and 2.5×10^2 CFU/g at 4°C and −20°C after 40 days of storage (Figure 4).

In addition, *Enterobacteriaceae*, *E. coli*, *S. aureus*, *Salmonella* spp., and *C. perfringens* were not detected in any of the samples at any time or condition of storage.

The initial pH level of shrimp hydrolysate was 4.95 ± 0.02, and after 40 days of storage, pH increased up to 5.02 ± 0.01 at room temperature, 5.04 ± 0.01 at 4°C and 4.97 ± 0.01 at −20°C, showing no significant differences ($p > .05$) between all the conditions.

TABLE 1 Protein content, specific protease activity and the effect of specific inhibitors on crude extracts of different body regions of *Pleoticus muelleri*

	Soluble protein (mg/ml)	Protease activity (U/mg protein)	Percentage inhibition	
			SBTI	TLCK
Head	3.0 + 0.15[a]	0.3 ± 0.02[a]	73.2 ± 12.97[a]	31.6 ± 11.39[a]
Midgut gland	3.8 ± 0.61[b]	0.4 ± 0.04[b]	69.1 ± 11.99[a]	30.0 ± 3.36[a]
Abdomen	4.1 ± 0.55[c]	N/R	N/R	N/R

Note: Values are means of triplicate assays ± standard error. Different letters in the same column show statistical differences ($p \leq .05$).

Abbreviations: N/R, not registered; SBTI, trypsin inhibitor from soybean; TLCK, N-α-tosyl-L-lysyl-chloromethyl-ketone.

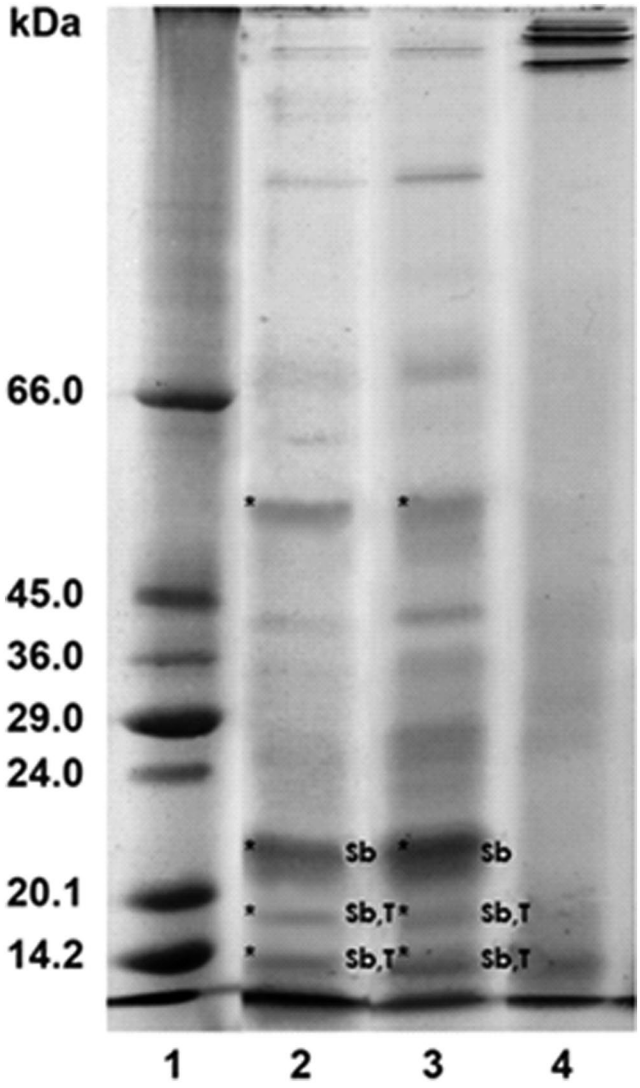


FIGURE 1 Composition of proteins in head, midgut gland, and abdomen of *Pleoticus muelleri* after separation by electrophoresis. Lane 1, protein molecular weight standard; Lanes 2, 3, and 4 refer to proteins from head, midgut gland, and abdomen, respectively. Sb, serine proteinase; T, trypsin; *proteinases with proteolytic activity

4 | DISCUSSION

Shrimp *P. muelleri* represents one of the most important fisheries in the Argentine Sea (Bertuche et al., 2000), and the commercial processing of this crustacean produces a large amount of wastes (Pereira et al., 2020). Several authors have investigated the evaluation and biotechnological uses of fishing industry wastes (Caruso, 2015; D'Ambrosio et al., 2003; Djellouli et al., 2020; Gunasekaran et al., 2015; Heu et al., 2003; Ketnawa et al., 2016; Mao et al., 2017; Rossano et al., 2011). Their research focuses on employing marine raw material taken from those wastes, evaluating the enzymes and proteins present in it, and providing an alternative management of wastes generated by commercial fishing.

In the present study, the head and midgut gland of shrimps exhibited alkaline protease activity, while the abdomen did not show such activity. This result was predictable because the midgut gland is located in cephalothorax, and it synthesizes digestive enzymes, whereas in the abdomen, part of midgut and hindgut are present, but they do not produce any enzyme. Proteolytic enzymes of *P. muelleri* displayed a maximum activity at pH 8, evidencing high activity in neutral and alkaline conditions according to Garbari (2018). Previous research has documented that *Pleuroncodes planipes* and *Pacifastacus astacus* reached the highest protease activity between pH 6 and 8 (García-Carreño & Haard, 1993), and *Munida* exhibited the optimum enzyme activity at pH 7 (D'Ambrosio et al., 2003; Rossano et al., 2011). However, the *Panulirus interruptus* enzymes showed two activity peaks at pH 8 and 9 (Celis-Guerrero et al., 2004).

The enzyme extract of *P. muelleri* was highly stable over a wide pH range, maintaining about 100% of their activities between pH 7 and 11 after one h of incubation at 25°C. Similar results were found in *P. interruptus*, which was highly active in the alkaline pH range and was also stable over a wide pH range (Celis-Guerrero et al., 2004).

In the present study, the optimum temperature for alkaline protease activity was determined to assess its suitability for biotechnological applications. Thus, the results obtained show that proteases were active at temperatures of 20–80°C, and the optimum temperature for *P. muelleri* proteases was 60–70°C. This value is similar to that of *P. planipes*, *P. astacus* (García-Carreño & Haard, 1993), and *P. interruptus* (Celis-Guerrero et al., 2004). However, the alkaline proteases extracted either from the whole body or midgut gland of *Munida* displayed their maximum activity at 55–60°C and 40°C, respectively (D'Ambrosio et al., 2003; Rossano et al., 2011).

Also, the results showed that proteases from *P. muelleri* were thermostable between 20°C and 50°C. However, at higher temperatures (60–80°C), these enzymes were inactivated after 40–20 min of incubation respectively. Similar results were observed in enzymes of *P. interruptus* which were stable and remained fully active between 10°C and 50°C, even after 60 min of incubation (Celis-Guerrero et al., 2004). Therefore, these results suggest that *P. muelleri* enzymes would be a potential source of alkaline proteases for certain industrial applications that require high alkaline conditions at temperatures below 50°C.

On the other hand, the effect of specific inhibitors on total proteolytic activity (SBTI and TLCK) was tested, and the results showed that the serine proteases were inhibited in around 70% at head and midgut gland, where 30% correspond to trypsin activity. These results coincided with Lemos et al. (2000), Fernández Gimenez et al. (2001), and Muhlia-Almazán and García-Carreño (2003), who observed that trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of penaeid shrimps.

Electrophoresis is a sensitive and rapid assay method that detects low quantities of protein. In order to estimate the number of proteases and their molecular mass of enzyme extracts of *P. muelleri*, samples were separated by SDS-PAGE and their proteolytic activities were revealed by casein zymography activity staining. It has been found that enzyme extracts of head and midgut gland showed several clear bands of protease activity with different molecular weights, while in the abdomen, this kind of enzyme activity was not registered. Some of these bands were hindered by specific

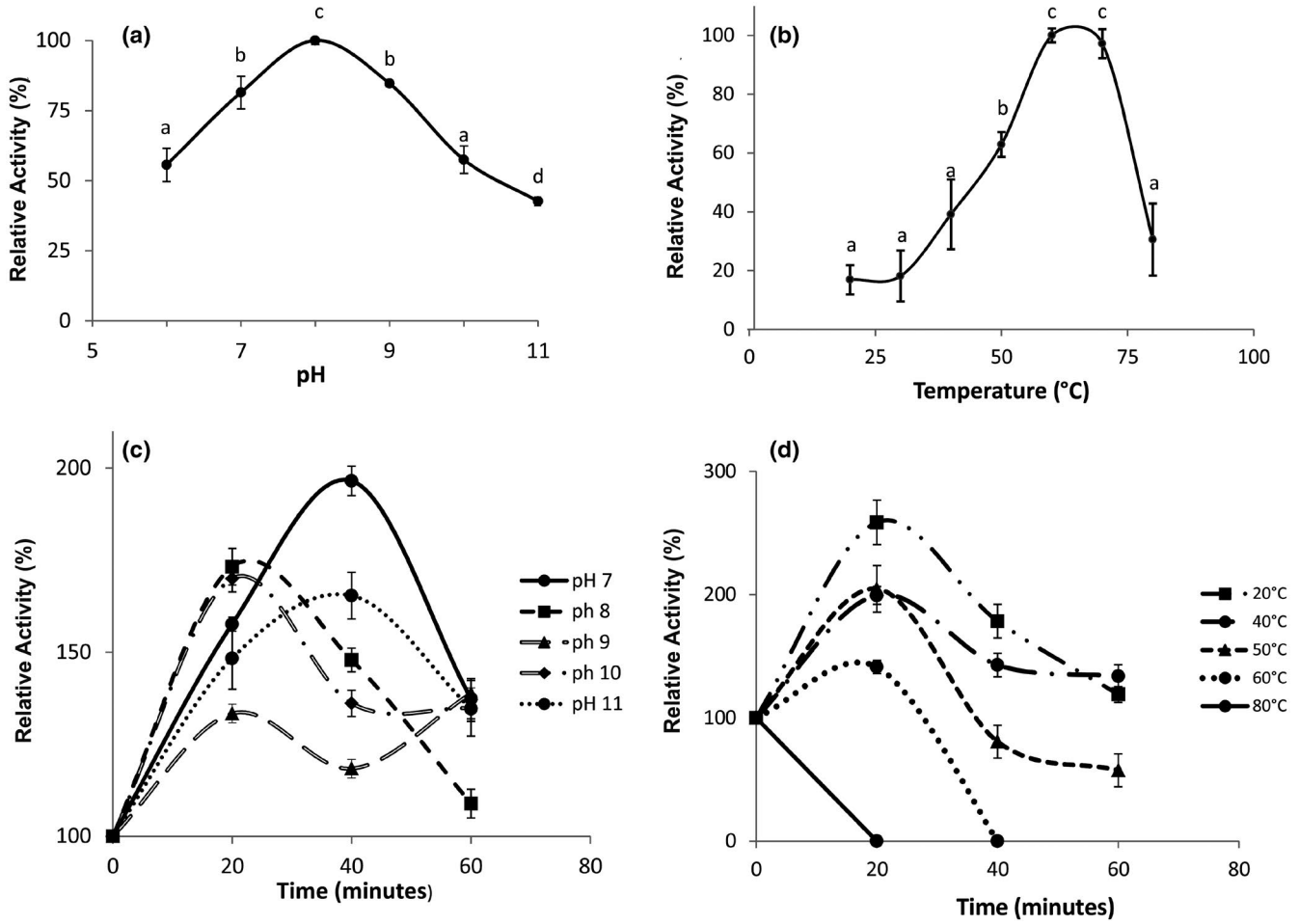


FIGURE 2 The effect of pH and temperature on the enzyme activity of *Pleoticus muelleri*. (a) Optimal pH at 25°C. (b) Optimum temperature at pH 8. (c) Stability at different pH during one hour. (d) Stability at different temperatures during one hour. Values are means of three replicates. Different letters indicate significant differences between treatments ($p < .05$)

inhibitors, suggesting the presence of trypsin and serine proteases in H and MG. These results are consistent with Fernández Gimenez et al. (2001), and similar to other results observed in crustaceans such as *Litopenaeus setiferus* (Lovett & Felder, 1990), *P. planipes* and *P. astacus* (García-Carreño & Haard, 1993), *Farfantepenaeus paulensis* (Lemos et al., 2000), *Pandalus borealis*, and *Trachypena curvirostris* (Heu et al., 2003).

The wastage remaining tissues from the shrimp industry can be recovered by hydrolysis and serve as a useful source of protein and flavoring in food formulations, mainly due to their high level of free and essential amino acids (Heu et al., 2003). Previous studies indicate that *Litopenaeus vannamei* head hydrolysates are composed of 44% protein, with a high content of essential amino acids (Leal et al., 2010). Additionally, Karuppasamy et al. (2014) have reported high levels of arginine, lysine, and leucine for *Peneaus monodon*. In this work, *P. muelleri* hydrolysate presented a high content of essential amino acids such as lysine, valine, and methionine and non-essential amino acids, such as glutamic acid, glycine, ornithine, taurine, and hydroxyproline. Our results demonstrate that the hydrolysis by endogenous shrimp proteases represents a feasible method due to the presence of proteases with strong autolytic capacity, which can hydrolyze the high protein content in shrimp heads, releasing peptides and amino acids. Additionally, extract shrimp enzyme appears to be able to reach a high DH at low concentration levels, which produces a hydrolysate with low concentration of tryptophan (bitter-tasting amino acid), in contrast with the tryptophan values registered in hydrolysates of *L. vannamei* and *P. monodon* (Table 2).

A previous study has differentiated protein hydrolysates in two groups: hydrolysates with a low DH (1%–10%), and extensive hydrolysates with a DH exceeding 10% (Benítez et al., 2008). Following this criterion, the SPH obtained in this work corresponds to an extensive hydrolysate.

Generally, protein hydrolysates of marine species are made with commercial enzymes such as Alcalase® 2.4 L, Protamex®, and Flavourzyme® 1,000 L, obtaining extensive hydrolysates with 60% DH reached after 60 min of reaction (Guerard et al., 2002; Liceaga-Gesualdo, & Li-Chan, 1999; Ovissipour et al., 2012; Piotrowicz & Mellado, 2015; Thiansilakul et al., 2007). However, in this work, these values were exceeded (DH 71.5%) by adding 2% (H2) of *P. muelleri* enzymes after 30 min of reaction. Garbari (2018) performed *Engraulis anchoita* protein hydrolysates by utilizing Alcalase 2.4 L-FG® and Neutrase 0.8L® enzymes as well as enzymes drawn from *P. muelleri*

TABLE 2 Amino acids profile (%) of protein hydrolysate of *Pleoticus muelleri*, *Litopenaus vannamei*, and *Penaeus monodon*

	Pleoticus muelleri	*Litopenaeus vannamei*[1]	*Penaeus monodon*[2]
Essential amino acids			
Arginine	1.0	7.3	7.9
Histidine	1.8	2.3	2.3
Isoleucine	3.7	4.3	3.4
Leucine	7.6	7.4	7.9
Lysine	12.9	7.2	8.3
Methionine	3.0	2.7	2.8
Phenylalanine	3.8	5.1	4.0
Threonine	2.1	4.5	4.8
Thryptophan	0.1	1.4	1.3
Valine	4.9	4.8	4.9
Total	40.9	47.0	47.5
Non-essential amino acids			
Alanine	6.2	6.6	8.2
Aspartic acid	7.4	9.1	9.5
Cystine/cysteine	0.9	0.9	1.3
Glutamic acid	16.8	12.4	7.7
Glycine	11.0	6.2	3.9
Hydroxiproline	0.3	ND	ND
Ornithine	6.6	ND	ND
Proline	3.3	6.3	4.1
Serine	1.6	4.3	10.5
Taurine	4.1	ND	7.3
Tyrosine	0.9	7.2	ND
Total	59.1	53.0	52.5

Abbreviation: ND, data not determined.

[1]Santos et al. (2013).

[2]Karuppasamy et al. (2014).

shrimp, *Merluccius hubbsi* hake and anchovy wastes, and she obtained a higher DH on hydrolystates drawn from *M. hubbsi* (DH 60.2%) and *P. muelleri* enzymes (DH 56.8%). This could be due to the fact that enzymes drawn from these species are mixtures of endopeptidases and exopeptidases in contrast with Alcalase 2.4L-FG ®, which only cleavage internal peptide bonds (Dos Santos et al., 2011). However, Liébana (2018), who developed *P. muelleri* shrimp hydrolystate via autolysis, reached a 10% DH at 45°C after 10 min (intensive hydrolysate), which remained constant for 90 min. These differences in DH could be attributed to the disparity in the initial activity of the enzymes drawn from raw materials (2.96 ± 0.145 U/ml present study 3.59 ± 0.61 U/ml in Garbari (2018) and 0.14 ± 0.01 U/ml in Liébana (2018). Thus, the variability in the DH values obtained at different times for each species may be related to the action of the enzyme on protein chains as well as the substrate upon which the enzyme will act in relation with the final DH value (Bellido et al., 2011). The rate of DH is greater during the first 10 min of reaction, and after this time, it slows down until it reaches a plateau at 30 min. Guerard et al. (2002) suggest that this reduction of the hydrolysis rate is due to the limitation of the activity of the enzyme and the decrease in concentration of available substrate. In this research, the types of specific bonds that could be hydrolyzed by the *P. muelleri* enzymes at 30 min for H0 and H1 and while for H2 could be already wholly hydrolyzed.

Free radical-scavenging is a primary mechanism by which antioxidants inhibit oxidative processes. The DPPH radical-scavenging assay is a widely used method for evaluating the ability of hydrolysates to scavenge free radicals generated from DPPH reagent. When DPPH radical encounters a proton-donating substrate such as an antioxidant, the radical is scavenged, and the absorbance is reduced (Rajapakse et al., 2005). The decrease in absorbance is taken as a measure for radical scavenging activity. The presence of antioxidant activity in SPH may be attributed to different factors: the type of enzyme and substrate, their concentrations and the reaction medium during the process of hydrolysis, as well as composition, structure, hydrophobicity, or the presence of the amino acid in position within the sequence forming the peptide (Chen et al., 1996; Rajapakse et al., 2005). Previous studies have registered that the *P. muelleri's* extracts have strong DPPH radical scavenging activity, suggesting the presence of natural antioxidants barriers (Díaz et al., 2004; Díaz et al., 2014). Therefore, these qualities are expected to be reflected in the ability of the protein hydrolysates to scavenge DPPH radical, since they are made from enzymes and shrimp tissues.

Several authors registered significant differences in DPPH radical scavenging capability between fish or crustaceans hydrolysates, elaborated with different enzymes and reaction times. This may be due to several factors, like the type (hydrophobicity or hydrophilicity), amount and sequence of amino acids, or the DH and molecular weights of peptides produced (Ambigaipalan & Shahidi, 2017). Also, aromatic amino acid residues (Tyr, His, Trp, Phe) can donate protons to electron deficient radicals via resonance stabilization, which improves the radical-scavenging properties of the amino acid residues (Rajapakse et al., 2005). The hydrolysate of carp *Ctenopharyngo donidellus* presented a value of 49.5% of scavenger ability, using the enzyme Alcalase® 2.4 L with 2 hr of reaction (Li et al., 2012), while the hydrolysate of Yellow stripe *Selaroides leptolesis* made with Flavourzyme® 1,000 L in 20 min obtained scavenger capability values close to 80%. Jun et al. (2004) reported that yellowfin sole hydrolysate using pepsin at lowest DH (22%) had a higher antioxidant activity than those produced using other enzymes, such as Alcalase® 0.6 L. Ambigaipalan and Shahidi (2017) observed a significant increment of hydroxyl radical scavenging activity in protein hydrolysates from shrimp shell discards in comparison to raw shrimp shells. Similar results were reported in others studies for Alcalase-assisted hydrolysed products of shrimp shell processing discards (Guerard et al., 2007; Sila et al., 2014). In this work, we observed a DPPH scavenging activity at the initial time in all treatments. This could be explained because the shrimp tissue presents a high antioxidant activity as previously demonstrated by Díaz et al. (2004), Díaz et al. (2014). However, H1 and H2 had an initial activity significantly higher than

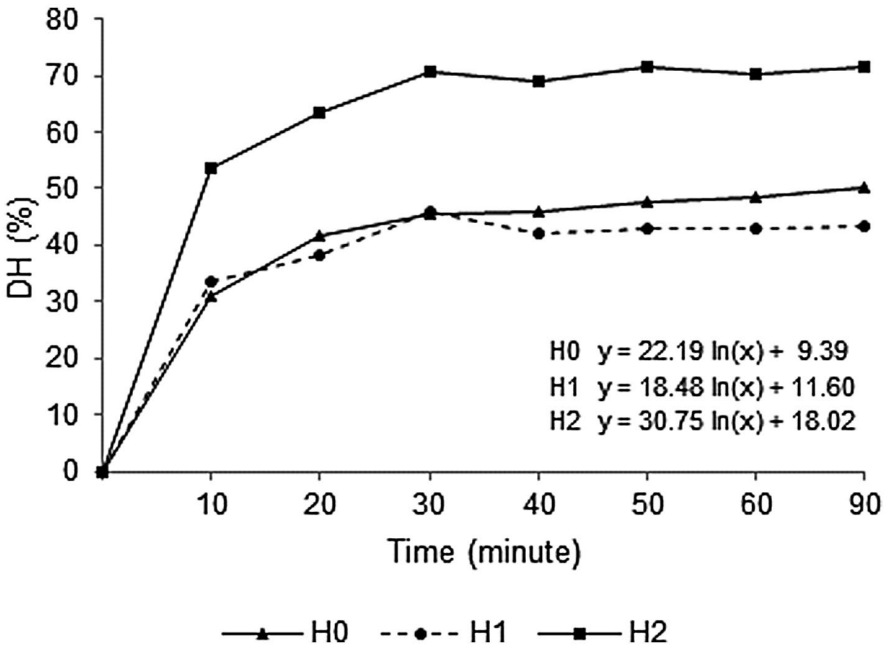


FIGURE 3 Degree of hydrolysis of *Pleoticus muelleri* with supplement of 0 (H0), 1 (H1), and 2 vol/vol (H2) of extract enzymes of same species

H0. Such antioxidant activity was possibly complemented by the enzymatic extract of the midgut gland, which might contribute by adding its hepatopancreatic antioxidative enzymes (Chirawithayaboon et al., 2020). After 10 min of hydrolysis, H0 and H1 maintained their antioxidant activity constant, while H2 continued increasing.

Additionally, protein hydrolysates can be used as a functional food ingredient or a flavor enhancer. In this study, a high content of flavor enhancers were observed in shrimp hydrolysate, such as glutamic acid, aspartic acid, glycine, and alanine, which may improve food palatability and low tryptophan levels which guarantee a no production of bitter taste. Furthermore, it was found that SPH contains high levels of lysine, a precursor in the production of carnitine, which plays a role in converting fatty acids into energy and in regulating cholesterol levels (Cao et al., 2008). Thus, it could be used as dietary supplement for animal nutrition. Another important implication of this study's findings is that shrimp hydrolysate could be utilized in specialized nutrition for humans, as a result of its high-quality protein. As regards clinical applications, hydrolysates with high DH and antioxidant capability are also useful for patients with gastrointestinal activity deficiency (Crohn's disease) due to their excellent solubility, digestibility, and intestinal absorption (Kushner, 1992), or in cases where the digestive capacity is reduced as in cystic fibrosis or pancreatitis (Farrell et al., 1987).

No inhibitory activity was observed for shrimp hydrolysate against *E. coli*, *S. aureus*, or *P. larvae*. Djellouli et al. (2020) did not report antimicrobial activity from shrimp hydrolysate, although the glycation of some of this hydrolysate gave place to the formation of different molecules possessing different antibacterial compounds.

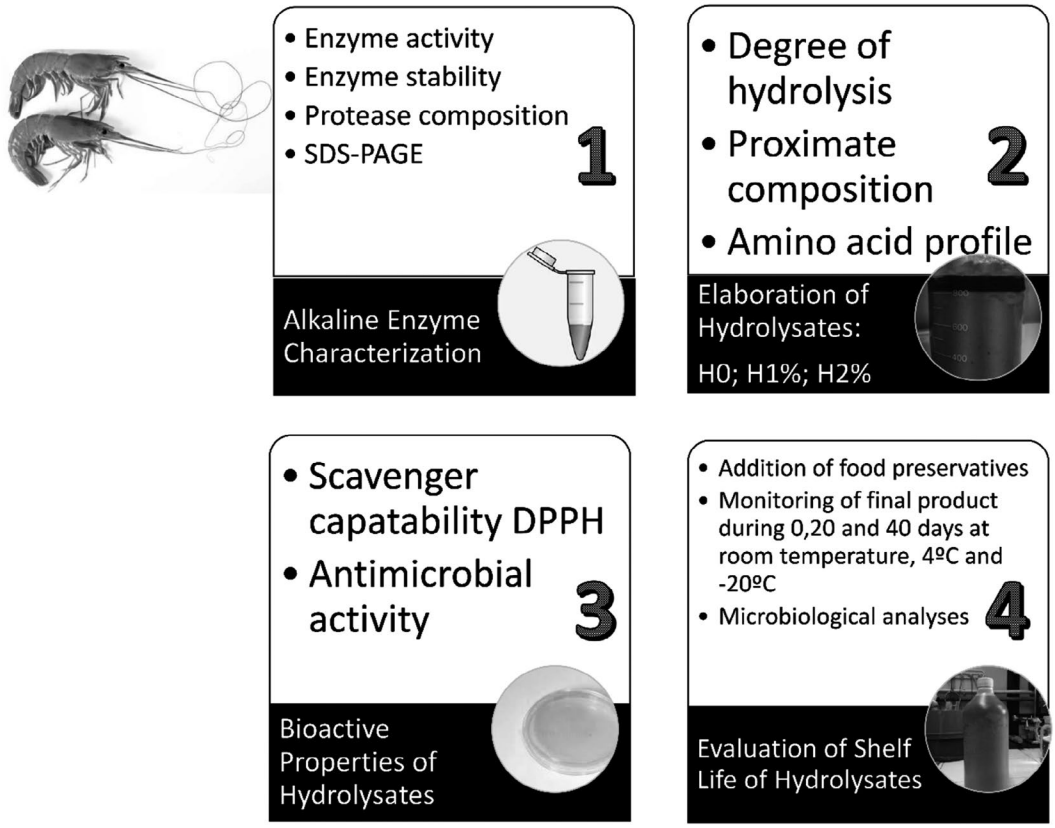
To assess the consequences of the different conditions of storage on SPH, the aerobic mesophilic bacteria counts were measured in samples at room temperature, 4°C and −20°C. The microbiological analyses of the SPH showed aerobic mesophilic bacteria counts within the limits set by Food Standards Australia New Zealand (FSANZ, 2018) for ready-to-eat products that imposes a maximum of satisfactory counts not higher of 10^4 CFU/g and marginal of 10^6 CFU/g, for all storage conditions after 40 days. Aerobic mesophilic bacteria counts were also below values reported by He et al. (2015) 6.2×10^2 CFU/g) in fish protein hydrolysates (FPH) and Kpoclou et al. (2013), who reported aerobic mesophilic bacteria counts of 1.4×10^4 and 3.2×10^4 CFU/g for entire smoked shrimp and smoke shrimp powder, respectively, collected in some local markets in Ghana.

TABLE 3 Scavenger capability of *Pleoticus muelleri's* hydrolysates (H0, H1 and H2), at different hydrolysis times

		Time		
Treatment	**Initial**	**10 min**	**20 min**	**30 min**
H0	10.25 ± 2.16^{aA}	58.06 ± 2.32^{aB}	58.68 ± 1.20^{aB}	63.06 ± 4.80^{aB}
H1	24.02 ± 2.89^{bA}	54.93 ± 1.72^{aB}	57.28 ± 1.84^{aB}	69.44 ± 7.59^{aB}
H2	28.23 ± 4.25^{bA}	54.01 ± 2.33^{aB}	$57.17 \pm 2.99^{aB,C}$	68.87 ± 7.30^{aC}

Note: Values are means and standard error of three replicates. Different lower case letters in the same column indicate significant difference between the hydrolysates obtained with different doses of *Pleoticus muelleri's* enzyme. Different capital letters in the same row indicate a significant difference between the hydrolysates obtained at different times ($p < .05$).

-Scheme of the tests carried out:



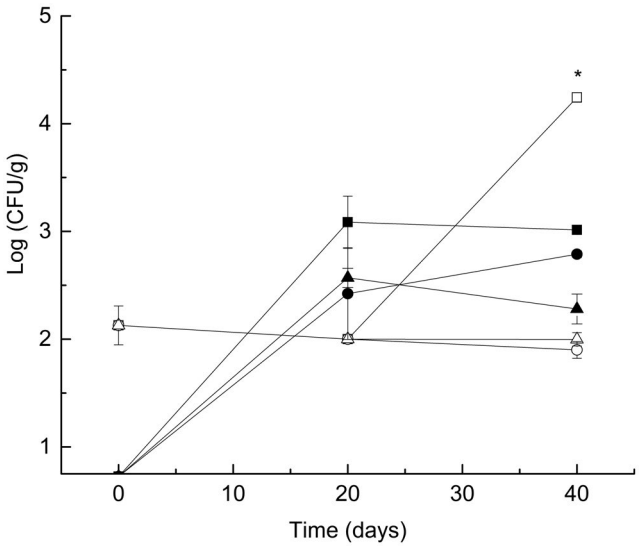


FIGURE 4 Aerobic mesophilic bacteria (open) and yeast and mould (fills) counts (Log CFU/g) of samples stored at room temperature (squares), 4°C (circles) and −20°C (triangle). An asterisk indicates statistically significant difference ($p < .05$) between counts of control and treated samples at the same time of storage

In addition, samples stored at 4°C and −20°C showed no differences throughout the storage time. Other researchers have also observed a similar maintenance in the total populations of aerobic mesophilic bacteria when shrimp samples are treated with materials containing benzoic and sorbic acid preservatives (Einarsson & Lauzon, 1995) after 60 days of storage.

The initial counts of molds and yeasts was down below the values reported by He et al. (2015) who indicated counts of mold and yeast of 6.2×10^2 CFU/g for FPH or (Akuamoah et al., 2018) with 10^5 CFU/g for smoke dry shrimp. There was a significant increase ($p > .05$) in growth of molds and yeasts in shrimp hydrolysates samples stored 20 and 40 days at room temperature and 40 days at 4°C, with maximum values of 1×10^3 CFU/g. However, there is not a set limit for molds and yeast in ready-to-eat food. Therefore, this increase does not affect the acceptability of the sample.

E. coli was not present in any condition or time of storage. *Enterobacteriacea* (including coliforms) in ready-to-eat foods has maximum limits of less than 10^4 CFU/g, with satisfactory values of 10^2/g CFU/g, while *E. coli* values must be lower than 10^2 CFU/g. While total coliforms have been detected in fresh shrimp in a range of 10^2–10^5 CFU/g, total fecal coliform in a range of 10^2–10^4 CFU/g and *E. coli* between 10^2–10^3 CFU/g (Nilla et al., 2012), FPH from *Yellowtail kingfish* have showed less than 10 CFU/g (He et al., 2015). The absence of these microorganisms in the hydrolysate would be due to their treatment to/under 100°C during 10 min to inactivate the enzyme.

In addition, *S. aureus*, *Salmonella* spp., and *C. perfringens* were not detected in any of the samples at any time or condition of storage. According to international standards, samples containing less than 10^2 CFU/g of *S. aureus* and *C. perfringens* are satisfactory while the absence of *Salmonella* spp. in 25 g of sample is mandatory. According to this quality requirement, the SPH is acceptable in all storage conditions.

Evaluation of pH during storage showed a slight increase of pH, although this increment was not significant. The increase in pH might be due to the production of ammonia and volatile bases as a result of proteolysis and the increase of microbial counts (Fangio & Fritz, 2014).

5 | CONCLUSION

The results of the present study show that endogenous proteases of *P. muelleri* shrimp can be used for the production of hydrolysates with variable degrees of hydrolysis, antioxidant capability, and richness in certain amino acids. Additionally, the wastes of the Argentine shrimp industry are an excellent source of protein and enzymes with high biotechnological potential. This SPH could be used for feed formulation enhancement, as a functional food ingredient and flavor enhancer. Furthermore, the SPH prepared with food additives is suitable for food application with respect to its microbiological safety profile. It is for all these reasons that the present work promotes the use of the whole marine resource, which reduces adverse environmental impact effects produced by the fishery industry and supports environmental sustainability.

ACKNOWLEDGMENTS

We are grateful to CONICET (National Council of science and technology of Argentina), for covering scholarships costs for Dr. Pereira, Dra. Rodriguez and Lic. Bonadero.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

AUTHOR CONTRIBUTIONS

Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing: Nair de los Angeles Pereira. *Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing*: Maria Florencia Fangio. *Conceptualization; Formal analysis; Investigation; Software; Validation; Writing-review & editing*: Yamila Eliana Rodriguez. *Investigation; Software; Validation*: Maria Cecila Bonadero. *Formal analysis; Investigation; Validation*: Nora Selma Haran. *Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Writing-review & editing*: Analía Verónica Fernández-Gimenez.

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REFERENCES

Abd-El-Aziz, N. A., & Moharram, Y. G. (2016). Microbiological quality of imported frozen shrimp in Egypt. *Annals of Agricultural Sciences*, *61*(1), 35–40. https://doi.org/10.1016/j.aoas.2016.04.002

Akuamoah, F., Odamtten, G. T., & Kortei, N. K. (2018). Impact of gamma radiation on the microbiological quality of smoke dry shrimp (*Penaeus notialis*) from three different water sources in Ghana. *Cogent Food & Agriculture*, *4*(1), 1–12. https://doi.org/10.1080/23311932.2018.1484200

Ambigaipalan, P., & Shahidi, F. (2017). Bioactive peptides from shrimp shell processing discards: Antioxidant and biological activities. *Journal of Functional Foods*, *34*, 7–17. https://doi.org/10.1016/j.jff.2017.04.013

AOAC. (1953). *Official methods of analysis 950.46. Moisture in meat*. Association of Official Analytical Chemists.

AOAC. (2008). *Official methods of analysis 940.25. Nitrogen (Total) in seafood*. Association of Official Analytical Chemists.

AOAC. (2013). *Official methods of analysis 938.08. Fish and marine products. Ash of seafood*. Association of Official Analytical Chemists.

Baek, H. H., & Cadwallader, K. R. (1995). Enzymatic hydrolysis of crayfish processing by-products. *Journal of Food Science*, *60*(5), 929–935. https://doi.org/10.1111/j.1365-2621.1995.tb06264.x

Bellido, J. M., Santos, M. B., Pennino, M. G., Valeiras, X., & Pierce, G. J. (2011). Fishery discards and bycatch: Solutions for an ecosystem approach to fisheries management? *Hydrobiologia*, *670*(1), 317–333. https://doi.org/10.1007/s10750-011-0721-5

Benítez, R., Ibarz, A., & Pagan, J. (2008). Hidrolizados de proteína: Procesos y aplicaciones Resumen. *Acta Bioquímica Clínica Latinoamericana*, *42*(2), 227–237.

Bertuche, D., Fischbach, C., Roux, A., Fernández, M., & Piñero, R. (2000). Langostino Pleoticus muelleri. *Pesquerías De Argentina*, 179–190. https://www.inidep.edu.ar/wp-content/uploads/Langostino.pdf

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Cao, W., Tian, S., Wang, H., Zhang, C., & Yuan, J. (2019). Release principle of peptides and amino acids during the autolysis of shrimp head from *Litopenaeus vannamei* after UV-C irradiation stress. *Food Science & Nutrition*, *8*(1), 170–178. https://doi.org/10.1002/fsn3.1288

Cao, W., Zhang, C., Hong, P., & Ji, H. (2008). Response surface methodology for autolysis parameters optimization of shrimp head and amino acids released during autolysis. *Food Chemistry*, *109*(1), 176–183. https://doi.org/10.1016/j.foodchem.2007.11.080

Caruso, G. (2015). Fishery wastes and by-products: A resource to be valorised. *Journal of Fisheries Sciences Com*, *9*, 80–83.

Celis-Guerrero, L. E., García-Carreño, F. L., & Navarrete Del Toro, M. A. (2004). Characterization of proteases in the digestive system of spiny lobster (*Panulirus interruptus*). *Marine Biotechnology*, *6*(3), 262–269. https://doi.org/10.1007/s10126-003-0032-6

Chen, H.-M., Muramoto, K., Yamauchi, F., & Nokihara, K. (1996). Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein. *Journal of Agricultural and Food Chemistry*, *44*(9), 2619–2623. https://doi.org/10.1021/jf950833m

Cheung, I. W. Y., & Li-Chan, E. C. Y. (2010). Angiotensin-I-converting enzyme inhibitory activity and bitterness of enzymatically-produced hydrolysates of shrimp (*Pandalopsis dispar*) processing byproducts investigated by Taguchi design. *Food Chemistry*, *122*(4), 1003–1012. https://doi.org/10.1016/j.foodchem.2010.03.057

Chirawithayaboon, P., Areechon, N., & Meunpol, O. (2020). Hepatopancreatic antioxidant enzyme activities and disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) fed diet supplemented with garlic (*Allium sativum*) extract. *Agriculture and Natural Resources*, *54*(4), 377–386.

Clinical and Laboratory Standards Institute. (2015). *M07-A10 Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard* (10th ed.). Clinical and Laboratory Standards Institute.

D'Ambrosio, A., Rossano, R., Ungaro, N., & Riccio, P. (2003). Proteolytic and milk clotting activities in extracts obtained from the crustaceans Munida. *Journal of Molecular Catalysis B: Enzymatic*, *22*(3–4), 145–150. https://doi.org/10.1016/S1381-1177(03)00026-2

Díaz, A. C., Gimenez, A. V. F., Mendiara, S. N., & Fenucci, J. L. (2004). Antioxidant activity in hepatopancreas of the shrimp (*Pleoticus muelleri*) by electron paramagnetic spin resonance Spectrometry. *Journal of Agricultural and Food Chemistry*, *52*(10), 3189–3193. https://doi.org/10.1021/jf0346962

Díaz, A. C., Velurtas, S. M., Espino, M. L., & Fenucci, J. L. (2014). Effect of dietary astaxanthin on free radical scavenging capacity and nitrite stress tolerance of postlarvae shrimp, *Pleoticus muelleri*. *Journal of Agricultural and Food Chemistry*, *62*(51), 12326–12331. https://doi.org/10.1021/jf503754q

Dingman, D. W., & Stahly, D. P. (1983). Medium promoting sporulation of Bacillus larvae and metabolism of medium components. *Applied and Environmental Microbiology*, *46*(4), 860–869.

Djellouli, M., López-Caballero, M. E., Arancibia, M. Y., Karam, N., & Martínez-Alvarez, O. (2020). Antioxidant and antimicrobial enhancement by reaction of protein hydrolysates derived from shrimp by-products with glucosamine. *Waste and Biomass Valorization*, *11*(6), 2491–2505. https://doi.org/10.1007/s12649-019-00607-y

dos Santos, S. D., Martins, V. G., Salas-Mellado, M., & Prentice, C. (2011). Evaluation of functional properties in protein hydrolysates from bluewing searobin (*prionotus punctatus*) obtained with different microbial enzymes. *Food and Bioprocess Technology*, *4*(8), 1399–1406. https://doi.org/10.1007/s11947-009-0301-0

Einarsson, H., & Lauzon, H. L. (1995). Biopreservation of brined shrimp (*pandalus borealis*) by bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology*, *61*(2), 669–676. https://doi.org/10.1128/AEM.61.2.669-676.1995

Fangio, M. F., & Fritz, R. (2014). Potential use of a bacteriocin-like substance in meat and vegetable food biopreservation. *International Food Research Journal*, *21*(2), 677–683.

Farrell, P. M., Mischler, E. H., Sondel, S. A., & Palta, M. (1987). Predigested formula for infants with cystic fibrosis. *Journal of the American Dietetic Association*, *87*(10), 1353–1356.

Fernández Gimenez, A. V., García-Carreo, F. L., Navarrete del Toro, M. A., & Fenucci, J. L. (2001). Digestive proteases of red shrimp *Pleoticus muelleri* (Decapoda, Penaeoidea): Partial characterization and relationship with molting. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology*, *130*(3), 331–338. https://doi.org/10.1016/S1096-4959(01)00437-7

FSANZ. (2018). *Compendium of microbiological criteria for food*. Food Standards Australia New Zealand. https://www.foodstandards.gov.au/publications/Pages/Compendium-of-Microbiological-Criteria-for-Food.aspx.

Garbari, M. D. (2018). *Hidrolizados de anchoita engraulis anchoita elaborados con enzimas provenientes de subproductos pesqueros (Tesis de Grado)*. Universidad Nacional de Mar del Plata.

García-Carreño, F. L. (1992). Protease inhibition in theory and practice. *Biotechnology Education*, *3*, 145–150.

García-Carreño, F. L., & Haard, N. F. (1993). Characterization of protease classes in langostilla (*pleuroncodes planipes*) and crayfish (*pacifastacus astacus*) extracts. *Journal of Food Biochemistry*, *17*, 97–113.

Gavio, M. A., & Boschi, E. E. (2004). Biology of the shrimp Artemesia longinaris Bate, 1888 (*Crustacea*: *Decapoda*: *Penaeidae*) from Mar del Plata coast, Argentina. *Nauplius*, *12*(2), 83–94.

Gudmundsdóttir, Á., & Pálsdóttir, H. M. (2005). Atlantic cod trypsins: From basic research to practical applications. *Marine Biotechnology*, *7*(2), 77–88. https://doi.org/10.1007/s10126-004-0061-9

Guerard, F., Guimas, L., & Binet, A. (2002). Production of tuna waste hydrolysates by a commercial neutral protease preparation. *Journal of Molecular Catalysis B: Enzymatic*, *19–20*, 489–498. https://doi.org/10.1016/S1381-1177(02)00203-5

Guerard, F., Sumaya-Martinez, M. T., Laroque, D., Chabeaud, A., & Dufossé, L. (2007). Optimization of free radical scavenging activity by response surface methodology in the hydrolysis of shrimp processing discards. *Process Biochemistry*, *42*(11), 1486–1491. https://doi.org/10.1016/j.procbio.2007.07.016

Gunasekaran, J., Kannuchamy, N., Kannaiyan, S., Chakraborti, R., & Gudipati, V. (2015). Protein hydrolysates from shrimp (*Metapenaeus dobsoni*) head waste: Optimization of extraction conditions by response surface methodology. *Journal of Aquatic Food Product Technology*, *24*(5), 429–442. https://doi.org/10.1080/10498850.2013.787134

He, S., Franco, C., & Zhang, W. (2015). Fish protein hydrolysates: Application in deep-fried food and food safety analysis: Fish protein hydrolysates. *Journal of Food Science*, *80*(1), E108–E115. https://doi.org/10.1111/1750-3841.12684

Heu, M. S., Kim, J. S., Shahidi, F., Jeong, Y., & Jeon, Y. J. (2003). Extraction, fractionation and activity characteristics of proteases from shrimp processing discards. *Journal of Food Biochemistry*, *27*(3), 221–236. https://doi.org/10.1111/j.1745-4514.2003.tb00278.x.

Hou, Y., Wu, Z., Dai, Z., Wang, G., & Wu, G. (2017). Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. *Journal of Animal Science and Biotechnology*, *8*(1), 1–13. https://doi.org/10.1186/s40104-017-0153-9

Ideia, P., Pinto, J., Ferreira, R., Figueiredo, L., Spínola, V., & Castilho, P. C. (2020). Fish processing industry residues: A review of valuable products extraction and characterization methods. *Waste and Biomass Valorization*, *11*(7), 3223–3246. https://doi.org/10.1007/s12649-019-00739-1

Jun, S. Y., Park, P. J., Jung, W. K., & Kim, S. K. (2004). Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein. *European Food Research and Technology*, *219*(1), 20–26. https://doi.org/10.1007/s00217-004-0882-9

Karuppasamy, P. K., Priyadarshini, R. S. S., Ramamoorthy, N., Sujatha, R., & Ganga, S. (2014). *Comparison of proximate, amino and fatty acid composition of Penaeus monodon (H. Milne Edwards, 1837) and Aristeus virilis (Bate, 1881) of Nagapattinam landing centre, Tamil Nadu*. *55* (December 2013). https://doi.org/10.6024/jmbai.2013.55.2.01783-0x

Ketnawa, S., Martínez-Alvarez, O., Gómez-Estaca, J., del Carmen Gómez-Guillén, M., Benjakul, S., & Rawdkuen, S. (2016). Obtaining of functional components from cooked shrimp (*Penaeus vannamei*) by enzymatic hydrolysis. *Food Bioscience*, *15*, 55–63. https://doi.org/10.1016/j.fbio.2016.05.005

Kpoclou, Y. E., Anihouvi, V., Azokpota, P., Mohamed, S., Daube, G., Douny, C., & Hounhouigan, D. (2013). Microbiological and physicochemical quality of smoked shrimp, an expanding food condiment in beninese local markets. *Food and Public Health*, *3*, 273–283. https://doi.org/10.5923/j.fph.20130306.03

Kushner, R. F. (1992). Should enteral nutrition be considered as primary therapy in acute Crohn's disease? *Nutrition Reviews*, *50*(6), 166–169. https://doi.org/10.1111/j.1753-4887.1992.tb01314.x

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage t4. *Nature*, *227*(5259), 680–685. https://doi.org/10.1038/227680a0

Leal, A. L. G., Castro, P. F., Lima, J. P. V., Souza Correia, E., & Souza Bezerra, R. (2010). Use of shrimp protein hydrolysate in Nile tilapia (*Oreochromis niloticus*, L.) feeds. *Aquaculture International*, *18*(4), 635–646. https://doi.org/10.1007/s10499-009-9284-0

Lemos, D., Ezquerra, J. M., & Garcia-Carreño, F. L. (2000). Protein digestion in penaeid shrimp: Digestive proteases, protease inhibitors and feed digestibility. *Aquaculture*, *186*(1–2), 89–105. https://doi.org/10.1016/S0044-8486(99)00371-3

Li, X., Luo, Y., Shen, H., & You, J. (2012). Antioxidant activities and functional properties of grass carp (*Ctenopharyngodon idellus*) protein hydrolysates. *Journal of the Science of Food and Agriculture*, *92*(2), 292–298. https://doi.org/10.1002/jsfa.4574

Liceaga-Gesualdo, A. M., & Li-Chan, E. (1999). Functional properties of fish protein hydrolysate from herring (*Clupea harengus*). *Journal of Food Science*, *64*(6), 1000–1004. https://doi.org/10.1111/j.1365-2621.1999.tb12268.x

Liébana, C. (2018). *Evaluación de la calidad de harina de arveja amarilla suplementada con hidrolizado proteico de langostino en alimentos para cultivo de tilapia del Nilo Oreochromis niloticus* (Tesis de Grado). Universidad Nacional de Mar del Plata.

Lovett, D. L., & Felder, D. L. (1990). Ontogenetic change in digestive enzyme activity of larval and postlarval white shrimp *penaeus setiferus* (*crustacea*, *decapoda*, *penaeidae*). *The Biological Bulletin*, *178*(2), 144–159.

MAGyP. (2021). http://www.magyp.gob.ar/sitio/areas/pesca_maritima/desembarques/lectura.php?imp=1&tabla=especie_flota_2020

Mao, X., Guo, N., Sun, J., & Xue, C. (2017). Comprehensive utilization of shrimp waste based on biotechnological methods: A review. *Journal of Cleaner Production*, *143*, 814–823. https://doi.org/10.1016/j.jclepro.2016.12.042

Muhlia-Almazán, A., & García-Carreño, F. (2003). Digestion physiology and proteolytic enzymes of crustacean species of the Mexican Pacific Ocean. *Contribution O the Study of East Pacific Ccrustaceans*, *2*, 303.

Nilla, S. S., Mustafa, M. G., Ahsan, D. A., Rahman Khan, M. M., & Rahman Khan, M. A. (2012). Bacterial abundance in indian white shrimp, *penaeus indicus* collected from two different market conditions of dhaka city. *Dhaka University Journal of Biological Sciences*, *21*(1), 29–38. https://doi.org/10.3329/dujbs.v21i1.9742

Ovissipour, M., Safari, R., Motamedzadegan, A., & Shabanpour, B. (2012). Chemical and biochemical hydrolysis of persian sturgeon (*Acipenser persicus*) visceral protein. *Food and Bioprocess Technology*, *5*(2), 460–465. https://doi.org/10.1007/s11947-009-0284-x

Pardio, V. T., Waliszewski, K. N., & Zuñiga, P. (2011). Biochemical, microbiological and sensory changes in shrimp (*Panaeus aztecus*) dipped in different solutions using face-centred central composite design: Shrimp quality after dipping in additives. *International Journal of Food Science & Technology*, *46*(2), 305–314. https://doi.org/10.1111/j.1365-2621.2010.02474.x

Pereira, N. D. L. A. & Fernández-Gimenez, A. V. (2017). Exogenous enzymes in dairy technology: Acidic proteases from processing discards of shrimp *Pleoticus muelleri* and their use as milk-clotting enzymes for cheese manufacture. *International Journal of Food Science & Technology*, *52*(2), 341–347. https://doi.org/10.1111/ijfs.13285

Pereira, N. D. L. A., Haran, N. S., Rodríguez, Y. E., & Fernández-Gimenez, A. V. (2020). *Propuesta de un Desarrollo Industrial Sustentable Post Covid19: Obtención de Coagulante Lácteo a partir de restos del eviscerado de especies pesqueras mediante tecnología verde (Primera Edición)*. Editorial Académica Española.

Piotrowicz, I. B. B., & Mellado, M. M. S. (2015). Antioxidant hydrolysates production from Argentine anchovy (*Engraulis anchoita*) with different enzymes. *International Food Research Journal*, *22*(3), 1203–1211.

Rajapakse, N., Mendis, E., Jung, W. K., Je, J. Y., & Kim, S. K. (2005). Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Research International*, *38*(2), 175–182. https://doi.org/10.1016/j.foodres.2004.10.002

Ramírez-Navas, J. (2007). Liofilización De Alimentos. *Revista ReCiTeIA*, *6*, 1–39.

Rodriguez, Y. E., Pereira, N. D. A., Haran, N. S., Mallo, J. C., & Fernández-Gimenez, A. V. (2017). A new approach to fishery waste revalorization to enhance Nile tilapia (*Oreochromis niloticus*) digestion process. *Aquaculture Nutrition*, 1–11. https://doi.org/10.1111/anu.12510

Rossano, R., Larocca, M., & Riccio, P. (2011). Digestive enzymes of the crustaceans Munida and their application in cheese manufacturing: A review. *Marine Drugs*, *9*(7), 1220–1231. https://doi.org/10.3390/md9071220

Santos, J. F., Castro, P. F., Leal, A. L. G., de Freitas Júnior, A. C. V., Lemos, D., Carvalho, L. B., & Bezerra, R. S. (2013). Digestive enzyme activity in juvenile Nile tilapia (*Oreochromis niloticus*, L) submitted to different dietary levels of shrimp protein hydrolysate. *Aquaculture International*, *21*(3), 563–577. https://doi.org/10.1007/s10499-012-9589-2

Shimada, K., Fujikawa, K., Yahara, K., & Nakamura, T. (1992). Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry*, *40*(6), 945–948. https://doi.org/10.1021/jf00018a005

Sila, A., Sayari, N., Balti, R., Martinez-Alvarez, O., Nedjar-Arroume, N., Moncef, N., & Bougatef, A. (2014). Biochemical and antioxidant properties of peptidic fraction of carotenoproteins generated from shrimp by-products by enzymatic hydrolysis. *Food Chemistry*, *148*, 445–452. https://doi.org/10.1016/j.foodchem.2013.05.146

Thiansilakul, Y., Benjakul, S., & Shahidi, F. (2007). Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (*Decapterus maruadsi*). *Food Chemistry*, *103*(4), 1385–1394. https://doi.org/10.1016/j.foodchem.2006.10.055

Velurtas, S. M., Diaz, A. C., Fernandez Gimenez, A. V., & Lino Fenucci, J. (2011). Influence of dietary starch and cellulose levels on the metabolic profile and apparent digestibility in penaeoid shrimp. *Latin American Journal of Aquatic Research*, *39*(2), 214–224. https://doi.org/10.3856/vol39-issue2-fulltext-3

How to cite this article: Pereira NDLÁ, Fangio MF, Rodriguez YE, Bonadero MC, Harán NS, Fernández-Gimenez AV. Characterization of liquid protein hydrolysates shrimp industry waste: Analysis of antioxidant and microbiological activity, and shelf life of final product. *J Food Process Preserv*. 2021;00:e15526. https://doi.org/10.1111/jfpp.15526