

Research Article

Partial biochemical characterization of digestive proteases present in the gastric juices of two Chilean crustaceans, *Lithodes santolla* (Molina, 1782) and *Cancer edwardsii* (Bell, 1835)

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ABSTRACT. The objectives of the present study were to characterize biochemically the proteases present in gastric juices from adults of the southern king crab (*Lithodes santolla*) and the Chilean rock crab (*Cancer edwardsii*), to determine the classes of proteases using specific inhibitors, as well as to evaluate the effect of temperature and pH that affect the proteolytic activity. The enzyme activity analysis revealed non-significant differences between the activities of serine proteases (trypsin and chymotrypsin) in both species. On the other hand, acid protease activity in *C. edwardsii* was significantly higher than in *L. santolla*. The activity of aspartate proteases was inhibited by Pepstatin A in both species, with no significant differences in the extent of inhibition between them. The maximal enzyme activity detected for both species occurred at alkaline pH. The optimum pH for proteolytic activity was ranged between 7 and 9 for *C. edwardsii*, and between 7 and 11 for *L. santolla*. Moreover, the highest proteolytic activity was recorded at 60°C in both species. However, at this temperature, enzyme activity was unstable after 20 min. Finally, collagenase-like activity in the gastric juices was detected for both species; this activity deserves further investigation, considering its biotechnological potential.

Keywords: *Lithodes santolla*, *Cancer edwardsii*, digestive physiology, proteases, protein inhibitors, subpolar crabs.

INTRODUCTION

Crabs, such as stone crabs and lithodids, are economically valuable fishery resources from Polar and Subpolar regions (Calcagno *et al.*, 2005; Lovrich & Tapella, 2014). The southern king crab (*Lithodes santolla*) and the Chilean rock crab (*Cancer edwardsii*) are species that inhabit the southern tip of South America (Paschke *et al.*, 2013; Vinuesa *et al.*, 2013). Their natural populations have plummeted in recent years, and aquaculture production has become an option to meet the commercial demand for these products as well as to reduce the fishery overexploitation of these resources (Tapia *et al.*, 2008; Paschke *et al.*, 2013).

Grow-out nursery technology for *L. santolla* is relatively new compared to other crustacean species,

and therefore requires research studies focused on nutrition and feeding aspects. Nowadays, the feeding of lithodids and crabs in captivity is based on the use of natural feeds such as small pelagic fishes (Woll & Berge, 2007) and fresh mollusk meat from squids and mytilids (Calcagno *et al.*, 2004). However, there are reports on the use of species-specific manufactured feed to maintain high survival and growth rates of the edible crab *Cancer pagurus* (Berge & Woll, 2006) and the red king crab *Paralithodes camtschaticus* (James *et al.*, 2013; Siikavuopio & James, 2015). Nevertheless, it is well known that to successfully achieve a sustainable aquaculture for crustaceans, the development of formulated diets should be investigated (Cuzon *et al.*, 1994).

Investigations focused on digestive physiology provide essential knowledge about feeding habits

(Figueiredo & Anderson, 2009) and digestive capacity (Buarque *et al.*, 2010; Fernández-Gimenez, 2013) of organisms, and could be applicable to formulate species and stage-specific feeds. Thus, diet formulation should be performed considering feedstuffs that are suitable for digestion (Lindner *et al.*, 1995), containing nutrients with a proper assimilation potential (bioavailability) (Márquez *et al.*, 2013), and lacking anti-nutritional factors such as enzyme inhibitors, among many other factors. Numerous studies on digestive enzymes in marine crustaceans from tropical and temperate waters have been published; however, there is limited information on the biochemical characterization of cold-water crustacean species (Buchholz & Vetter, 1993; Freese *et al.*, 2012; Rojo *et al.*, 2013), changes in digestive capacity through ontogeny (Saborowski *et al.*, 2006), and the effects of starvation on digestive enzymes (Comoglio *et al.*, 2008). It has been considered that analysis of digestive enzymes could be used as an indicator of the nutritional status in different ontogenetic stages, as well as the conditions of cultures in relation to feeding and other environmental variables.

Juveniles of *L. santolla* are macrophage and opportunistic omnivorous predators (Vinuesa *et al.*, 2013), with a highly diverse diet composed of several species of algae, protists, and invertebrates. On the other hand, *C. edwardsii* are scavengers and omnivorous, preferably feeding on various species of mussels and other mollusks (Tapia *et al.*, 2008). Considering the feeding habits, proteases are the most conspicuous enzymes involved in digestive processes in omnivorous species with a carnivory tendency such as *L. santolla* and *C. edwardsii*. Previously, Saborowski *et al.* (2006) and Galgani & Nagayama (1987) analyzed the presence and activity of different digestive enzymes in *L. santolla* in early ontogenetic stages and in adults; nevertheless, to date a biochemical characterization of proteases evaluating the effect of various physical and biochemical conditions on enzyme activity has not been reported. For *C. edwardsii*, this is the first report on the biochemical characterization of proteases contained in the gastric juices of these species.

Therefore, the aim of the present work was to compare the partial biochemical characterization of proteases contained in the gastric juices of the southern king crab (*L. santolla*) and the Chilean stone crab (*C. edwardsii*). The findings derived from this study provide basic information about the digestive physiology of both crustacean species and will be useful in further nutritional and biotechnological research.

MATERIALS AND METHODS

Specimens and sampling

This research was performed using live adults in intermolt stage of *C. edwardsii* (body weight: 403 ± 61 g; carapace width: 8.4 ± 0.4 cm) and *L. santolla* (body weight: 774 ± 222 g; carapace width: 9.9 ± 1.2 cm) provided by the Laboratory of Crustacean Ecophysiology (LECOFIC) at the Universidad Austral de Chile, Chile. Crabs were maintained in tanks with a continuous flow of filtered seawater and were fed twice daily at 10:00 and 16:00 h with fresh meat of Chilean mussels, *Mytilus chilensis*. Twenty-four hours after the last meal and previous to the first feeding horary (10:00 AM), gastric juices were obtained separately from 12 starved specimens of each species. Then, three pools of gastric juices were formed (4 individuals per pools) for further analysis. Individual crabs were sampled by introducing a flexible Teflon tube through the esophagus to reach the gastric chamber and aspirating approximately 1 to 1.5 mL of gastric juices per individual with a micropipette (Saborowski *et al.*, 2006; Rojo *et al.*, 2013).

The pH of three pools (4 individuals per pool) of gastric juices was measured using a potentiometer immediately after extraction. Juices were immediately transferred to cool vials ($<4^{\circ}\text{C}$) and centrifuged at $10,000\times g$ for 10 min at 4°C to remove potential debris. Supernatants were stored at -80°C until use for enzyme analysis.

Enzyme assays

Soluble protein concentration was determined according to the method by Bradford (1976) using the Quick StartTM (BIO RAD) protein assay kit, using bovine serum albumin as a protein standard. Alkaline proteolytic activity was quantified, as described by Sarath *et al.* (1989), using 2% (w/v) azocasein in 50 mM Tris-HCl buffer with 10 mM CaCl_2 pH 9 as substrate. Calcium chloride was used to maintain the stability of proteases (Tremacoldi *et al.*, 2007). The concentration of azocasein here used has been previously used in crustacean studies (Figueiredo *et al.*, 2001). The reaction was initiated by adding 100 μL of gastric juice to 150 μL of substrate solution and incubated for 30 min at 37°C . The reaction was stopped by adding 750 μL of 10% (w/v) trichloroacetic acid and incubating final volume (1000 μL) for 30 min at 4°C . The undigested substrate and enzyme precipitates were separated by centrifugation at $19,000 g$ for 5 min. Supernatant absorbance was recorded at 366 nm (Lemos *et al.*, 2000). One unit of alkaline proteolytic activity was defined as the amount of enzyme required to cause an increase of 0.1 absorbance per minute. Acid

proteolytic activity was determined according to Sarath *et al.* (1989) using 300 μL of 2% (w/v) bovine hemoglobin in 0.2 M glycine-HCl pH 3.0 as substrate. Both gastric juices (100 μL) and substrate were incubated at 37°C, enzyme reaction stopped by adding 600 μL of 5% (w/v) trichloroacetic acid and the absorbance of reaction products was measured at 280 nm. One unit of enzyme activity was defined as 1 μg tyrosine released per minute, using the molar extinction coefficient of 0.005 $\text{mL } \mu\text{g}^{-1} \text{cm}^{-1}$.

Trypsin activity (EC 3.4.21.4) was measured according to the method by Erlanger *et al.* (1961), previously used on marine decapods by García-Carreño *et al.* (1994). Thus, 500 μL of 1 mM BAPNA (N- α -benzoyl-DL-arginine-*p*-nitroanilide) dissolved in DMSO was brought to 50 mL with a buffer with 50 mM Tris-HCl and 20 mM CaCl_2 pH 8.2 was used as the substrate. The enzyme reaction was initiated by adding 80 μL of gastric juices and stopped with 160 μL of 30% (w/v) acetic acid. The absorbance of reaction products was measured at 410 nm. One unit of enzyme activity was defined as 1 μmol of *p*-nitroanilide released per minute, using a molar extinction coefficient of 8800 $\text{cm}^{-1} \text{M}^{-1}$.

Chymotrypsin activity (EC 3.4.21.1) was measured according to continuous method reported by Hummel (1959), and previously used on crabs (Galgani & Nagayama, 1986), using 630 μL BTEE (0.56 mM N-benzoyl-L-tyrosine-ethyl ester) dissolved in a buffer of 0.12 M Tris-HCl with 20 mM CaCl_2 pH 8.1 as substrate, 70 μL of gastric juices and absorbance of reaction products on final volume reaction (700 μL) was measured at 256 nm. One unit of activity was defined as 1 μmol of nitroanilide released per minute, using a molar extinction coefficient of 964 $\text{cm}^{-1} \text{M}^{-1}$.

Leucine aminopeptidase (LAP, EC 3.4.11.1) activity was measured using 1.2 mM L-leucine-*p*-nitroanilide dissolved in 50 mM Tris-HCl pH 8.0 as a substrate, as suggested by Appel (1974). Then, 80 μL of gastric juices were added to the substrate and posteriorly stopped enzyme reaction with 200 μL of 30% (w/v) acetic acid. The absorbance of reaction products was measured at 405 nm. One unit of enzyme activity was defined as 1 μmol of *p*-nitroanilide released per minute, using a molar extinction coefficient of 8.2 $\text{cm}^{-1} \text{M}^{-1}$.

Collagenase activity (EC 3.4.24.7) was measured according to the method reported by García-Carreño *et al.* (1994) for marine decapod. Briefly, collagen from bovine Achilles tendon (4.8 mg mL^{-1}) as substrate was suspended in 0.1 M tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer with 0.35 mM CaCl_2 pH 7.5. The reaction was initiated by adding 25 μL of the enzyme preparation to 1.25 mL of the

substrate solution. Five hours later, the reaction mixture was centrifuged at 4000 g for 5 min. Then, a 200 μL sample of supernatant was transferred to test tubes with 500 μL of 1.5% ninhydrin solution. The mixture was incubated at 100°C for 15 min and then cooled to room temperature, and 2.5 mL of 50% ethanol was added with mixing. Then, the absorbance was recorded at 600 nm. One unit of collagenase activity was defined as the amount of enzyme required to cause an increase of 0.1 absorbances per minute.

For all enzyme assays, previous tests were performed to evaluate a suitable reaction time where the rate of product formation was linear. pH values considerate to evaluate the activity of different specific proteases were chosen according to standard methods previously reported.

The specific activity in gastric juices was determined using the following formula: Activity units (U/mg protein) = $[(\Delta\text{Abs} \times \text{mL reactions mix} \times \text{dilution factor}) / (\text{MEC} \times \text{time (min)} \times \text{mL extract volume} \times \text{mg soluble protein})]$; where ΔAbs represents the increased absorbance at a specific wavelength, and MEC are the theoretical molar extinction coefficients for the reaction product.

Effect of pH and temperature on proteolytic activity and thermal stability of proteases

The effect of pH and temperature to maximize protease activity in gastric juices from both crustacean species was evaluated. The effect of pH ranging from 2 to 11 on proteases was evaluated using a universal buffer (Stauffer, 1989), which contained similar volume of 25 mM acetic acid, 25 mM phosphoric acid and 25 mM boric acid, and pH adjusted with 25 mM sodium hydroxide; 2% azocasein was used as protein substrate for the assessment at a pH range of 6 to 11. Separately, 0.5% hemoglobin was used as a substrate for acid proteases at a pH range from 2 to 5. Assay conditions were used as described above.

To determine the effect of temperature on protease activity, gastric juices from both species were incubated separately at different reaction temperatures following the standard protocol described above, at 5, 10, 20, 30, 40, 50, 60 and 70°C, pH 9.0. For the thermal stability of enzyme activity on gastric juices, aliquots were previously incubated at different temperatures (5, 15, 30, 45 and 60°C) for 0, 5, 10, 20, 40, 60 and 90 min prior to the enzyme reaction assays. Then, the residual proteolytic activity of gastric juices was measured using 2% azocasein as substrate at 37°C and pH of 9.0, as described above. Residual enzyme activity was calculated as a percentage (%) of peak activity measured at time zero with no incubation.

Effect of inhibitors

The protease classes present in the gastric juices were evaluated by incubating the enzymatic reaction mixtures with different mixtures of specific inhibitors. Different concentrations of protease inhibitors were used according to recommendations of García-Carreño (1992) and Alarcón *et al.* (1998). Thus, 0.25 mM type II-S soybean trypsin inhibitor (SBTI) was dissolved in distilled water and 0.1 M phenylmethylsulfonyl fluoride (PMSF) was dissolved in ethanol both were used separately to inhibit serine proteases. To inhibit chymotrypsin activity, 5 mM N α -p-tosyl-L-phenylalanine chloromethyl ketone (TPCK) dissolved in ethanol was used. To inhibit metalloprotease activity, 0.5 M ethylenediaminetetraacetic acid (EDTA) disodium salt dissolved in distilled water was used. Finally, to inhibit aspartic protease activity, 29 mM Pepstatin A dissolved in dimethyl sulfoxide (DMSO) was used. Briefly, 20 μ L of gastric juice was mixed with 0.5 mL 50 mM Tris-HCl, 10 mM CaCl₂ buffer pH 9 and 10 μ L of a specific inhibitor, and incubated for 60 min at 37°C. Then, the mixture was assayed for alkaline protease (for SBTI, PMSF, TPCK, and EDTA) or aspartic protease (for Pepstatin A) activities using 2% azocasein or 2% hemoglobin, respectively, as described above. The percent relative inhibition or residual activity was reported as the loss of enzyme activity compared to a control with no inhibitor (as 100%). All assays were run in triplicate samples.

Statistical analysis

The results of all assays are reported as the mean $\pm$ standard deviation (SD). The enzyme activity for specific proteases and the effect of specific inhibitors were analyzed between species using a Student's *t*-test. The effect of pH and temperature on the alkaline proteolytic activity for both crustacean species was analyzed separately using a one-way ANOVA. Percent data from inhibition assays were previously transformed to arcsin of square-root from variable to comply with the assumptions of normality and homos-

cedasticity (Zar, 1996). When significant differences were found between mean values, a Tukey's multiple comparison test was performed. Differences were considered significant at $P < 0.05$. All statistical analyses were performed using the graphical and statistical program SigmaPlot, Version 11.0 (Erkrath, Germany).

RESULTS

pH, soluble protein, and specific proteolytic activities

Non-significant differences ($P = 0.057$) were detected in the slightly acid pH values measured in gastric juices of *L. santolla* (6.0 ± 0.06) and *C. edwardsii* (5.9 ± 0.06). The amount of soluble protein measured in the gastric juices (32.2 ± 2.4 and 31.0 ± 5.9 mg mL⁻¹ for *L. santolla* and *C. edwardsii*, respectively) showed no statistical differences ($P = 0.731$) between species.

Alkaline and acid protease activity was significantly lower ($P = 0.025$ and $P < 0.001$, respectively) in *L. santolla* relative to *C. edwardsii*. Trypsin activity showed non-significant differences ($P = 0.315$) between species. However, collagenase activity in *L. santolla* was significantly lower ($P = 0.03$) than in *C. edwardsii*. Conversely, chymotrypsin and LAP activities were significantly higher ($P = 0.007$ and $P = 0.002$, respectively) in *L. santolla* than in *C. edwardsii* (Table 1).

pH and temperature effect on total proteolytic activity and thermal stability of proteases

The effect of pH on the proteolytic activity of gastric juices of *L. santolla* and *C. edwardsii*, is shown in Figure 1. The gastric juices of *L. santolla* (Fig. 1a) showed a high variability of proteolytic activity ($34\text{--}44$ U mg protein⁻¹) at a pH range of 6 to 11, with non-significant differences ($P = 0.135$) between values. However, a significant reduction ($P < 0.001$) of proteolytic activity (from 3.8 to 0 U mg protein⁻¹) was observed concomitant to the drop of pH in the reaction mixture, from 5 to 2 (Fig. 1a).

Table 1. Protease activity and soluble protein in the gastric juice of adults of southern king crab *L. santolla* and Chilean rocky crab *C. edwardsii*. Values (mean $\pm$ SD) in the same row with different superscript are significantly different ($P < 0.05$).

Proteases	<i>L. santolla</i>	<i>C. edwardsii</i>
Alkaline proteases (U mg protein ⁻¹)	15.1 $\pm$ 0.33 ^b	20.7 $\pm$ 2.77 ^a
Acid proteases (U mg protein ⁻¹)	25.9 $\pm$ 0.91 ^b	35.9 $\pm$ 0.79 ^a
Trypsin (mU mg protein ⁻¹)	0.45 $\pm$ 0.06	0.52 $\pm$ 0.09
Chymotrypsin (mU mg protein ⁻¹)	0.007 $\pm$ 0.001 ^a	0.003 $\pm$ 0.001 ^b
Collagenase (U mg protein ⁻¹)	0.045 $\pm$ 0.002 ^b	0.052 $\pm$ 0.01 ^a
Leucine-aminopeptidase, (mU mg protein ⁻¹)	0.07 $\pm$ 0.01 ^a	0.02 $\pm$ 0.01 ^b
Soluble protein (mg protein mL ⁻¹)	32.2 $\pm$ 2.4	31.0 $\pm$ 5.9

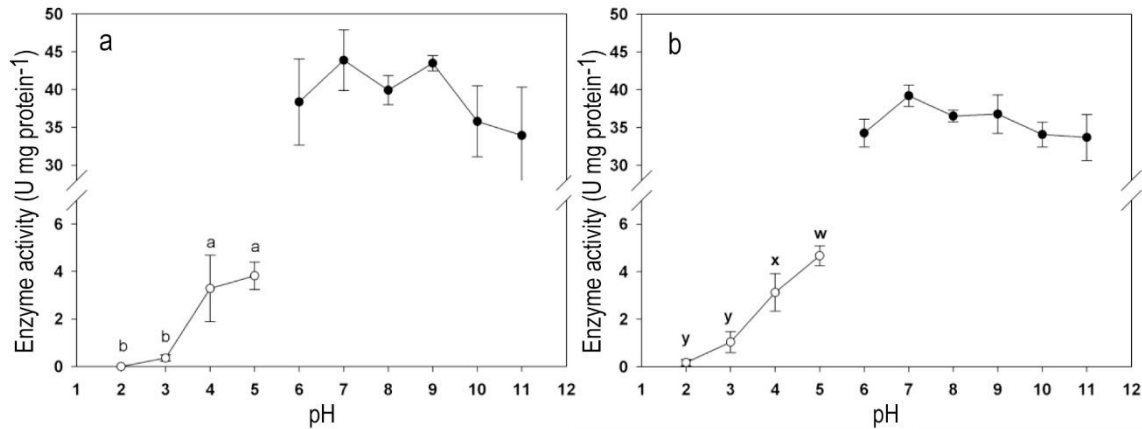


Figure 1. Effect of pH on specific enzyme activity from the gastric juices of adults of a) southern king crab *L. santolla*, and b) Chilean rocky crab *C. edwardsii*. The substrates used were hemoglobin (pH 2-5) and casein (pH 6-11). Values (mean $\pm$ SD) with different letter are significantly different ($P < 0.05$).

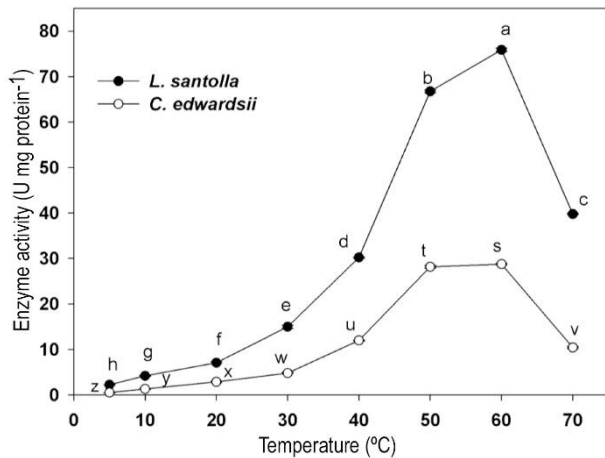


Figure 2. Effect of temperature on specific enzyme activity in the gastric juices of adults of southern king crab *L. santolla* and Chilean rocky crab *C. edwardsii*. Values (mean $\pm$ SD) with different letters are significantly different ($P < 0.05$).

The gastric juices of *C. edwardsii* showed a range of 34–39 U mg protein⁻¹ on proteolytic activity at pH 6 to 11, with non-significant differences between values (Fig. 1b). Similar to *L. santolla*, a significant reduction of proteolytic activity of the gastric juices of *C. edwardsii* was observed ($P < 0.001$ for both species) at an acid pH (pH 2 to 5), which was almost nil at pH 2.

The effect of temperature on the total proteolytic activity of gastric juices of *L. santolla* and *C. edwardsii* at pH 9 is shown in Figure 2. A similar trend was observed in both crustacean species for specific enzyme activity at different reaction temperatures, where proteolytic activity increased significantly from 5 to 60°C. Thus, for both species, the maximum activity was observed at 60°C; however, *L. santolla* showed a higher

proteolytic activity (75.9 ± 0.4 U mg protein⁻¹) compared to *C. edwardsii* (28.8 ± 0.14 U mg protein⁻¹). On the other hand, proteolytic activity dropped abruptly to nearly 15% of the peak activity in both species when the enzymatic reaction was performed at 70°C.

The thermal stability of the total alkaline proteolytic activity in *L. santolla* (Fig. 3a) was maintained above 90% when gastric juices were incubated at 30°C for 10 min, with an abrupt subsequent drop of the proteolytic activity; while the total activity was above 80% when the gastric juice was incubated at 5°C for 90 min. For *L. santolla*, a residual proteolytic activity between 50 and 60% was observed after incubation at 30°C and 45°C for 90 min (Fig. 3a).

Moreover, in the case of *C. edwardsii*, a proteolytic activity >90% was maintained (Fig. 3b) when gastric juices were incubated at temperatures as high as 45°C for 20 min. Also, the gastric juices of *C. edwardsii* showed a high proteolytic activity (residual activity >90%) when the gastric juice was incubated at 15°C for 90 min, and the activity was >80% when gastric juices were incubated at 45°C for 60 min. However, a marked loss of activity (residual activity <20%) was observed in both *L. santolla* and *C. edwardsii* (Figs. 3a–3b) after 10 min when gastric juices were incubated at 60°C.

Effect of specific inhibitors on total protease activity

Different specific inhibitors were employed in order to identify the contribution of individual protease classes to the total proteolytic activity of the gastric juice of both crustacean species (Table 2). The total proteolytic activity (at pH 9) of gastric juices of *L. santolla* and *C. edwardsii* was highly and similarly inhibited by SBTI (64.6% and 59.8% inhibition, respectively; $P = 0.27$)

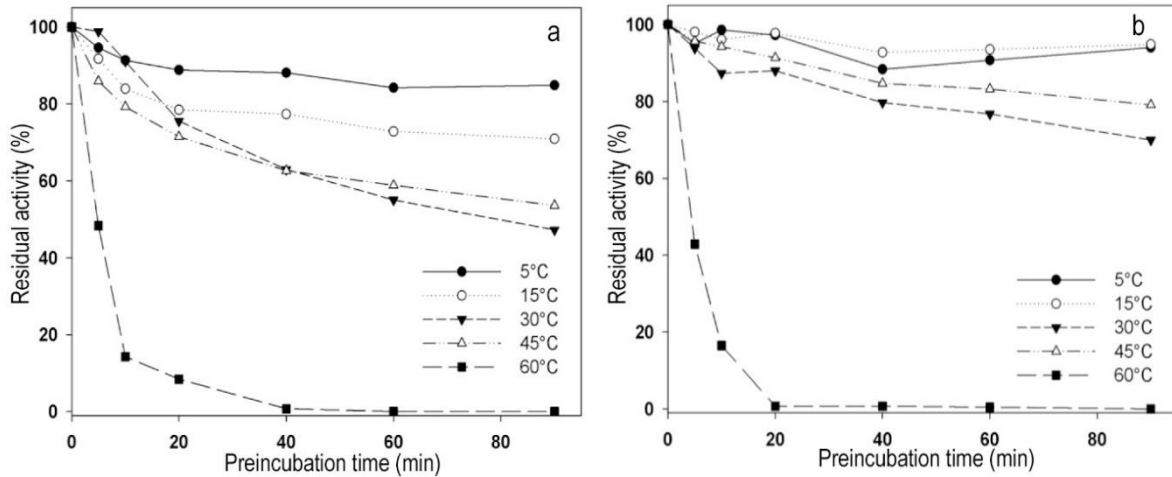


Figure 3. a) Thermostability of total alkaline protease activity from the gastric juice of adults of southern king crab *L. santolla*, and b) Chilean rocky crab *C. edwardsii*. Activity was normalized to 100% for maximum activity recorded.

Table 2. Effect of specific inhibitors on total activity of alkaline protease and aspartic protease contained in the gastric juice of adults of southern king crab *L. santolla* and Chilean rocky crab *C. edwardsii*. Values (mean $\pm$ SD) in the same row with different superscripts are significantly different ($P < 0.05$).

Inhibitor	Stock concentration (mM)	Concentration in reaction mixture (mM)	Percentage of inhibition (%)	
			<i>L. santolla</i>	<i>C. edwardsii</i>
Total alkaline protease				
SBTI	0.25	0.002	64.6 ± 2.5	59.8 ± 4.2
PMSF	100	0.962	54.7 ± 5.5	61.3 ± 6.9
TPCK	5	0.048	21.1 ± 0.8 ^a	13.1 ± 3.7 ^b
EDTA	500	4.850	8.9 ± 2.9 ^a	2.5 ± 2.0 ^b
Total acid protease				
Pepstatin A	29	0.280	31.0 ± 2.2	35.5 ± 3.0

and PMSF (54.7% and 61.3% inhibition, respectively; $P = 0.13$). On the other hand, TPCK, a specific chymotrypsin inhibitor, reduced proteolytic activity to a significantly higher ($P = 0.02$) extent in the southern king crab ($21.1 \pm 0.8\%$ inhibition) versus the Chilean rocky crab ($13.1 \pm 3.7\%$ inhibition). EDTA, a metalloprotease inactivator, achieved a significantly higher ($P = 0.003$) reduction of the proteolytic activity in *L. santolla* (8.9% inhibition) versus *C. edwardsii* (2.5% inhibition). The activity of total acid proteases (at pH 3) contained in the gastric juices of *L. santolla* and *C. edwardsii* was similarly inhibited (31% and 35.5% inhibition, respectively; $P > 0.05$) by Pepstatin A. SBTI, soybean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; TPCK, N-p-tosyl-L-phenylalanine chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid. Values (mean $\pm$ SD) within the same row with different superscript are significantly different ($P < 0.05$).

DISCUSSION

The use of gastric juices for the biochemical analysis of digestive enzymes is a technique commonly used for medium- and large-sized crustaceans such as crabs (Saborowski *et al.*, 2004, 2006; Díaz-Tenorio *et al.*, 2006; Navarrete del Toro *et al.*, 2006), lobsters (Celis-Guerrero *et al.*, 2004; Bibo-Verdugo *et al.*, 2016), and crayfish (Coccia *et al.*, 2011). Compared to the use of tissue homogenates, the utilization of gastric juice entails some advantages, namely: 1) to reduce the presence of soluble non-enzymatic tissue protein on enzyme sources, leads to a more accurate estimation from the specific enzyme activity (U mg of protein⁻¹), 2) prevent the death of the study specimen, hence allowing to perform sequential studies using the same specimen under various experimental conditions (*e.g.*, temperature, food types, molt stages, etc.).

Crustacean gastric juices are a cocktail of protease zymogens synthesized by F-cells in the digestive gland (Ceccaldi, 1989), which are then secreted into the lumen of tubules along with other digestive enzymes (e.g., esterases, lipases, amylases, glycosidases, chitinases, and other) into the gut cavity. Thus, in the present work, we used gastric juices to determine the trypsin-, chymotrypsin-, collagenase- and leucine-aminopeptidase activities, in addition to the total acid and alkaline proteolytic activity, in the southern king crab and the Chilean rock crab using alive and fasted specimens.

A study on the partial biochemical characterization of digestive enzymes in *L. santolla* during different ontogenic stages using semi-quantitative and quantitative techniques has been previously reported by Saborowski *et al.* (2006). However, the present study supplements the enzymatic biochemical characterization by evaluating the effect of different physical (temperature) and chemical (pH) factors, as well as by determining the activity of other protease classes and the effect of specific enzyme inhibitors on the digestive proteolytic activity in *L. santolla* and *C. edwardsii*. Moreover, in the case of the *C. edwardsii*, this is the first report related to the biochemical characterization of its gastric proteases.

Trypsin is a ubiquitous protease in decapod crustaceans (Muhlia-Almazán *et al.*, 2008). This study found similar trypsin activities in both species. In *L. santolla*, high trypsin activity levels have been reported in the gastric juice of adult crabs, but not for eggs and larval stages (Saborowski *et al.*, 2006). In this research, chymotrypsin activity was detected in both species, with lower levels in *C. edwardsii* relative to *L. santolla*. In other studies with crustaceans, nil chymotrypsin activity has been reported. For instance, lithodid species such as *Lithodes aequispinus*, *Paralithodes camtschaticus*, *P. brevipes*, *P. neolithodes* and *Nipponensis multispina*, chymotrypsin activity was not detected using succinyl-*p*-nitroanilide as substrate (Galgani & Nagayama, 1987). In *Cancer pagurus*, a high chymotrypsin activity was detected on gastric juice, produced by a single isoform (Saborowski *et al.*, 2004). In another study, Saborowski *et al.*, (2006) detected chymotrypsin-type activity in eggs, larvae, juveniles and adults of *L. santolla* using N-succinyl-ala-ala-pro-phe *p*-nitroanilide as a substrate, but not when used N-glutaryl-phenylalanine-2-naphthylamide. Therefore, the absence of activity of a digestive enzyme in an analysis, rather than the lack of production by the organism, could be due to both, the incompatibility with the utilized substrate, and to the particular digestive enzyme analyzed (Saborowski *et al.*, 2006; Pereda *et al.*, 2009); another factor influencing the low

activity of a specific class of proteases, could be the lack of biochemical and/or physical conditions suitable for the enzymatic kinetics.

Collagenolytic activity was observed in both crustacean species studied, being significantly higher in *C. edwardsii* than in *L. santolla*. Collagenases are enzymes belonging to the serine-protease group, and this kind of activity has been reported in predator and scavenger crustaceans, including lithodid crabs (Galgani & Nagayama, 1987) that preying on organisms in which collagen is the main connective tissue component (Díaz-Tenorio *et al.*, 2006). Both *C. edwardsii* and *L. santolla* are opportunistic omnivorous predators. In this regard, *C. edwardsii* is carnivorous to a greater extent (Tapia *et al.*, 2008) relative to *L. santolla*, as coralline algae, echinoderms, mollusks, and other invertebrates have been found in the gut content of the latter (Vinueza *et al.*, 2013). This characteristic could be related to the higher collagenolytic, alkaline and acidic proteolytic activity observed in *C. edwardsii* relative to the proteolytic activity detected in *L. santolla*. On the other hand, collagenases of cold-water invertebrates, such as lithodid crabs (Klimova & Chebotarev, 2000; Semenova *et al.*, 2008), have been widely studied due to their biotechnological importance for food, cosmetics, and pharmaceutical industries, among others.

In gastric juices and midgut-gland extracts of the red claw crayfish *Cherax quadricarinatus*, Figuereido *et al.* (2001) detected leucine aminopeptidase activity, as the pH (7.0) was optimal for this enzyme. In *L. santolla*, Saborowski *et al.* (2004) recorded alanine-aminopeptidase activity from the egg to the adult stages. Similarly, aminopeptidase activity has been reported for various ontogenic stages of crustaceans such as *Pleuroncodes planipes* and *Pacifastacus astacus* (García-Carreño *et al.*, 1994), *Munida* spp. (Rossano *et al.*, 2011), *Farfantepenaeus subtilis* (Buarque *et al.*, 2010), and *Maja brachydactyla* (Andrés *et al.*, 2010). The presence of leucine aminopeptidase activity in both crustacean species evaluated in this research indicates that these organisms are adapted to digest peptides either in the diet or derived from protein digestion, because while trypsin and chymotrypsin (proteinases) cleave the inner peptide bonds, aminopeptidases (peptidases) complete protein digestion by making it more efficient (Figuereido *et al.*, 2001).

The pH values measured in the gastric juices of *L. santolla* (6.0) and *C. edwardsii* (5.9) are similar to the slightly acid values reported for other crustacean species such as *C. pagurus*, 5.8 (Saborowski *et al.*, 2004), *Cherax quadricarinatus*, 5.8 (Figuereido *et al.*, 2001), *Callinectes arcuatus*, 6.1; *Panulirus interruptus*,

6.0 (Navarrete del Toro *et al.*, 2006) and penaeid shrimps, 5.7 (Navarrete del Toro *et al.*, 2011).

For the species studied in this work, proteolytic activity was observed in both, alkaline and acid pH. In addition, no significant differences were observed in the total proteolytic activity evaluated over a wide pH range (6 to 11). Highest total proteolytic activities at a pH range of 5 to 8 have been reported for other crustacean species (Galgani & Nagayama 1987; Figuereido *et al.*, 2001; Díaz-Tenorio *et al.*, 2006; Saborowski *et al.*, 2006; Figueiredo & Anderson, 2009), as was also reported for *C. pagurus* (Saborowski *et al.*, 2004). In other lithodid crab species, the optimal pH for proteolytic activity lied within a narrow pH range of 7 to 7.8 (Galgani & Nagayama, 1987). Nevertheless, having proteolytic activity in a wide pH range gives an organism a greater catalytic potential to digest more efficiently food nutrients under different physiological conditions (pH) in the gastric chamber. As observed, in the present study we have not found a match between pH of gastric juices and pH-profile of proteolytic activity, which is in agreement with previous reports (Figueiredo *et al.*, 2001; Celis-Guerrero *et al.*, 2004; Saborowski *et al.*, 2004). Moreover, different pH values were used to measure trypsin, chymotrypsin, LAP and collagenase activities which also does not correspond to pH measured in gastric juices for both species, but were chosen according to reports of standard methods (García-Carreño *et al.*, 1994); however, we suggest to realize further studies to evaluate the effect of pH as well as pH-stability of different specific proteases present in gastric juices of *C. edwardsii* and *L. santolla*.

For both crustacean species studied, the highest proteolytic activity was observed at 60°C. Optimal temperatures reported for other lithodid and brachyuran crabs range from 45 to 55°C (Galgani & Nagayama, 1987; Saborowski *et al.*, 2004). However, these "optimal" temperatures observed *in vitro* are far above of the maximum temperatures tolerated by *L. santolla* and *C. edwardsii*. According to Calcagno *et al.* (2005), *L. santolla* juveniles are well adapted to subarctic marine environments (5-10°C), but high mortality rates have been observed at 12 to 15°C, despite the fact that these temperatures lead to accelerated growth and maturation rates.

Results of the thermal stability of proteases present in the gastric juices reveal a lower thermal stability for *L. santolla* relative to *C. edwardsii*. For *L. santolla*, 80% and 50% of the total proteolytic activity remained after incubating the gastric juices at 15 and 30°C, respectively, prior to the enzymatic reaction. In contrast, for *C. edwardsii* over 70 and 90% of the total proteolytic activity was recorded at the same incubation

conditions. The proteolytic activity of *C. edwardsii* disappeared after incubating the gastric juices at 60°C for 20 min. Similar results were observed for cold-water crustaceans such as *C. pagurus* (Saborowski *et al.*, 2004), *Homarus americanus* and *Homarus gammarus* (Rojo *et al.*, 2013), where proteases were almost deactivated after incubation at 60°C for 20 min. Proteases from psychrophilic organisms such as *C. edwardsii* and *L. santolla* are thermolabile due to their limited structural stability (Gerday *et al.*, 2000; Rojo *et al.*, 2013), which is related to the weak hydrophobic interactions at the inner protein structure (Oh *et al.*, 2000). In contrast, proteases from the gastric juices of crustaceans that inhabit in tropical waters exhibit high thermostability (García-Carreño & Haard, 1993; Oh *et al.*, 2000). Thus, the proteolytic activity of both studied crustaceans species was deactivated using even mild temperatures (60°C). This feature is relevant for potential technological applications (Rossano *et al.*, 2011), mainly in food processing industry (*e.g.*, manufacture of cheese, roe processing, recovery of pigments in seafood, among others).

In the present study, specific inhibitors were used to characterize the protease classes in the gastric juices analyzed. Serine proteases (mainly trypsin-like and chymotrypsin-like enzymes) result important for the protein digestion process in both studied species. Approximately, 55 and 60% of proteolytic activity from *C. edwardsii* and *L. santolla*, respectively, was inhibited when PMSF was used. García-Carreño & Haard (1993), and Buarque *et al.* (2010), also reported an inhibition of more than 50% on proteolytic activity when PMSF was used in different species of omnivore decapod crustaceans. Likewise, SBTI led to 60% of total proteolytic activity in the digestive juices of *L. santolla* and *C. edwardsii*. Both SBTI and PMSF are serine-protease inhibitors, but SBTI is more specific for trypsin-like enzymes. Therefore, trypsin represents the main serine protease contained in the analyzed gastric juices, which is a feature shared with other decapod species (Celis-Guerrero *et al.*, 2004; Perera *et al.*, 2008; Coccia *et al.*, 2011). Chymotrypsin participated to a lesser extent in protein digestion, since the inhibitor TPCK led to an 18% reduction of total proteolytic activity in both species. However, the inhibition of proteases using TPCK has been inefficient in other crustacean species (García-Carreño & Haard, 1993; García-Carreño *et al.*, 1994; Fernández-Gimenez *et al.*, 2002; Perera *et al.*, 2008; Buarque *et al.*, 2010), suggesting that the use of other specific chymotrypsin inhibitors (*e.g.*, chymostatin) should be further evaluated to corroborate the findings obtained in this research. Also, the sum of inhibition caused by SBTI and TPCK not necessarily are similar with those values

found for PMSF inhibition, since TPCK also could to inhibit some cysteine proteases (Oppert *et al.*, 2003) contained in gastric juices. With regard to the inhibition of metalloproteases, such as leucine aminopeptidase, EDTA led to a 2.5% and 8.9% inhibition of proteolytic activity in the gastric juice of *C. edwardsii* and *L. santolla*, respectively. Values of metalloproteases contributing to total alkaline proteolytic activity for both species in the present study are low when compared to those reported for other crustacean species such as *P. planipes* and *P. astacus* (García-Carreño & Haard, 1993), for which inhibitions between 18% and 32% of the total proteolytic activity have been reported. However, to date and compared with other proteases, studies on crustaceans where inhibition of metalloproteases has been reported are scarce.

In crustaceans, not all the digestive proteolytic activity is performed only by serine proteases alone. Activity of aspartic and cysteine proteases has been reported in gastric juices and extracts of the midgut gland of decapods, which act in an acid-to-neutral pH range and play an important role in the digestive process of protein (Hu & Leung, 2007; Andrés *et al.*, 2010; Rojo *et al.*, 2010, 2013). In species such as *H. americanus* (Laycock *et al.*, 1989), *Crangon crangon* and *Crangon allmani* (Teschke & Saborowski, 2005), cysteine proteases such as cathepsins can even account for more than 60% of the total proteolytic activity. Also, aspartic proteases have been detected in gastric juices of *P. interruptus*, *C. pagurus*, *C. arcuatus* and *Callinectes bellicosus* (Navarrete del Toro *et al.*, 2006), *H. gammarus* and *H. americanus* (Rojo *et al.*, 2013), as well as in enzyme extracts of *Munida* (D'Ambrosio *et al.*, 2003). In the present study, acid proteases activity was measured at pH 3.0 to ensure that such activity mainly comes from aspartic proteases. In some crustacean species, serine proteases still remain actives at a pH close to 5 (Saborowski *et al.*, 2004; Bibo-Verdugo *et al.*, 2015). For both crab species evaluated in this study, Pepstatin A (a specific inhibitor of aspartic proteases) inhibited around 30% of the acid proteolytic activity (pH 3) in gastric juices. The contribution of aspartic proteases to acid proteolytic activity is variable among crustaceans, since for *P. planipes* and *P. astacus* these contribute with 9% and 15% at pH 4.5, respectively (García-Carreño *et al.*, 1993); in contrast, in *H. gammarus* and *P. interruptus* these proteases account for more than 70% of the activity at pH 3 (Navarrete del Toro *et al.*, 2006).

Data on the activity of total alkaline and acid proteases from the gastric juice of *C. edwardsii* and *L. santolla* confirm the importance of these enzymes in the digestive process of the organisms studied. Our results regarding inhibition studies revealed that the

proteolytic activity of the gastric juice of *C. edwardsii* and *L. santolla* is largely related to the presence of serine and aspartic proteases. Peak protease activities in both organisms studied were recorded at pH 6. Moreover, the thermal stability of gastric proteases of *C. edwardsii* was higher than the one exhibited by proteases of *L. santolla*. However, proteases of gastric juices from *C. edwardsii* and *L. santolla* result in thermolabile respect to proteases from mesophilic animals (Rojo *et al.*, 2013). This behavior points to the potential application of gastric proteases as a biotechnological aid in processes that require mild temperatures to deactivate proteolytic activity. In order to explore specific biotechnological/aquaculture applications of proteases from *C. edwardsii* and *L. santolla* gastric juices, further studies about the isolation and individual characterization of collagenases, serine, and aspartic proteases are highly recommended.

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