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Characterization and comparison of digestive proteinases of the Cortez swimming crab, *Callinectes bellicosus*, and the arched swimming crab, *Callinectes arcuatus*

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Abstract. Protease activity in the midgut gland, gastric chamber, and gastric juice from the crabs *Callinectes bellicosus* and *Callinectes arcuatus* was characterized by several methods, confirming that the composition of digestive proteases is the same in the gastric juice and the midgut gland. Gastric juice was suitable for the identification and characterization of the proteinases trypsin and chymotrypsin. Such enzymes were presented as isotrypsins and isochymotrypsins. Proteinase composition evaluated by SDS-PAGE and substrate-SDS-PAGE showed differences between species, but not between gender. Proteinases were thermostable at 40°–50°C for 1 h and showed maximum activity at pH 6–8, making the use of digestive proteinases for evaluations of protein digestibility by the pHstat method possible. We propose using gastric juice as a source of digestive enzymes for *in vitro* studies of enzymes in digestibility assays and characterization procedures.

Additional key words: proteases, pHstat, trypsin, chymotrypsin, Portunidae

Studies of decapods are often motivated by two interests, as members of diverse marine environments and as food for humans. Recent focus has been on farming for food production (Provenzano 1985). Whatever the concern, understanding digestion capabilities is paramount for a broad knowledge of crustacean physiology regarding handling, digestion, and assimilation of food (García-Carreño et al. 1997). Studies on the subject have focused on two aspects: (1) understanding how digestion is accomplished including the organs and molecules involved (Brockerhoff et al. 1970; Bliss 1982; Dall & Moriarty 1983; McGaw & Reiber 2000; Fernández Gimenez et al. 2001, 2002) and (2) evaluating bioavailability of food proteins, digestibility, and assimilation of released peptides as these relate to nutrition (García-Carreño 1992; Ezquerra et al. 1997; García-Carreño et al. 1997).

To obtain useful results, researchers have developed *in vitro* and *in vivo* assays. The first is less expensive, faster, and simpler, as organisms remain in the laboratory for shorter time periods, tests are evaluated instrumentally, and only digestive enzymes are used (Tonglet et al. 2001). However, in other experiments, animals are killed to dissect digestive organs and these use a great number of specimens as replicas. This kind of experiment requires model species that can be sampled for the duration of the experiment, avoiding variation among individuals, and costs related to the number of organisms.

Crabs around the world have received attention in ecological, physiological, and biochemical studies; however, few studies on their digestive mechanisms have been published. Of particular interest are digestive enzymes, mostly proteinases (endoproteases) because they are implicated in rapid autolysis of muscle protein after the crab's death. This affects its suitability for human consumption (Ezquerra et al. 1997).

For its possible uses in ecology, biotechnology, and food technology, digestive enzymes in decapods became important (Díaz-López & García-Carreño 2000; Gilberg et al. 2000). Gibson & Barker (1979) studied several dozen members of the taxa. Recent studies in crab digestion include those of McGaw & Reiber (2000) of physiological responses to feeding of the Atlantic blue crab *Callinectes sapidus* Rathbun 1896. This was important for its description of the route and residence time of food in the digestive system, and its relationship to oxygen uptake and cardiovascular characteristics. Reports of the digestive proteinases in crabs include the deep-sea crab *Chaceon affinis* A. Milne-Edwards & Bouvier 1894, beni-zuwai crab *Chionoecetes japonicus* Rathbun 1932 (Galgani & Nagayama 1988), and

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C. sapidus (Dendinger 1987). These studies were carried out in the midgut gland and revealed the presence of serine proteinases, such as trypsin and chymotripsin.

Callinectes bellicosus Stimpson 1859 and *Callinectes arcuatus* Ordway 1863 are species of crabs found along the Pacific coast of Mexico in tropical and temperate waters with muddy or sandy bottoms. Both species are the main fisheries crabs in Mexico (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación 2002). In the Gulf of California, *C. bellicosus* is more abundant than *C. arcuatus*. Both species are benthic predators feeding on molluscs, small fish, and other crabs. They are a significant link in the food web (Brusca 1980) and an important part of the natural marine ecology and fishing economy of the northwestern coast of Mexico. Here, we experimentally evaluated whether proteinases in the gastric juice are the same as those found in the midgut gland of *C. bellicosus* and *C. arcuatus*. Further, we offer evidence that gastric juice can be used for *in vitro* digestibility studies instead of midgut gland extracts, thereby reducing the number of specimens sacrificed in these types of studies. Proteinases from gastric juice and digestive organs were characterized and compared between both species and evaluated for digestive ability for a standard protein, casein, by using the pHstat method. Our intent is to generate an alternative measure of protein digestibility and avoid traditional *in vivo* methods that are tedious, destructive, and expensive.

Methods

Organisms

Twenty specimens of each species were collected in the lagoon Ensenada de La Paz (24.2°N, 110.4°W) located on the south shore of the sand bar El Mogote that separates the lagoon from Bahía de La Paz. *Callinectes bellicosus* was represented by seven females and 13 males. *Callinectes arcuatus* was represented by six females and 14 males. Specimens were kept in aquaria for 15 d to acclimate. Salinity was 35 ppt at 27°–28°C. Seawater was exchanged manually at a rate of 80% daily. Two animals of the same species, separated by a metal screen (mesh 1.27 cm), were kept in each 70 L plastic aquarium and fed daily with freshly cut squid (*Dosidicus gigas* D'Orbigny 1835).

Sampling

Gastric juice was extracted with a 1.6 mm plastic tube attached to a 5 mL syringe. The volume of gastric juice at each sampling varied from 100 to 1200 μL. Gastric juice was centrifuged to eliminate food debris (10,000 *g* at 4°C for 30 min) and the supernatant was freeze dried. Gastric juice powder was maintained at −20°C. After the last sampling, organisms were killed by chilling in a freezer. The gastric chamber and midgut gland were extracted by dissection and they were weighed and stored individually at −20°C until analysis.

Enzymatic extracts from dissected organs

Enzymatic extracts were prepared from each specimen's midgut gland or hepatopancreas and digestive chamber or stomach, as described by García-Carreño (1992). Tissue was homogenized in distilled water (1:2 weight/volume) with a mechanical tissue grinder homogenizer (Vir Tis, SP Industries, Inc., Gardnier, NY) for midgut glands and a pressure tissue grinder for digestive chambers. Samples were centrifuged as indicated above to eliminate tissue debris and lipids. The supernatant was kept at 4°C and analyzed for protein concentration and proteolytic activity. Protein concentration was evaluated by the method of Bradford (1976) using bovine serum albumin (fraction V) as the standard.

Enzyme activity

From each gastric juice or enzymatic extract, total proteolytic activity was evaluated in triplicate following the technique described by García-Carreño (1992) using azocasein as the substrate at 25°C. Ten microliters of enzymatic extracts or reconstituted gastric juice (5 mg gastric juice powder in 100 μL distilled water) and 500 μL buffer 1 (20 mmol L^{-1} Tris-HCl, pH 8) were mixed. The reaction was started by the addition of 500 μL of 1% azocasein in buffer 1. After 10 min, 500 μL of 20% trichloroacetic acid (TCA) was added to stop the reaction. Samples were maintained at 0°C for 10 min and then centrifuged at 10,000 *g* for 5 min. Absorbance of the supernatant was recorded at 366 nm in a LamdaBio20 spectrophotometer (Perkin-Elmer Corp., Norwalk, CT), adjusting to zero with buffer 1. For the blank, TCA was added before the substrate was added. The specific activity was calculated by using the following formula: specific activity (U mg^{-1} protein) = Abs 366 nm min^{-1} mg^{-1} protein.

Characterization of class and type of proteinases

Evaluation of the classes of proteinase present in gastric juice was based on the method described by García-Carreño & Haard (1993). Enzymes contained

in gastric juice were incubated with class and type-specific protease inhibitors. *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) was used as a specific inhibitor of trypsin. *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*-carbobenzoxy-L-phenylalanine chloromethyl ketone (ZPCK), and chymostatin were used as specific inhibitors of chymotrypsin. Phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor (SBTI) were used as inhibitors of proteinases belonging to the serine class. Ethylenediaminetetraacetic acid (EDTA) was used as an inactivator of metallo-proteinases. For incubation, 10 mmol L^{-1} TLCK in 1 mmol L^{-1} HCl, pH 3, 5 mmol L^{-1} TPCK in methanol, 5 mmol L^{-1} ZPCK in methanol, 100 mmol L^{-1} chymostatin in dimethyl sulfoxide (DMSO), 100 mmol L^{-1} PMSF in methanol, 250 μM SBTI in distilled water, or 0.4 mmol L^{-1} EDTA in distilled water were separately mixed with the enzyme preparations from gastric juice and incubated at 25°C for 60 min. Distilled water instead of the inhibitor solution was used as controls. Enzyme activity was then evaluated as above. Activity measured in the absence of an inhibitor was considered as 100%. Assays were performed in triplicate.

Trypsin activity was measured with 1 mmol L^{-1} *N*-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA) in 50 mmol L^{-1} Tris-HCl, pH 8, 20 mmol L^{-1} $CaCl_2$. BAPNA was dissolved in 1 mL DMSO and increased to 100 mL with buffer 2 (50 mmol L^{-1} Tris-HCl, pH 8, 20 mmol L^{-1} $CaCl_2$). Three assays of gastric juice were added to 750 μL substrate solution at 32°C (spectrophotometer internal temperature). Changes in absorbance at 410 nm were recorded for 3 min (Erlanger et al. 1961). Chymotrypsin activity was evaluated as above, with 100 μmol L^{-1} succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAPNA) in buffer 2 as substrate. Triplicates of gastric juice samples were mixed with 750 μL of substrate solution. Absorbance at 410 nm was recorded for 3 min (del Mar et al. 1979). Each assay included commercial enzymes (porcine trypsin type IX and bovine chymotrypsin type II) as positive controls. Trypsin and chymotrypsin specific activities were calculated as follows: activity units = (Abs 410 nm min^{-1} × 1000 × reaction mixture in mL)/8800 × mg protein in the enzyme solution, where 8800 is the extinction coefficient of *p*-nitroaniline.

Electrophoresis analysis

Sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) without using a reducing agent or boiling the samples. For protein band analysis, a volume of enzyme preparation containing 20 μg protein was mixed with two volumes of sample buffer and then loaded into the gel (10% acrylamide). For proteinase activity, a volume of enzyme preparation containing 7 mU azocaseinolytic activity was mixed with two volumes of sample treatment buffer and loaded into a twin gel. Electrophoresis was performed in a Hoefer E-260 mini-gel electrophoresis unit (Hoefer Pharmacia Biotech, Inc., San Francisco, CA). Gels were run at 15 mA per gel at 4°C. After electrophoresis, the gel for protein band analysis was fixed and stained with Coomassie solution (0.05% Coomassie brillant blue R-250, 40% methanol, 7% acetic acid) for at least 2 h and then destained using the same solution without dye. The twin gel for proteolytic activity was treated by substrate-SDS-PAGE (s-SDS-PAGE) (García-Carreño et al. 1993). After electrophoresis, the gel was soaked for 30 min in a 3% casein solution with buffer 1 at 5°C to allow the substrate to diffuse into the gel. Then, temperature was raised to 25°C for 90 min. The gel was washed with distilled water, and then immediately fixed and stained, as above. Clear bands over a dark blue background are separated proteins with proteolytic activity.

To characterize the class and type of proteinase, enzyme extracts were incubated with proteinase inhibitors. One volume of inhibitor solution, such as TLCK, TPCK, PMSF, SBTI, and chymostatin, was mixed separately with four volumes of enzyme solution containing 7 mU azocaseinolytic activity, and incubated at 25°C for 60 min. Distilled water was used instead of inhibitor solution in controls. Samples were mixed with sample buffer and loaded into the gels. After electrophoresis, control and inhibition lanes were stained for activity, as described previously. Bands of enzymes with inhibitors were compared with controls to identify the class or type of the enzyme in the band.

Operational variables

Enzyme preparations were assayed for optimum pH and temperature using azocasein as substrate. To generate different pH values with the same salt composition, universal buffer (Stauffer 1989) was used at pH 4–9. For optimum temperature, activity was evaluated at 5°–75°C for a 10 min reaction at pH 7.

Thermostability

To evaluate thermostability, proteases were incubated at 40°–80°C for 0–60 min. Then total protease, trypsin, and chymotrypsin activities were measured

as described previously. Data were expressed in percent of remained activity after treatment.

In vitro protein digestibility assay

Protein digestibility was evaluated by degree of hydrolysis (DH) following the method described by García-Carreño et al. (1997). For this purpose, a pHstat 718 Stat Tritino was interfaced with a PC with Metrohmdata software (Metrohm Ion Analysis, Metrohm, Ltd., Herisan, Switzerland). This apparatus titrates the α-amino groups protonated after peptide bond cleavage by proteolysis. As the control, a four-protease cocktail was used; it contained bovine chymotrypsin, porcine trypsin, aminopeptidase, and pronase. Three units of azocaseinolytic activity were assayed. The substrate was $8\,mg\,mL^{-1}$ casein solution. During the assay, 0.1 N NaOH was used to maintain the pH at 8.

Data were analyzed with Statistica software (StatSoft Inc. 1995) using a one-way analysis of variance (ANOVA) design. A *post hoc* test was performed by a least significant differences (LSD) test ($\alpha = 0.05$) (Sokal & Rohlf 1981).

Results

In *Callinectes bellicosus,* the final carapace length of females and males was 11.97 ± 1.70 cm and 13.50 ± 1.04 cm, respectively. The final carapace length of *C. arcuatus* was 9.80 ± 1.04 cm for females and 10.88 ± 1.00 cm for males. Female crabs were not gravid.

Protein and proteinase composition by SDS-PAGE was determined for individual specimens of each species and gender, allowing comparison among them. Electrophoregraphs of representative specimens show that both species possess several proteins with proteinase activity in the gastric juice and the midgut gland extract (Fig. 1). Figure 1A,C shows the composition of proteins in gastric juice and midgut gland of both species. No differences were seen among individuals of the same species.

The protein composition is species specific, with important differences between species but slight differences between gender. Zymograms (Fig. 1B,D) show that most proteins in the samples are proteinases, except for those weighing more than 66 kDa. Since proteinases presented in the gastric juice perfectly match those found in the midgut gland, we corroborated the idea that enzymes are synthesized in the midgut gland and secreted to the gastric chamber.

Total proteinase, chymotrypsin, and trypsin-specific activity showed differences ($p < 0.05$) between species and gender (Table 1). To evaluate the contribution of individual proteinases to the total activity of gastric juice, each sample was incubated with inhibitors and the residual activity was evaluated with azocasein as substrate. Table 2 shows the percentage of inhibition of this assay. PMSF reduced the proteinase activity in all samples, including porcine trypsin and bovine chymotrypsin. SBTI reduced enzyme activity better than PMSF. The inhibition by both inhibitors confirms the presence of serine proteinases. TLCK reduced the enzyme activity in all samples, including porcine trypsin, confirming the presence of serine proteinases in crab samples. TPCK and ZPCK fail to reduce the enzyme activity in the crab samples; differences under 10% of inhibition are considered as no inhibition, provided it failed by the error of the technique. Chymostatin reduced the enzyme activity in all samples, with reduction significantly higher in females. This confirms the presence of chymotrypsin in the digestive system. EDTA did not reduce the enzyme activity in gastric juice; hence metallo-proteinases are not detected in gastric juice by this technique.

The zymograms of samples incubated with inhibitors before electrophoresis show that several bands of activity were inhibited, indicating the class or type of proteinase comprising such bands. ZPCK and EDTA did not affect any activity band (data not shown). We confirmed that TPCK did not affect any protease band (Fig. 2). In *C. bellicosus,* bands of 20–25 kDa (trypsins) were inhibited by TLCK, SBTI, and PMSF, and two bands of 33 and 37 kDa (chymotrypsin) were inhibited by PMSF and chymostatin (Fig. 2A,B). Two bands of 39 and 44 kDa were only identified as serine proteinases and three bands (47, 49, and 57 kDa) were not identified. In *C. arcuatus,* trypsin was identified as bands weighing 20–25 kDa and chymotrypsin weighing 28 and 31 kDa. Two bands weighing 41 and 43 kDa were identified as serine proteinases and two bands (54 and 63 kDa) were not identified (Fig. 2C,D).

For both species, the optimum pH of total proteases was 7, with no significant differences in the range pH 6–8 between species (Fig. 3). Also in both species, maximum activity for a 10 min reaction time occurred at 50°C (Fig. 4).

The thermostability of total proteases (Fig. 5A,B), trypsin (Fig. 5C,D), and chymotrypsin (Fig. 5E,F) showed that all enzyme activities remained without changes at 40°C. A loss of 20% activity was found after 60 min at 50°C. All activities were completely lost at 70°C and 80°C. At 60°C, total proteases and chymotrypsin activities showed the same pattern of loss of activity, but trypsin was highly affected by increased temperature. This suggests a resistance to

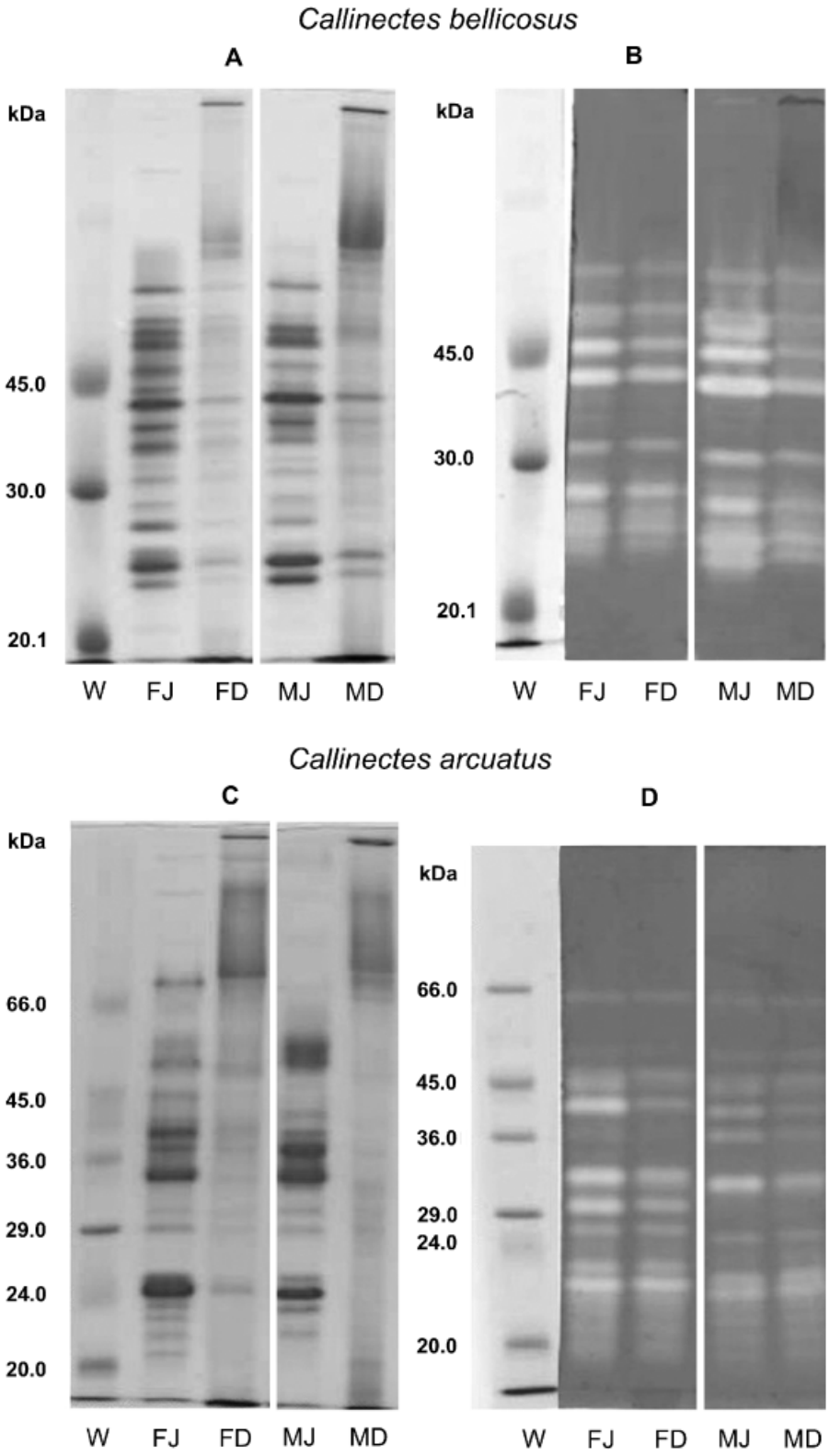

Fig. 1. Sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) (**A**, **C**) for proteins and substrate-SDS-PAGE (s-SDS-PAGE) (**B**, **D**) for proteinases from *Callinectes bellicosus* and *Callinectus arcuatus*. D, midgut gland; F, female; J, gastric juice; M, male; W, molecular weight marker.

Table 1. Total protease, trypsin, and chymotrypsin activities from specimens of *Callinectes* spp. Specific activity expressed in U/mg protein. Different lowercase letters in the same row indicate statistical differences among groups. Ca, *Callinectes arcuatus;* Cb, *Callinectes bellicosus;* F, female; M, male; NA, not analyzed.

	Control	Cb/F	Cb/M	Ca/F	Ca/M
Total protease activity	NA	4.10 ± 0.57^{a}	4.58 ± 0.88^{a}	5.27 ± 1.93^{b}	3.64 ± 1.25^{c}
Tryptic activity	3.27 ± 0.32	0.54 ± 0.01^{d}	0.45 ± 0.07^{e}	0.47 ± 0.03^{f}	0.41 ± 0.04^{g}
Chymotryptic activity	27.70 ± 1.10	2.26 ± 0.06^{h}	2.14 ± 0.16^{i}	2.43 ± 0.21^{j}	1.87 ± 0.20^{k}

high temperature in chymotrypsin, and the main protease in total activity, up to 60°C.

No statistical differences were found in enzyme assays to digest casein in the two species. *Callinectes bellicosus* yielded 13.04% DH and *C. arcuatus* yielded 13.75% DH. Controls, including a cocktail of four pure commercial proteases, yielded 20.46% DH. Crabs and control assays showed significant differences.

Discussion

Enzymes present in the gastric juice are synthesized in the midgut gland (Bliss 1982; Dall & Moriarty 1983; Icely & Nott 1992; McGaw & Reiber 2000) and are secreted anteriorly into the gastric chamber. Enzymes in the gastric juice mix with masticated food to yield chyme. This was confirmed by comparing proteinase patterns from the midgut gland extract and the gastric juice, concluding that both contain the same proteinases. Protein digestion starts in the gastric chamber with midgut gland enzymes.

The methods used in this investigation allowed identification of trypsin and chymotrypsin as the main proteases synthesized in the midgut gland of both species. Results confirm that decapods, including the shrimp *Pleoticus muelleri* Bate 1888 and *Artemesia longinaris* Bate 1888 (Fernández Gimenez et al. 2001, 2002), langostilla *Pleuroncodes planipes* Stimpson 1860, and crayfish *Pacifastacus* spp. Bott 1950 (Garcia-Carreño & Haard 1993), possess the same enzymes. Specimens of *Callinectes bellicosus* express three isotrypsins at 26.9, 24.9, and 23.6 kDa, while five isotrypsins were found in specimens of *Callinectes arcuatus* at 25.8, 24.7, 23.1, 21.6, and 20.2 kDa. In *C. bellicosus,* isochymotrypsins were found at 42.6, 34.9, and 33.2 kDa and in *C. arcuatus* two isochymotrypsins at 30.8 and 28.4 kDa were present. In both species, we found four unknown proteases, two of them are serine proteinases and one probably is a collagenase, according to information from Klimova et al. (1990) and Galgani & Nagayama (1988). This enzyme is important in carnivores and scavengers because their prey contains collagen as the main component of connective tissue.

Optimal temperature and pH should be considered as operational parameters, provided they depend on reaction time. Usually, they do not coincide with values at physiological levels. Enzymes can show greater activity at higher temperatures and pH than at normal physiological conditions, indicating that enzymes at physiological values work at suboptimal conditions. The higher activity may be because their structure is better suited for activity or conditions

Table 2. Effect of proteinase inhibitors on total proteinase activity. Ca, *Callinectes arcuatus;* Cb, *Callinectes bellicosus;* Chy, chymotrypsin; EDTA, ethylenediaminetetraacetic acid; F, female; M, male; NA, not analyzed; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Try, trypsin; ZPCK, *N*-carbobenzoxy-L-phenylalanine chloromethyl ketone.

	Percentage inhibition					
	Cb/F	Cb/M	Ca/F	Cb/M	Try	Chy
Control	0.00	0.00	0.00	0.00	0.00	0.00
TLCK	20.26	17.37	29.78	24.13	88.69	0.00
TPCK	7.04	6.03	7.12	2.73	9.96	92.91
ZPCK	3.46	0.14	6.30	5.16	NA	NA
Chymostatin	12.62	10.27	19.23	14.75	0.00	93.91
PMSF	24.20	33.24	41.40	36.94	93.59	93.65
SBTI	44.98	43.60	56.68	46.84	97.32	43.45
EDTA	2.94	5.83	1.91	8.63	0.00	0.00

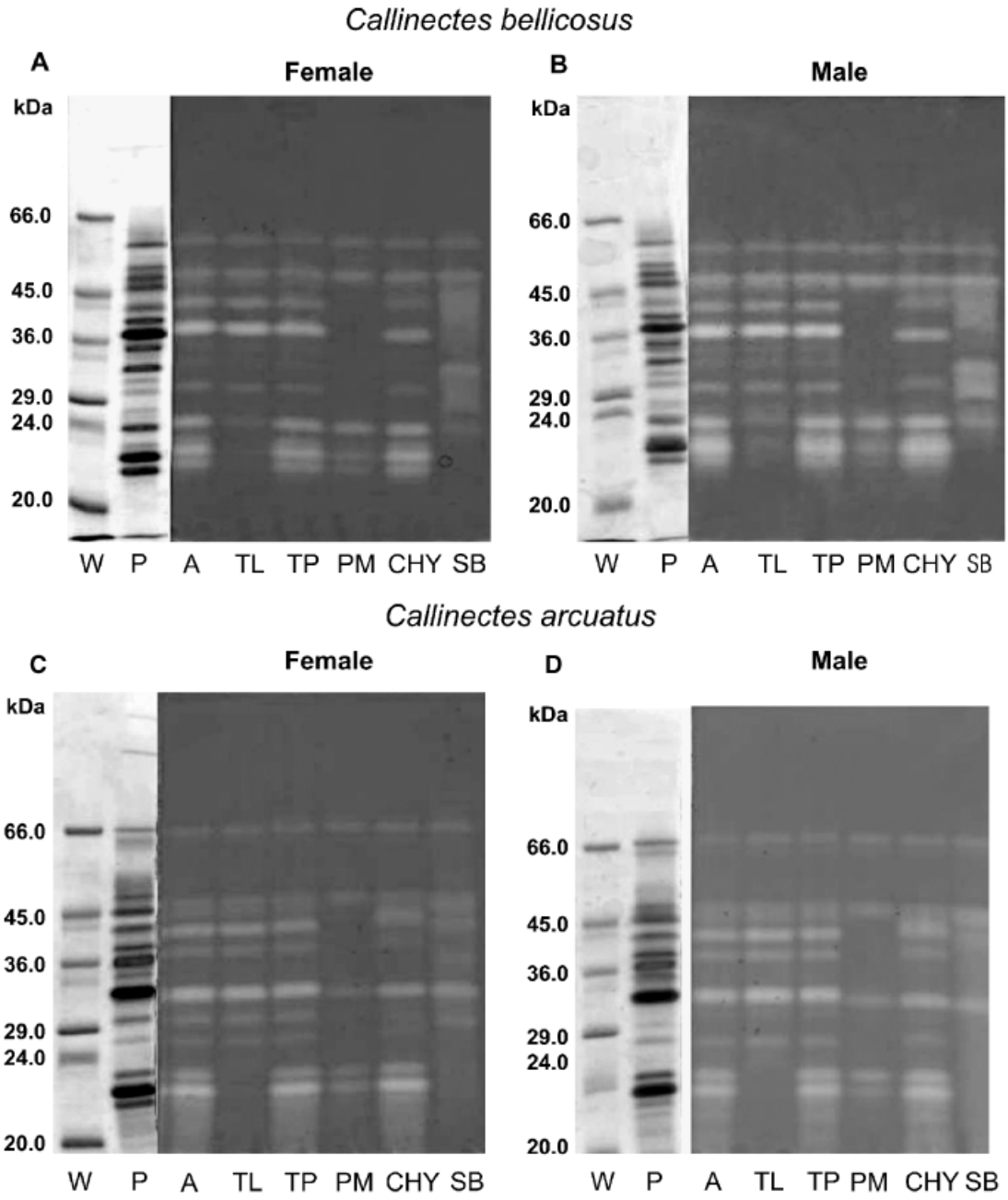

Fig. 2. Effect of proteinase inhibitors. Substrate-SDS-PAGE (s-SDS-PAGE) of *Callinectes bellicosus* (**A**, **B**) and *Callinectus arcuatus* (**C**, **D**) gastric juice. A, proteinase activity (control); CHY, chymostatin; P, proteins; PM, PMSF; SB, SBTI; TL, TLCK; TP, TPCK; W, molecular weight marker.

that promote a better enzyme–substrate interaction (Whitaker 1994). This is an advantage when considering these enzymes as reagents for biotechnologies. For example, digestive proteinases from lepidopterans and coleopterans show higher activity at pH 10–11 and 6–7 (Purcell et al. 1992; Ortego et al. 1996). American lobster (*Homarus americanus* H. Milne-Edwards 1837) and Japanese spiny lobster (*Panulirus japonicus* Von Sieblod 1824) have an optimum pH range in their midgut gland proteases at 7–8 (Brockerhoff et al. 1970; Galgani & Nagayama 1987). Reports about gastric juice proteases from *Panulirus interruptus* Randall 1840 were found (Celis-Guerrero et al. 2004), where optimum pH was at 9 for alkaline proteases and at 3 for acid proteases. Digestive proteases in crabs, such as the deep-sea crab (*Chaceon affinis*) and beni-zuwai crab (*Chionoecetes japonicus*), show an optimum pH at 6–7 (Galgani &

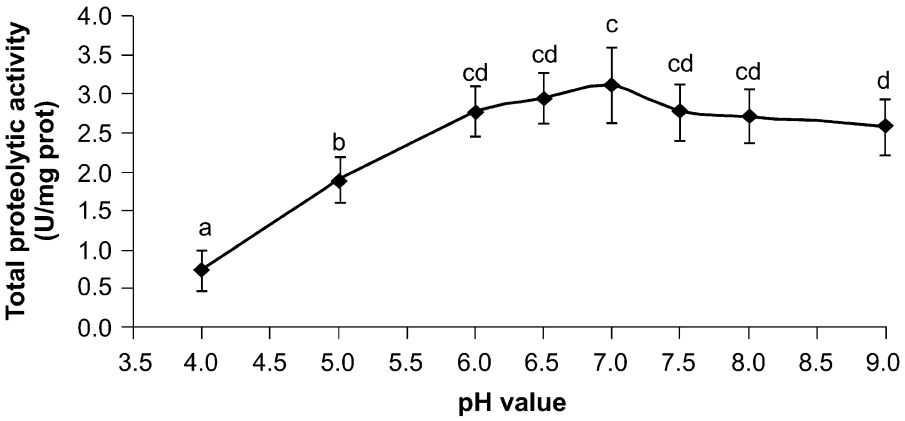

Fig. 3. Effect of pH on total protease activity of genus *Callinectes*. Different lowercase letters indicate statistical differences. The graph is made with the average of both species and genders.

Nagayama 1988). Dendinger (1987) found that proteases from the Atlantic blue crab (*Callinectes sapidus*) midgut gland had higher activity at pH 6.2, but it still conserved 90% of activity at pH 7.5. The results in our article suggest that the optimal pH range (6–8) for gastric juice proteinases from the specimens of the genus *Callinectes* is similar in most decapods.

Temperature is an important factor in an enzymatic reaction because protein structure is highly affected. An optimal value represents the rate of protein denaturalization and increment in reaction rate by addition of energy ($Q_{10} \approx 2$). Other decapods are reported to have a high optimal temperature. Galgani & Nagayama (1987) found maximum activity at 45°–55°C in the midgut gland of *C. affinis* and *Chionoecetes* sp. The authors found optimal activity at 60°C in specimens of *P. japonicus* (Galgani & Nagayama 1988). Proteases from gastric juice in specimens of *P. interruptus* were optimum at 50°C (Celis-Guerrero et al. 2004). In our study, decapod proteases catalyze best around 55°C. Thermostability results indicate that chymotrypsin is more stable than trypsin because SAPNA activity remains after incubation at 60°C, whereas BAPNA activity does not occur at this temperature. Those enzymes can maintain activity under varied conditions of evaluation, both in test tube, as in zymography, and the more stressing conditions of digestibility by the pHstat method.

Information about optimal pH was useful for *in vitro* digestibility assays of this genus because it provides information about the range in which enzymes maintain more than 90% activity. Crab enzymes remain highly active at pH 8 for evaluation of digestibility by the pHstat method (Tonglet et al. 2001). This value is only slightly higher than that found in gastric juice and is suitable for evaluation of digestibility. This allows evaluation of crab enzymes for digestibility of natural foods or fabricated feeds intended for farm cultivation. Under the conditions of digestion in the pHstat method, DH achieved by the digestive enzymes in the gastric juice is acceptable for nutrition purposes, around 13%, meaning that 13 out of 100 peptide bonds were hydrolyzed, yielding peptides significantly smaller than the length of the original protein and preparing for action by

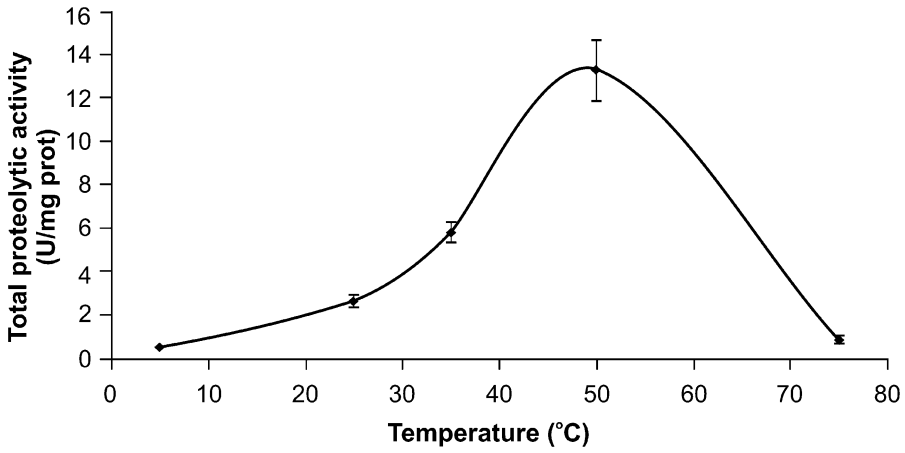

Fig. 4. Effect of temperature on total protease activity of genus *Callinectes*. The graph is made with the average of both species and genders.

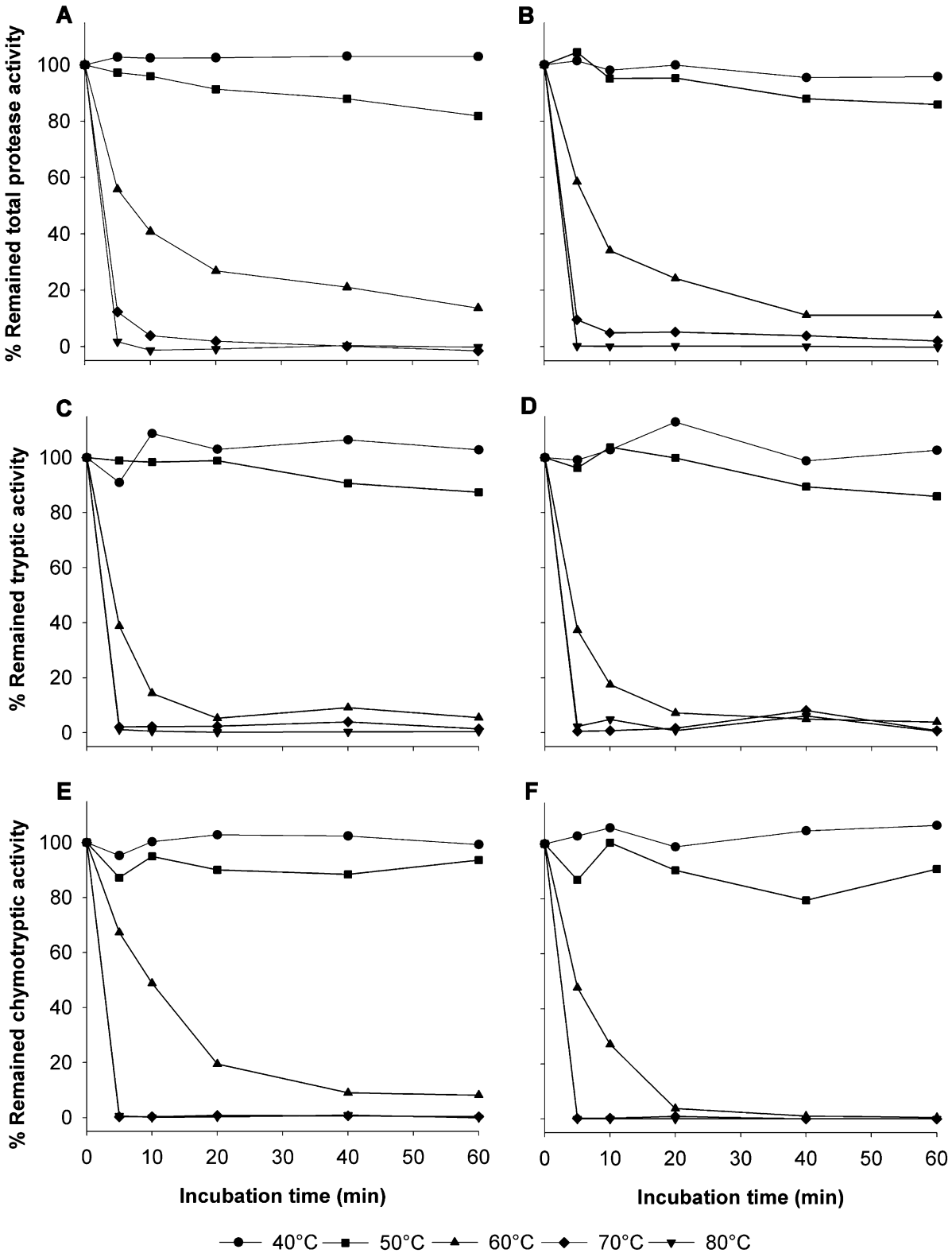

Fig. 5. Thermostability of total protease, trypsin, and chymotrypsin activities from the gastric juice of *Callinectes bellicosus* (**A**, **C**, **E**) and *Callinectus arcuatus* (**B**, **D**, **F**).

exopetidases. DH is similar to that found in other decapods, such as the European green crab (*Carcinus maenas* LINNAEUS 1758), edible crab (*Cancer pagurus* LINNAEUS 1758), and shrimp (*Penaeus* spp. FABRICIUS 1798) (M.A. Navarrete del Toro & H. Palafox-Carlos, unpubl. data). This information will generate a further understanding of how crabs process natural food and how to manufacture feed for farming of similar species.

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