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Source: Journal of Shellfish Research, 35(4):1067-1074.
Published By: National Shellfisheries Association
DOI: http://dx.doi.org/10.2983/035.035.0429
URL: http://www.bioone.org/doi/full/10.2983/035.035.0429

Journal of Shellfish Research, Vol. 35, No. 4, 1067–1074, 2016.

A SYNERGISTIC PEPTIDASE NETWORK MEDIATES FOOD PROTEIN DIGESTION IN THE AMERICAN LOBSTER *HOMARUS AMERICANUS* (EDWARDS, 1837)

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ABSTRACT The American lobster [*Homarus americanus* (Edwards, 1837)] is an omnivore decapod with generalist and opportunistic feeding habits, in which gastric fluid cysteine and aspartic peptidases are the main proteolytic enzymes. Using *in vitro* assays, the network of concerted acting peptidases belonging to aspartic and cysteine catalytic mechanisms that hydrolyze bovine serum albumin (BSA), hemoglobin, and actin was elucidated. The role of each class of peptidases in protein digestion was evaluated by monitoring the hydrolysis of each substrate in the presence of single or mixed specific peptidase inhibitors. Peptidases of the gastric fluid can hydrolyze BSA, actin, and hemoglobin. Hemoglobin was hydrolyzed by cysteine and aspartic peptidases but not by serine ones. Results suggest that hydrolases act synergistically. Hydrolysis of BSA and actin is carried out by a multienzyme network mechanism. Although cysteine peptidases hydrolyze about 50% of BSA and actin, aspartic peptidases are required to fulfill the task; when aspartic and cysteine peptidase activity is absent, no hydrolysis of the substrates happens at all. Serine peptidases seem not to contribute to the analyzed substrates hydrolysis at the conditions of the assay.

KEY WORDS: *Homarus americanus*, aspartic peptidase, cysteine peptidases, crustacean protein digestion, American lobster

INTRODUCTION

Decapods are a diverse group of crustaceans that include shrimps, crabs, and lobsters (Siddall 2004) inhabiting in practically the entire range of marine environments, thus crustaceans have structural and physiological adaptations that allow them to thrive in the aqueous milieu at which they are exposed (Steele & Steele 1991, Childress & Seibel 1998, Linton & Greenaway 2007). In the digestive system, these adaptations are related to dietary preference, prey availability, and habitat (Dittrich 1992, Hervant & Renault 2002, Johnston & Freeman 2005). The crustacean's main digestive organ is the midgut gland (also known as hepatopancreas), where the synthesis and secretion of digestive enzymes, and absorption are carried out (Ceccaldi 1989). Among the enzymes synthesized by the midgut gland are the peptidases responsible for food protein digestion. Serine- (Celis-Guerrero et al. 2004), cysteine- (Stephens et al. 2012), aspartic- (Rojo et al. 2010a, 2010b), and metallopeptidases (Perera et al. 2008) are responsible for food protein digestion, being serine peptidases, with maximum activity at alkaline pH, the most frequently reported for Crustacea (Celis-Guerrero et al. 2004, Díaz-Tenorio et al. 2006, Buarque et al. 2009, Navarrete del Toro et al. 2011), this fact seems contradictory because Decapods' physiological pH of the gastric fluid is slightly acidic (Jordan, 1913; Navarrete del Toro et al. 2006); from 4.7 in *Homarus* sp. to 6.1 in *Callinectes* sp. the pH in which activity of cysteine and aspartic peptidases is highest. Both classes of peptidases have been found in the crustacean digestive tract (Laycock et al. 1991, Teschke & Saborowski 2005, Navarrete del Toro et al. 2006, Rojo et al. 2010a, 2010b). The physiological role and biochemical characteristics of many of these peptidases have been described (Celis-Guerrero et al. 2004, Pavasovic et al. 2004, Rojo et al. 2010a).

The American lobster *Homarus americanus* is a decapod of ecological and economic importance because of its trophic position in the West North Atlantic and value in the seafood market (Factor 1995). It is a cold-water benthic being and a generalist-opportunistic omnivore that preys on a variety of species, including other crustaceans, bivalves, gastropods, echinoderms, marine worms, algae, and carcasses (Elner & Campbell 1987). The pH of American lobster gastric fluid is 4.7, significantly more acidic than that of other crustaceans (Navarrete del Toro et al. 2006). Two catalytic classes of digestive peptidases have been reported: cysteine peptidases, three isocathepsin L «CatL» (Laycock et al. 1989), and one aspartic peptidase, cathepsin D1 «CatD1» (Rojo et al. 2010a).

In heterotrophs, either zoophagous or phytophagous, standard digestion of food protein includes coordinated and sequential interventions of peptidases. It is generally accepted that endopeptidases like trypsin (Try) or chymotrypsin (Chy) initiate the hydrolysis of protein-releasing polypeptides that are further hydrolyzed by other endopeptidases yielding smaller polypeptides available for exopeptidases; Try hydrolyzes the peptide bond formed by the carboxylic side of lysine or arginine, whereas Chy at voluminous hydrophobic amino acids like tyrosine, tryptophan, or phenylalanine. Then, amino and carboxy peptidases hydrolyze short polypeptides to release tri- and dipeptides and free amino acids. Decapods deviating from the standard composition of digestive peptidases model, like the American lobster, must have species-specific protein hydrolysis.

In some parasites and insects involving aspartic and cysteine peptidases in food proteins hydrolysis, an alternative mechanism of food protein digestion has been described (Brunelle et al. 1999, Delcroix et al. 2006, Horn et al. 2009, Williamson et al. 2004); in which peptidases act in a so-called

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DOI: 10.2983/035.035.0429

multienzyme pathway, an orchestrated mechanism in which one peptidase cannot hydrolyze the substrate by itself, and needs another peptidase that initiates the substrate hydrolysis, exposing previously buried peptide bonds, which become available for other classes of peptidases involved in the process. Because of the presence of aspartic and cysteine peptidases in the American lobster gastric fluid, it is hypothesized that they are also implicated in a multienzyme network. To defy the hypothesis, three different native proteins, including one naturally present in lobster diet, were hydrolyzed *in vitro* with the set of peptidases from the lobster gastric fluid; in parallel, assays including inhibitors were done to elucidate the role of each peptidase class in food protein digestion.

MATERIALS AND METHODS

Organisms and Sample Processing

Adult specimens of the American lobster were bought at a local market in San Francisco, CA. The gastric fluid was withdrawn from live organisms introducing a flexible plastic probe (5 cm long, 3 mm diameter) connected to a syringe into the esophagus to reach the gastric chamber. The gastric fluid was then transferred to a 2-ml microtube and centrifuged for 10 min at 4°C and 10,000× *g* to discard solids. The supernatant was freeze dried and stored at –20°C until used. The gastric fluid powder was suspended in distilled water at 20 mg/ml, and stored at –20°C in aliquots for further analysis. The protein concentration of the extract was quantified by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Total acid proteolytic activity was quantified as in Díaz-López et al. (1998), using 0.5% (w/v) bovine hemoglobin (Sigma-Aldrich H-2625) in 50 mM sodium acetate buffer pH 4.7 as substrates.

Peptidase Identification

To identify the composition and molecular mass of the peptidases from the gastric fluid, the substrate-sodium dodecyl sulfate polyacrylamide gel electrophoresis (S-SDS-PAGE) method was followed as in García-Carreño et al. (1993) at lobster's gastric physiological pH (4.7) and at alkaline pH (8.0). Before electrophoresis, 38 µl (equivalent to 5 mU of acid activity) gastric fluid was incubated with 35 µl of 45 mM Dithiothreitol (DTT, ICN 808376) for 10 min, at room temperature. Five microliters of the gastric fluid in reducing conditions were incubated for 1 h in the presence of 5 µl of 1 mM Pefabloc (Fluka 76307), 1 mM E-64 (Sigma-Aldrich E-3132), or 1 mM Pepstatin A (Sigma-Aldrich P-4265) in dimethyl sulfoxide (DMSO, Sigma-Aldrich D-5879), which are specific inhibitors for serine, cysteine, and aspartic peptidases, respectively. Gastric fluid was incubated with 5 µl of 0.15% (v/v) DMSO as control for inhibitor solvent. Each mixture was loaded in a SDS-PAGE 12% gel. Electrophoresis was performed at 15 mA per gel and at 4°C under water circulating bath. Once the protein was separated, the gels were rinsed three times with distilled water. For acid proteolytic activity, one gel was stabilized to pH 4.7 in 50 mM sodium acetate buffer before the incubation with 50 ml 0.25% (w/v) bovine hemoglobin in 50 mM sodium acetate buffer, pH 4.7. A replica gel was incubated with 50 ml 3% (w/v) bovine casein in 50 mM tris-HCl buffer, pH 8.0. Both gels were incubated in the substrate for 30 min in an ice bath under gentle orbital shaking. Then the temperature was raised to 25°C, after 1.5 h incubation, the gels were washed thoroughly with distilled water and stained with Coomassie staining solution (0.05% w/v Coomassie Brilliant Blue R, 40% methanol, 7% acetic acid), the staining time stopped when white bands on a blue background were observed, the white bands are proteins with proteolytic activity. Then the gels were distained with a solution mixture containing 40% methanol, 7% acetic acid. Then stored in 5% methanol, 7% acetic acid, 4% glycerol and documented in a Gel-EZ-Imager and analyzed with an Image Laboratory software (BIO-RAD).

Extraction of Actin from Clam Adductor Muscle

Specimens of the clam *Argopecten circularis* were obtained from a local market in La Paz, Baja California Sur, México, packed in sealable bags and placed on an ice bed and then transported to the laboratory; animals were dissected on arrival. The adductor muscle of the clams was separated and cut into pieces of 1 cm^3. Muscle proteins were extracted as Sáenz de Rodrigáñez methodology (2011) by grinding in a mortar with 100 mM Tris-HCl buffer, containing 0.25 mM sucrose and 1 mM Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich ED2SS). The homogenate was centrifuged at 10,000× *g* for 10 min at 4°C. The supernatant was collected and concentrated by ultrafiltration using a centrifugal filter, 10,000 Da molecular weight cutoff (Amicon, Millipore) at 4,000× *g* for 1 h at 4°C. Protein content was quantified by the Bradford method (1976). Appropriate dilution was done to adjust to 6 mg/ml protein concentration. Protein composition of muscle preparation was identified by SDS-PAGE in an 8% acrylamide gel, and compared with the muscle protein preparation obtained by Yamada et al. (2000). Subsamples of muscle protein preparation were stored at –20°C for further analysis.

In Vitro *Protein Hydrolysis*

The hydrolysis of substrates, BSA (Sigma-Aldrich B-4287), bovine hemoglobin, and actin from adductor muscle of *Argopecten circularis*, was done to evaluate the capability of peptidases from American lobster gastric fluid to hydrolyze the proteinaceous substrates and, at the same time, to determinate how long it takes to reach total hydrolysis of each substrate.

Hydrolysis reactions were done at room temperature. Each substrate was assayed as follows: in 0.7-ml microtube, 40 µl gastric fluid (5 U/ml activity) was poured and incubated for 10 min with 35 µl of 45 mM DTT and taken to 150 µl with distilled water, then 20 µl of 0.15% (v/v) DMSO, 20 µl of 350 mM sodium acetate buffer, pH 4.7 and 40 µl distilled water was added and mixed thoroughly. The hydrolysis reaction started by the addition of 40 µl of the substrate containing 240 µg protein and incubated for 20 h. To assess the behavior of the protein through the progress of hydrolysis, 15 µl of each mixture was sampled at 0.15, 1, 2, 4, 6, and 20 h and mixed with 15 µl of 2× DTT sample loading buffer and boiled for 10 min. In parallel, each substrate was combined with all reaction elements and using distilled water instead of gastric fluid as control. The products of hydrolysis were assessed by SDS-PAGE in a 12% acrylamide gel for BSA and actin, and 15% for hemoglobin. Each gel was digitalized and the image was analyzed as in the section Peptidase Identification, the Image

Laboratory software uses densitometry and allows the measure of the amount of remaining protein along the hydrolysis time course. All assays were done in triplicate.

In Vitro *Peptidase Inhibition Assay*

To identify the class of peptidases present in the gastric fluid of American lobster and its role in the hydrolysis of each substrate, inhibition assays were done using the specific serine, cysteine, and aspartic peptidase inhibitors (Pefabloc, E-64, and Pepstatin A, respectively) combined with the enzyme solution. All reactions were carried out at room temperature. Forty microliters of the gastric fluid (0.15 U/ml) were incubated for 10 min with 35 µl of 45 mM DTT and taken to 150 µl with distilled water. Then 10 µl of 350 mM sodium acetate buffer (pH 4.7), 10 µl of 0.15% (v/v) DMSO (except in reaction mixture having Pepstatin A) and 10 µl (1 mM) of individual inhibitor or in combination were added and mixed thoroughly. The hydrolysis reaction started by the addition of 20 µl substrate containing 120 µg protein. The reaction mixture was incubated for the time of maximum hydrolysis obtained in the section *In Vitro* Protein Hydrolysis. Controls with no inhibitor were considered as 100% hydrolysis. Hydrolysis progress of BSA and actin was followed by SDS-PAGE in a 12% acrylamide gel, and 15% acrylamide for hemoglobin. After electrophoresis, the protein bands of each substrate were analyzed by densitometry, measuring the amount of remaining protein using the Image Laboratory software (BIO-RAD). All assays were carried out in triplicate.

Statistical Analysis

Statistical analysis was done using the STATISTICA 7 software. Differences among means were analyzed by a one-way ANOVA followed by a Tukey's multicomparison test. Statistical differences were considered when $P < 0.05$. Different letters above bars (Fig. 4) of same color (substrate) indicate significant differences.

RESULTS

Gastric Fluid Peptidase Composition

The identification of peptidases of the gastric fluid of the American lobster was carried out by comparing the electrophoresis composition of control sample with the peptidase class-specific inhibitor-treated samples (Fig. 1). In the Substrate-SDS-PAGE, developed at acid pH (Fig. 1A), seven bands of activity were observed in the control (lane C). When the enzyme preparation was incubated with Pepstatin A the intensity of 36 kDa band was significantly reduced (Fig. 1A, lane P). Six activity bands with mass of 18.5, 37, 42, 48, 54, and 60 kDa, were no longer observed when the gastric fluid was incubated with E-64, an inhibitor for the cysteine peptidase catalytic class (Fig. 1A, lane E). The serine peptidase inhibitor did not affect any activity band (Fig. 1A, lane F). In the S-SDS-PAGE developing the activity at alkaline pH (Fig. 1B), nine bands of activity were observed in the control (Fig. 1B, lane C). The six bands inhibited by E-64 at acid pH (Fig. 1B, lane E) were also inhibited although in less extent at alkaline pH, suggesting that these enzymes are active at a wide range of pH. Two additional bands appeared at alkaline pH with a mass of 23.1 and 25 kDa; they were partially inhibited when the gastric fluid was incubated with Pefabloc (Fig. 1B, lane F), a specific inhibitor of catalytic class serine peptidases.

Argopecten Circularis Muscle Protein Composition

Protein composition of the adductor muscle of *Argopecten circularis* (Fig. 2) was identified by comparing the electrophoretic patterns of muscle proteins reported by Yamada et al. (2000). The bands of myosin (220 kDa, M in lane Me), paramyosin (111 kDa, P in lane Me), and actin (42 kDa, A in lane Me) were observed.

Hydrolysis of the Protein Substrates by Lobster Digestive Peptidases

The progress of hydrolysis of BSA, hemoglobin, and clam muscle proteins by the gastric fluid peptidases was assessed by tracking the changes in the intensity of the electrophoresis bands using densitometry and by comparison with the untreated substrates (Fig. 3B, lane C). The three assessed proteins were fully hydrolyzed by the whole set of peptidases of the gastric fluid of the lobster in a time-dependent manner. Hydrolysis of clam actin (42 kDa) was completed in 4 h, whereas for BSA (66 kDa) and hemoglobin (14.4 kDa) was completed in 6 h. Figure 3 shows the progress of the hydrolysis of the three proteinaceous substrates expressed as the percentage of remaining protein along the hydrolysis progress. The proteins BSA and hemoglobin are model substrates in protein hydrolysis assays and clam actin is a frequent proteinaceous component of the lobster diet.

Participation of Lobster Digestive Peptidases in Substrate Hydrolysis

The three proteinaceous substrates were incubated in individual reaction tubes with the lobster enzymatic preparation for 4 (actin) and 6 h (BSA and hemoglobin), the time at which the proteins were completely hydrolyzed as known from the previous assays. To uncover the role of each peptidase class in the hydrolysis of the proteinaceous substrates, class-specific peptidase inhibitors were used individually or in combination. The proteolysis products were separated by electrophoresis and the progress of the hydrolysis was followed by comparing the bands obtained in the enzyme-treated substrates with the untreated ones (Fig. 4). Lanes C show the untreated proteinaceous substrates; they are displayed on its prime. Lanes C+ are the substrates treated with the gastric fluid: significant hydrolysis is shown. Lanes P, E, and F represent the substrates incubated with the gastric fluid in the presence of Pepstatin A (P), E-64 (E), and Pefabloc (F). Bovine serum albumin (Fig. 4A) and actin (Fig. 4B) are hydrolyzed even in the presence of Pepstatin A and Pefabloc. A significant decrease on the hydrolysis of BSA and actin occurs when E and the inhibitor mixture E – F are present in the reaction. When the inhibitor mixtures P + E and P + E + F are used, BSA and actin are not hydrolyzed. In contrast, when F, P and the inhibitor mixture of P + F is used, both substrates are totally hydrolyzed. Indicating that cysteine peptidases are required for the hydrolysis of these substrates.

Hemoglobin was partially hydrolyzed when Pepstatin A, E-64, and Pefabloc were used individually in reactions (Fig. 4C). Apparently, Pefabloc had a milder effect in the reaction, although no statistical difference was found when enzyme

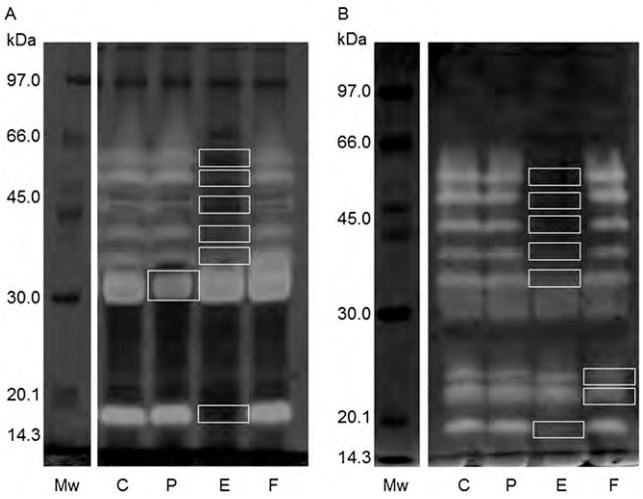

Figure 1. Zymograms that reveal peptidase content in the gastric fluid of American lobster at different pH, in presence of DTT as reducing agent. Peptidase activity at pH (A) 4.7 and (B) 8.0. Frames indicate the sites of peptidases affected by inhibitors. Mw, molecular weight; C, Gastric fluid in DMSO; and peptidases inhibited by class-specific inhibitors for P, Pepstatin A for aspartic peptidases; E, E-64 for cysteine peptidases; and F, Pefabloc for serine peptidases.

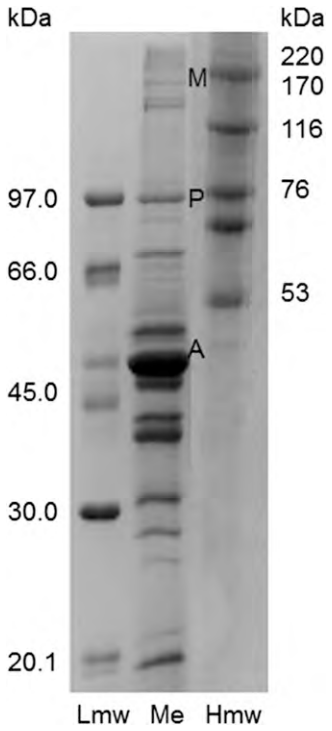

Figure 2. Protein composition of adductor muscle of *Argopecten circularis* clam. Lmw and Hmw are low and high molecular weight markers, respectively. Me, muscle extract; M, myosin, P, paramyosin, and A, actin.

mixture was treated with these inhibitors individually (Fig. 4D). When the inhibitor mixtures P + F and E + F were added to the reaction mixture, hemoglobin was also partially hydrolyzed and no statistical significant difference was found between these treatments and the individual treatments P, E, and F. It was only when the inhibitor mixtures of P + E and P + E + F were added to the reactions that the protein hydrolysis was completely inhibited. It is important to notice that only in the treatments P + E and P + E + F, the activity of aspartic and cysteine peptidases is targeted in a synergic manner, this indicates that both aspartic and cysteine peptidases are necessary to complete the hydrolysis of hemoglobin but in this assay its effect cannot be assessed when they are tested individually.

DISCUSSION

For an omnivore, preying on varied food items, to make the process rapid and effective, digestive systems may function as networks of peptidases to hydrolyze food protein. In vertebrates, food protein digestion is carried out by individual peptidases that hydrolyze the food proteins as it passes along the digestive system. The first acting peptidases are pepsins in the stomach and the task is completed in the duodenum by the remarkably conserved pancreatic enzymes of the catalytic mechanism, serine peptidases—all of them members of the Chy family S1. Recent advances in the field of invertebrate digestive peptidases have revealed a dissimilar process. Studies on insects and crustaceans uncovered the striking complex simultaneous participation of peptidases from different catalytic classes and families that allow the organism get access to amino acids needed to construct *de novo* protein for homeostasis.

In general, in the invertebrate digestive systems, two serine peptidases are involved Try and Chy. In some invertebrate species, digestive peptidases belonging to the catalytic mechanisms aspartic and cysteine peptidases predominate. In the phytophagous insect, Colorado potato beetle, molecular complex interactions take place between the insect and the plant observing the ''Red Queen hypothesis''; the food protein digestion is initiated by cathepsin D aspartic peptidase (Brunelle et al. 1999). In the parasitic helminth *Schistosoma mansoni*, digestion of food protein, host's hemoglobin and albumin, is ordered and occasionally redundant, and most of all, substrate specific; hydrolysis of hemoglobin starts with a cathepsin D and of albumin by a cathepsin B (Delcroix et al. 2006). Knowing

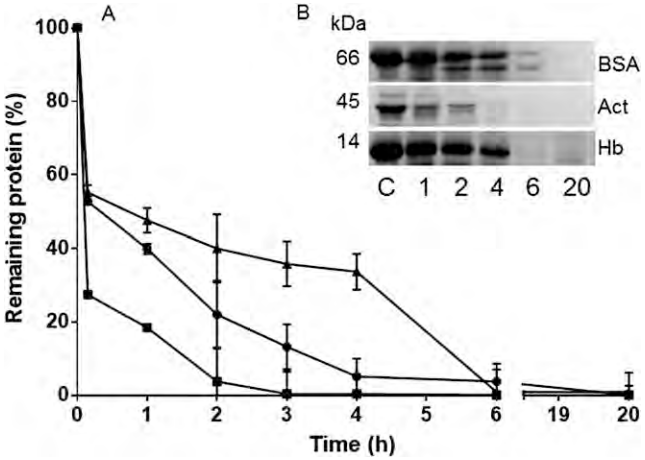

Figure 3. Protein hydrolysis by American lobster gastric fluid peptidases at physiological pH (4.7) through time, at room temperature. (A) Graphical representation of BSA (circles), Hb (triangles), and actin (squares) hydrolysis. (B) SDS-PAGE of the hydrolysis of BSA, actin, and hemoglobin by gastric fluid peptidases along different times. (C) untreated substrate; 1, 2, 4, 6, and 20 are the time in hours after incubation at room temperature. Each value is the average of triplicate.

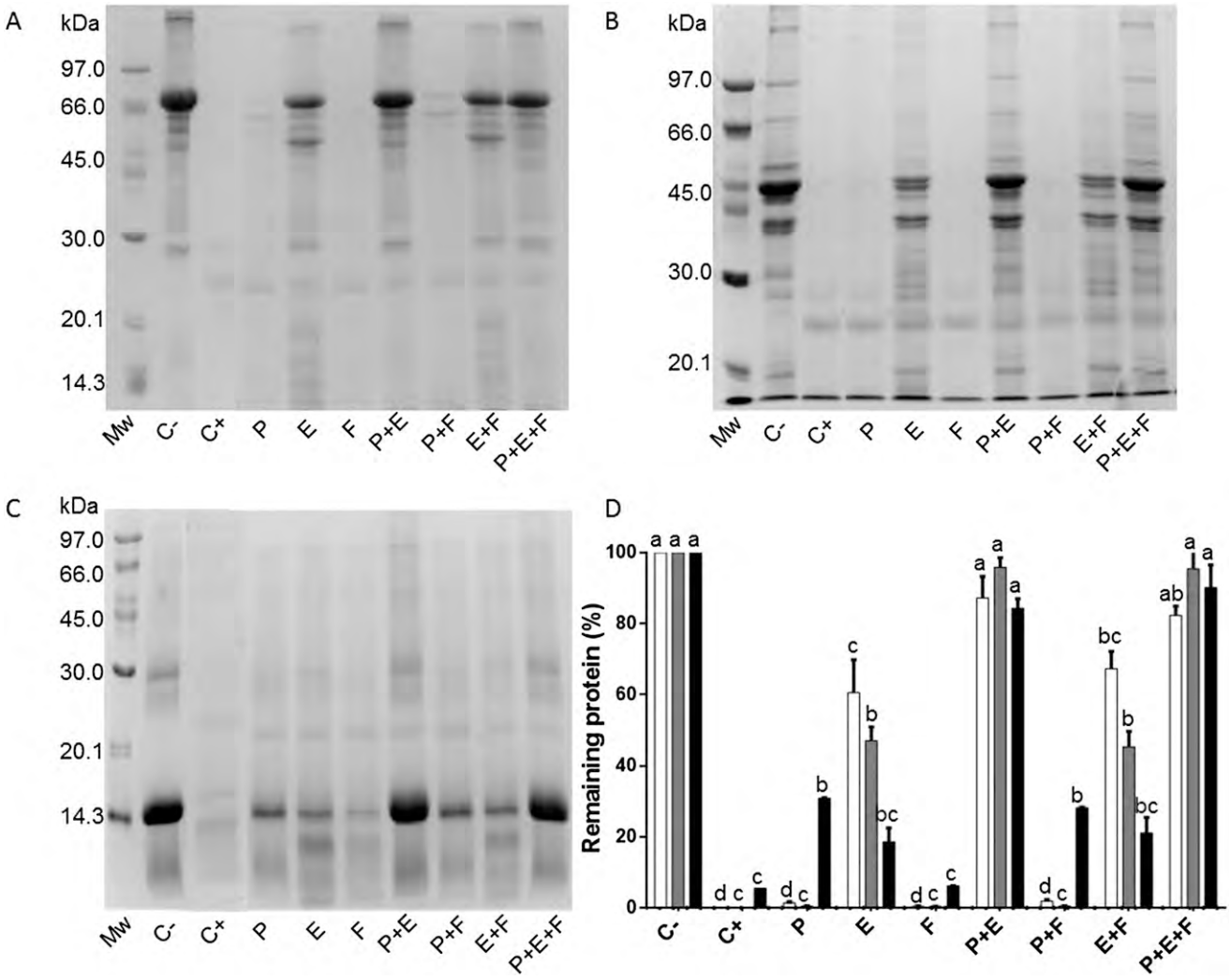

Figure 4. Effect of class-specific peptidase inhibitors in the hydrolysis of (A) BSA, (B) actin, and (C) hemoglobin by American lobster gastric fluid peptidases at physiological pH (4.7), at time of total substrate depletion in the absence of inhibitors (4 h for actin, and 6 h for hemoglobin and BSA). (D) Graphical comparison between hydrolysis of BSA (white bars), actin (gray bars), and hemoglobin (black bars) in the absence/presence of inhibitors. Bars marked with the same letter indicate nonsignificant statistical difference ($P < 0.05$) between treatments. Each value is the average of triplicate. Mw, molecular weight; C–, untreated proteinaceous substrate; and C+, hydrolysis of the proteinaceous substrate by the peptidase mixture of the gastric fluid. The gastric fluid peptidase mixture was treated with P, pepstatin A; E, E-64; and F, Pefabloc, and with the combination of these inhibitors (P + E), (P + F), (E + F), and (P + E + F). Each value is the average of triplicate. For each substrate, bars marked with the same letter indicate nonsignificant statistical differences ($P < 0.05$) between treatments.

how the mechanism of food protein hydrolysis occurs and how conserved such systems are, provides a deeper understanding of the functioning of a complex peptidase network and eventually, would find potential technologies to manipulate invertebrate digestive systems for the benefit of humankind.

In this work, we addressed the lobster digestive system network by using an advantageous biochemical concept that became a tool, inhibition of peptidases. Using specific peptidase inhibitors to reduce peptidase activity made possible to describe the functions and dynamic associations of lobster digestive peptidases.

Previously, an unbiased substrate profiling assay was used to uncover the global proteolytic substrate specificity or ''substrate signature'' in the lobster gastric fluid incubated in the presence of class-specific inhibitors, finding that most of the cleavage sites were sensitive to E-64 and 8 out of 140 cleavage sites to Pepstatin A (Bibo-Verdugo et al. 2016). Here, it is confirmed, *in vitro*, that digestive system of lobster is composed of several peptidases. The decrease in peptidase activity as a result of the addition of specific inhibitors to the gastric fluid assayed at its physiological acid pH (Fig. 1A) reveal two main catalytic mechanisms, those of aspartic and cysteine peptidases (Fig. 1, lanes P and E). The presence of digestive aspartic and/or cysteine peptidases has been referred as ''acidic proteolytic cocktail'' (Fuzita et al. 2015), and reported in other arthropods (Brunelle et al. 1999), platyhelminths (Delcroix et al. 2006) and molluscs (Martínez et al. 2011). The genes encoded for acidic proteolytic cocktail had been characterized and are present in the ancient arthropod lineages, *Trichoplax adhaerens* (Fuzita et al. 2015). So far, the evolution forces that have allowed the expression of such enzymes in American lobster remain to be addressed.

In most decapods, digestion of food protein rely on peptidases belonging to the serine peptidases catalytic class, in some species this dependence is total as in whiteleg shrimp (Sainz et al. 2004, Navarrete del Toro et al. 2011), spiny lobster (Celis-Guerrero et al. 2004), and swimming crabs (Díaz-Tenorio et al. 2006), whereas in some species, the dependency is partial as in caridean shrimps (Teschke & Saborowski, 2005), in which, the extent of participation of digestive serine peptidases vary within individuals and is not affected by the diet. For the clawed lobster, it seems that only aspartic and cysteine peptidases are responsible for hydrolysis of food protein. According to Laycock et al. (1989), based on an *in vitro* assay, up to 80% of the proteolytic activity in the lumen of the midgut gland is responsible of cysteine peptidases, specifically three isocathepsins L (CL1, CL2, and CL3; Laycock et al. 1991). Later, Navarrete del Toro et al. (2006) found, in the gastric fluid of the European lobster *Homarus gammarus*, a conspicuous activity at acid pH and Rojo et al. (2010a) demonstrated it is an aspartic peptidase, cathepsin D, functioning in the digestive system of European and American lobsters; although we found evidence of serine peptidases, those enzymes that are active in alkaline conditions (pH 8.0) far from the physiological pH (pH 4.7). Therefore it was concluded that for this species and this study, serine peptidases do not participate in the digestion of food protein.

From these findings, it is assumed that some decapod sub- and infraorders evolved independently to exploit different peptidase catalytic mechanisms, including the use of serine, cysteine, and aspartic peptidases for hydrolysis of peptide bonds in food proteins, the evolution forces leading to such variable digestive mechanisms remain unknown. It was demonstrated that the peptidases found in the American lobster gastric fluid accomplished hydrolysis of substrates of diverse nature and complexity including BSA, clam actin, and hemoglobin, which is not unexpected for a generalist feeder like the American lobster (Hudon & Lamarche 1989).

To uncover the mechanism by which digestive peptidases in clawed lobster achieve food protein digestion, the gastric fluid, containing at least seven peptidases (Fig. 1), was mixed with proteinaceous substrates and the hydrolysis was followed (Fig. 3). To assess the individual contribution of each class of peptidases, specific inhibitors for serine, cysteine, and aspartic peptidases were used to tamper with proteolytic activity. Inhibiting serine peptidases did not preclude the total hydrolysis of any of the assessed substrates, indicating that serine peptidases barely contribute to the process. When aspartic peptidases were inhibited, both BSA and clam actin were hydrolyzed, indicating that cysteine peptidases are responsible for the hydrolysis of such substrates, whereas 30% hemoglobin remained unhydrolyzed. When inhibiting cysteine peptidases, 30% of BSA, 50% of clam actin, and 80% of hemoglobin were hydrolyzed. The same results were observed when cysteine or aspartic peptidases were combined with serine peptidase inhibitors. This indicates that when cysteine peptidases are inactive, aspartic peptidases can partially hydrolyze the substrates. The combination of aspartic and cysteine peptidase inhibitors and the three inhibitors caused no hydrolysis of any substrate; 100% remaining substrate was observed. Well, what does this mean? Serine peptidases have no apparent participation in the digestion of food protein in American lobster, the 23.1 and 25 kDa alkaline activity bands identified by S-SDS-PAGE might correspond to Try and Chy activity reported in the lobster gastric fluid 46 y ago (Brockerhoff et al. 1970). The fact that these serine peptidases are active at a pH far from that of the lobster gastric fluid (pH 4.7) (Horn et al. 2009, Fuzita et al. 2015) suggests that serine peptidases are secreted in the American lobster midgut gland as vestigial character, as has been reported and proposed for other Arthropod's digestive enzymes; for example, in the *Ixodes* ticks, a switch in the diet preferences from scavenger to hematophagous was parallel to the changes in the salivary peptidase composition (Mans & Neitz 2004).

It was observed that the classes of peptidases comprising the American lobster gastric fluid differently hydrolyzed the proteinaceous substrates. The three isocathepsins L, partially hydrolyzed BSA and clam actin, whereas a cooperative hydrolysis with the aspartic peptidase, CD1, is required for hemoglobin hydrolysis, this is confirmed when cysteine and aspartic peptidases are inhibited simultaneously (Fig. 4C, lane P + E). This synergy has been reported in other food protein hydrolysis systems (Wallace 1985). Because CD1 alone did not fully hydrolyze BSA and actin (Fig. 4A and B, lane E), it is assumed that cathepsins L might trigger substrate hydrolysis and then, peptidases of the aspartic class continue in an orchestrated proteolytic mechanism. Such synergistic mechanism had been described in other vertebrate physiological processes such as control of infection, glucose uptake, and neuronal death (Lenarcic et al. 1991, Artal-Sanz & Tavernarakis 2005, Belman et al. 2014). As for digestion, analogous mechanism of hydrolysis had been identified in the digestive system of hematophagous parasites and some insects. Such orchestrated mechanism has been called ''a proteolytic multienzyme network'' (Delcroix et al. 2006) or ''multipeptidase network'' (Horn et al. 2009). This work is the first report of crustacean multienzyme protein hydrolysis by cysteine and aspartic peptidases, in which, a similar network, as the one reported before, might be acting.

For insects, different feeding habits had been linked to multipeptidase network in protein digestion. In the herbivorous Colorado potato beetle, the food protein hydrolysis is achieved by the initial participation of an aspartic peptidase, followed by serine, cysteine peptidases and later by exopeptidases (Brunelle et al. 1999); this adaptation helps them compensate for the presence of peptidase inhibitors in plant food (Zhu-Salzman et al. 2003, Ahn & Zhu-Salzman, 2009). Despite the lack of information about the peptidase inhibitor content in the organisms on which lobsters feed, in larval and juvenile stages, macroalgae constitute one of the main components of the lobster diet (Sainte-Marie & Chabot 2002). The fact that some macroalgae had been associated with the production of peptidase inhibitors (Perez-Lorenzo et al. 1998, Ahn et al. 2004) can be linked to the presence of a multienzyme network as an adaptive response for increasing the proteolytic efficiency of the American lobster peptidases even in the presence of inhibitors. This effect had been previously reported in *Daphnia magna* where an increased amount of food inhibitors somehow induces the presence of less-sensitive peptidase isoforms that increase the overall capability of protein digestion even in the presence of dietary peptidase inhibitors (Schwarzenberger et al. 2010).

The way in which protein hydrolysis happens is dependent on the complexity of the substrate (Sun et al. 2013). In the clawed

lobster, the hydrolysis of hemoglobin is partially achieved by aspartic and cysteine peptidases, the complete hemoglobin digestion is only possible when both enzymes are present, suggesting a synergic participation of such peptidases.

It was demonstrated that the American lobster gastric fluid peptidases digest proteinaceous substrates of different origin and structure. This can translate into the potential of such enzymes to hydrolyze proteins from diverse prey, in agreement with a feeding behavior that is closely related to generalist dietary habits (Johnston & Freeman 2005). Peptidases from gastric fluid from the American lobster have diverse amino acid specificity which is possibly useful of the hydrolysis of a wide diversity of substrates (Bibo-Verdugo et al. 2016). The presence of three CL with complementary activity and high promiscuity for a variety of amino acids is a result of evolutionary adaptations that allow the species to increase the hydrolytic efficiency (Khersonsky et al. 2006); CL2 and CL3 have affinity for different hydrophobic amino acids in the amino-terminal side of the scissile bond, CL3 for Leu and Ile, whereas CL2 has preference for Pro, Ile, and Val, in addition to affinity for hydrophobic amino acids and the CL1 cleavage in polar amino acids Gln and Thr too (Bibo-Verdugo et al. 2016). On the other hand, the presence of the aspartic peptidase CD1 with a preference for hydrophobic amino acid residues, mainly Phe (Bibo-Verdugo et al. 2016), indicates a wide range of digestibility from lobster peptidases.

CONCLUSIONS

In summary, the description of the mechanism for food protein hydrolysis in the American lobster has revealed the capability of promiscuous gastric fluid peptidases from this lobster to hydrolyze multiple substrates and, in the same way, was elucidated a previously unknown participation of peptidases in a multipeptidase synergistic network in crustacean digestion as a tool for increasing the protein digestibility. It is hypothesized that the American lobster evolved a diversity of mechanisms for protein digestion in response to diversification of its diet. Future studies will be required to elucidate the evolutionary forces that led to the American lobster to develop this complex system of food protein hydrolysis.

ACKNOWLEDGMENTS

We acknowledge Mexican Science and Technology Council (CONACyT) for funding grant no. 177954 to FGC and scholarships 277859 to BBA and 392201 to IRS.

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