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Partial Characterization of Digestive Proteases in the Common Snook *Centropomus undecimalis*

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Abstract

Common snook (*Centropomus undecimalis*) is a marine species with high aquaculture potential; although its digestive physiology is still unknown and knowledge of that could allow the development of a balanced feed for commercial culture of this fish. The objective of this study was to partially characterize the digestive proteases in *C. undecimalis* using electrophoretic and biochemical techniques. A total of 50 wild snook juveniles were used to determine the optimal values of pH stability and temperature as well as the effect of inhibitors on digestive, gastric and intestinal proteases. The optimal pH for gastric proteases was obtained to be 2 with stability obtained between 2 and 8; the optimal temperature was detected at 75ºC for in vitro test, and the thermal stability was between 25 and 45ºC. Intestinal proteases showed two peaks of activity at a pH of 7 and 11; meanwhile, the greatest stability was found between a pH of 4 and 10; the optimal temperature was at 65ºC, and the greatest stability was detected between 35 and 45ºC. Up to 86% of the gastric protease activity was inhibited by pepstatin A; meanwhile, the intestinal proteases TPCK, TLCK, 1-10 Phenanthroline, SBT1, EDTA, PMSF and ovalbumin reduced the activity by 17%, 68%, 85%, 41%, 40.5%, 60% and 59%, respectively.

Keywords: common snook, digestion, proteases, nutrition

1. Introduction

The nutrient requirement in a fish species varies depending on several factors and it is determined by the quantity and quality of macronutrients (proteins, lipids and carbohydrates) and micronutrients (vitamins and minerals) (Fenerci, & Sener, 2005). Digestion in fish starts in the digestive tract, where several proteases act and are responsible for protein hydrolysis (Tufan, Suzer, Taşbozan, & Tabakoğlu, 2008); however, the activity expressed in International Units (IU mg protein^{-1}) depends on the feeding habits and type of food, among other factors (Kuz'mina, 2008). Additionally, the protease activity could be affected by the quality of the ingredients composing the formula (Lazzari et al. 2010; Costa et al. 2011). Considering the above, the secretion of digestive enzymes depends on the physicochemical characteristics of the internal environmental, the quality and quantity of nutrients, and the presence of inhibitors in the feed (Lazo, Mendoza, Holt, Aguilera, & Arnold, 2007; Murashita et al. 2015). In this sense, the studies on protease characterization allow us to understand the capacity of fish to hydrolyze and metabolize proteins, which are necessary for adequate growth and development (Ma et al. 2005). For several years many studies have been conducted to characterize digestive proteases such as in common dentex (*Dentex dentex*), blue discus (*Symphysodon aequifasciata*), burbot (*Lota lota*) and common carp (*Cyprinus carpio*) (Alarcón, Díaz, Moyano, & Abellan, 1998; Chong, Natalia, & Hashim, 2002a; Izvekova, Solovyev, Kashinskaya, & Izvekov, 2013; Farhoudi, Abedian, Nazari, & Makhdoomi, 2013). In Mexico, the culture of

marine fish is based on a few species such as spotted rose snapper (*Lutjanus guttatus*) (Ibarra-Castro, & Alvarez-Lajonchère, 2011), totoaba (*Totoaba macdonaldi*) (Galaviz, et al. 2015) and red drum (*Sciaenops ocellatus*) (Gatlin III, 2002). In the State of Tabasco one of the most important commercial species is the common snook (*Centropomus undecimalis*), which is considered a euryhaline species with many adequate biological characteristics for its culture (Alvarez-Lajonchère, & Taylor, 2003); nevertheless, digestive physiology related to the changes of the digestive enzyme during initial ontogeny has been briefly studied (Jiménez-Martínez et al. 2012). Hence, the objective of this study was to characterize digestive proteases in *C. undecimalis* juveniles.

2. Materials and METHODS

2.1 Handling of Fish and Sampling

For the enzymatic characterization and *in vitro* digestibility bioassays, common snook juveniles were obtained from the wild from the Arroyo Verde community located in Comalcalco municipality borders, Tabasco, México. The fish were captured with conical nets, 15 meters in length and 3 meters deep. Once captured, the fish (50 juveniles, 13.98 ± 5.54 g average) were transported in aerated containers to the Laboratorio de Acuicultura Tropical at the División Académica de Ciencias Biológicas at the Universidad Juárez Autónoma de Tabasco, México, and fed with Nile Tilapia (*Oreochromis niloticus*) fry in plastic tanks of 2000 L during a period of 20 days until processing.

2.2 Multienzymatic Extracts Preparation

After the captivity adaptation period, the fish were starved for a 24-hour period and then exposed to an overdose of tricaine methanesulfonate (MS-222, 0.30 mg L^{-1}). The digestive apparatus was extracted from each fish, and the stomach and gut were separated and stored at 4ºC to avoid enzymatic denaturation. Gut samples were homogenized in a buffer of 50 mmol L^{-1} Tris-HCl and $CaCl_2$ 20 mmol L^{-1} at a pH of 7.5 (35 mg mL^{-1}). The stomachs were homogenized in a buffer of 100 mmol L^{-1} Glycine-HCl at a pH of 2.0. The samples were centrifuged at 16 000 *g* for 30 min at 4 °C to obtain the supernatant, placed in Eppendorf tubes and stored at -20 °C for further analysis.

2.3 Optimal pH and Temperature

Optimal pH was measured using the method described by Walter (1984), for the optimum pH for alkaline proteases (gut extracts) casein (1 g kg^{-1}) was used as the substrate, whereas for acid proteases (stomach extracts) the Anson (1938) technique was applied using hemoglobin (1 g kg^{-1}) as substrate. For pH adjustment the original buffers (50 mmol L^{-1} Tris-HCl and 20 $mmol^{-1}$ $CaCl_2$ with a pH of 7.5, and 100 mmol L^{-1} Glycine-HCl with a pH of 2.0) were substituted with the universal solution (Stauffer, 1989) to be adjusted to different pH levels for measuring the pH activities from 2 to 12. The optimal temperature for the stomach and gut proteases was measured with the techniques of Walter (1984), and Anson (1938), respectively, and modified at the temperatures of 25, 35, 45, 55 and 65 ºC. The incubation time in both cases were carried out for 5 minutes for acid proteases and 20 minutes for alkaline, and all assays were performed in triplicate.

2.4 Temperature and pH Stability

For pH stability, multi-enzymatic extracts were pre-incubated to the different pH levels for 0, 30, 60 and 90 min. The incubation was performed at room temperature. The optimal temperature for acidity and alkalinity was determined by modifying the incubation temperature within a 25-65 °C range with 10 °C intervals according to Anson (1938), and Walter (1984), respectively. To determine protease stability in relation to temperature changes, multi-enzymatic extracts were pre-incubated to the different temperatures previously mentioned for 0, 30, 60 and 90 min. For pH and temperature stability, the time of 0 min (no pre-incubation) was used as a control to determine the residual activity expressed as a percentage. All assays were performed in triplicate.

2.5 Effect of Inhibitors

The protease inhibition activity was determined as follows. For acidic proteases, pepstatin A inhibitor was used, and residual activity was determined with the method described by Anson (1938). In alkaline proteases, specific inhibitors were used as described by García-Carreño, Dimes, & Haard (1993). For residual activity on alkaline proteases, the technique described by Kunitz (1947) and modified by Walter (1984) was used. In both trials, a control with no inhibitor was used, and the residual activity was expressed as a percentage. All trials were conducted in triplicate. The analysis of the effect of inhibitors on the alkaline proteases was accompanied by SDS-PAGE electrophoresis in denaturalization processes using sodium dodecyl sulfate (SDS)-polyacrylamide gel. Four percent stacking gel and 10 g kg^{-1} running gel were used according to Laemmli (1970), with the protocol of Davis (1964) as modified by García-Carreño, Albuquerque-Cavalcanti, Navarrete del Toro, & Zaniboni-Filho (2002). Seven inhibitors were used as described by García-Carreño, Albuquerque-Cavalcanti, Navarrete del Toro,

& Zaniboni-Filho (1993), and they were pre-incubated in a 1:1 ratio (enzyme/inhibitor) for 1 h. A Bio Basic Inc. (Markham, Ontario, Canada) BM523 molecular weight marker was used along with Quantity One 1-D Analysis Software from Bio-Rad (Hercules, CA, USA) to calculate each molecular weight in each band.

To measure the effect of inhibitors on acidic protease activity, pepstatin A (1 mmol L^{-1}) was used, whereas for alkaline proteases the following specific inhibitors described by García-Carreño, Dimes, & Haard (1993) were used: phenylmethylsulfonyl fluoride (PMSF, 100 mmol L^{-1}), soy trypsin inhibitor (SBT1, 250 mmol L^{-1}), tosyl-phenylalanine chloromethyl ketone (TPCK, 10 mmol L^{-1}), tosyl-lysyl chloromethyl ketone (TLCK, 10 mmol L^{-1}), tetra acetate ethylene diamine (EDTA, 10 mmol L^{-1}), ovalbumin (250 mmol L^{-1}) and phenanthroline (10 mmol L^{-1}). The multi-enzymatic extracts were pre-incubated for an hour in a ratio of 1:1 with the different inhibitors, and the residual activity was measured in relation to an extract without pre-incubation and with no inhibitor. All assays were performed in triplicate.

2.6 Electrophoretic Analysis

Polyacrylamide electrophoresis was performed under denatured conditions (using sodium sulfate dodecyl) discontinuously for alkaline proteases following the protocol of Laemmli (1970), using a storer gel at 4% and slipping at 10%. The electrophoresis was performed in Mini PROTEAN III buckets (Bio-Rad) with two plates of vertical gels of 8×10×0.075 cm with a capacity of 20. The samples were prepared for the alkaline protease zymograms according to the method described by García-Carreño, Dimes, and Haard (1993) by running at 100 V at 5 ºC for 60 min. The gels were rinsed with distilled water and incubated for 30 min at 5 ºC in a casein solution of Hammerstein at 5% with a pH of 9. The inhibition analysis of the gut extracts was conducted with the same inhibitors mentioned earlier with preincubation for 1 hour. The gels were incubated for 90 min in the same solution at 37 ºC with no agitation. Finally, the gels were fixed with a TCA solution at 12% and stained with 0.1 % Coomassie R-250 brilliant blue, methanol, acetic acid and water (50:20:50) for 1 h and unstained with methanol-acetic acid-water (35:10:55). The clear zones indicated the presence of alkaline proteases after a period of 24 clearing hours; finally, the bands that appeared were compared with those of a control with no inhibitor.

For the analysis of acidic proteases continuous polyacrylamide electrophoresis was conducted under native conditions (10%). The electrophoresis conditions used were described by Walter (1984) and performed at 100 V and 64 mA by gel. To reveal the isoforms, the Díaz-López, Moyano-lópez, Alarcón-López, García-Carreño, & Navarrete-del Toro (1998) procedure was followed using a porcine pepsin solution (1 mg mL^{-1}, Sigma, 2,500-3,500 U mg of $protein^{-1}$) as a reference enzyme at a concentration of 30 µg of protein by well. After the electrophoresis, the gels were rinsed with HCl 0.1 mmol L^{-1} at a pH of 2.0 for acidic protease activation. After 15 minutes, the gels were incubated for 30 minutes at 4º C in a hemoglobin solution 0.25% in a buffer solution of glycine-HCl 100 mmol L^{-1} pH 2.0; after 90 minutes the same solution was used for a final incubation at 37 ºC. To evaluate the inhibition of the stomach extracts of *C. undecimalis*, pepstatin A was used in a 1:1 (enzyme/inhibitor) proportion; preincubation with the inhibitor was performed for 1 hour, and porcine pepsin used as the reference enzyme. Finally, the gels were rinsed with distilled water and fixed with a solution of TCA at 12 % for 15 minutes according to the aforementioned procedures with alkaline proteases.

2.7 Relative Migration Distance and Molecular Weight Calculation

A low-weight molecular weight marker was used (LRMWM) and applied for each SDS-PAGE with 5 µl added for the well. The LRMWM contained the following protein markers: phosphorylase b (97 kDa), serum bovine albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and tryptic soy inhibitor (20 kDa). The relative electromobility (Rf) was calculated for all of the zymograms (Igbokwe & Downe, 1978), and the molecular weight (MW) of each band with alkaline protease activity was calculated as the lineal adjustment between Rf and the decimal logarithm of the molecular weights of the proteins used as markers using the software Quality One V 4.6.5 (Hercules, CA).

3. Results

The optimal pH for gastric proteases was obtained at a pH of 2. It decreased drastically at a pH of 5 when the environment turned more alkaline (Figure 1a). The intestinal protolithic enzymes showed two peaks, the highest at a pH of 7, and its activity was reduced at the pH range from 8 to 10. The second peak of activity occurred at a pH of 11, decreasing at a pH of 12 (Figure 1b). The optimal temperature of the acidic proteases was shown at 75 ºC, decreasing at 85 ºC (Figure 1c); meanwhile, the optimal activity of the alkaline proteases was registered at 65 ºC; they showed high activity from 45 ºC until a drastic fall at 75 ºC (Figure 1d). The greater stability in acidic proteases was at a pH of 2 and 8, and it decreased with increasing pH (Figure 2a); meanwhile, the alkaline proteases could be more stable between the pH values of 4 and 10 at periods of preincubation of greater than 120 minute (Figure 2b). Acidic protease activity increased for 25, 35 and 45 ºC at 90 minutes of incubation (Figure

2c). Meanwhile, in alkaline proteases greater activity was exhibited at 35-45ºC after 90 minutes of incubation (Figure 2d).

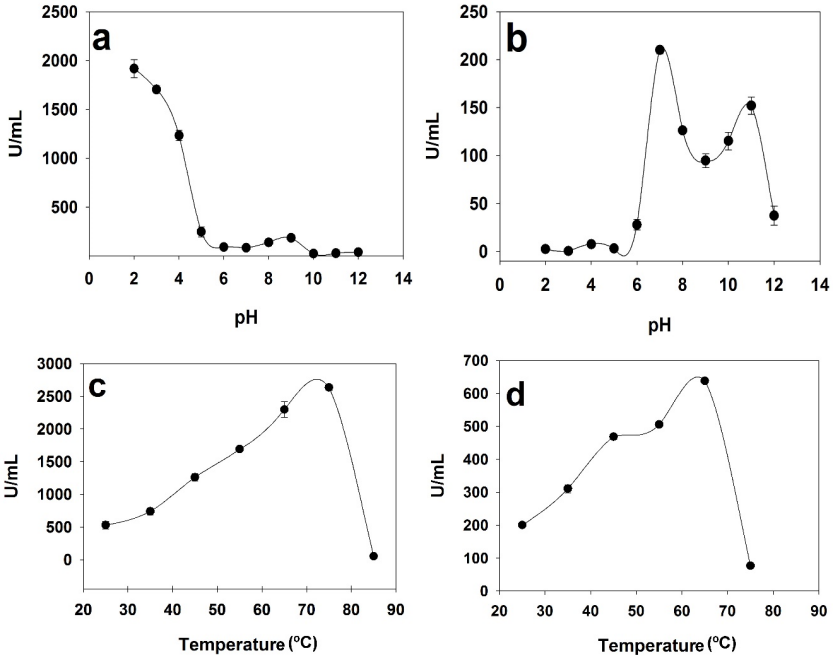

Figure 1. Optimum pH and temperature on digestive proteases of *Centropomus undecimalis* juvenile: (a) optimum pH acid proteases, (b) optimum pH alkaline proteases, (c) optimum temperature acid proteases, (d) optimum temperature alkaline proteases

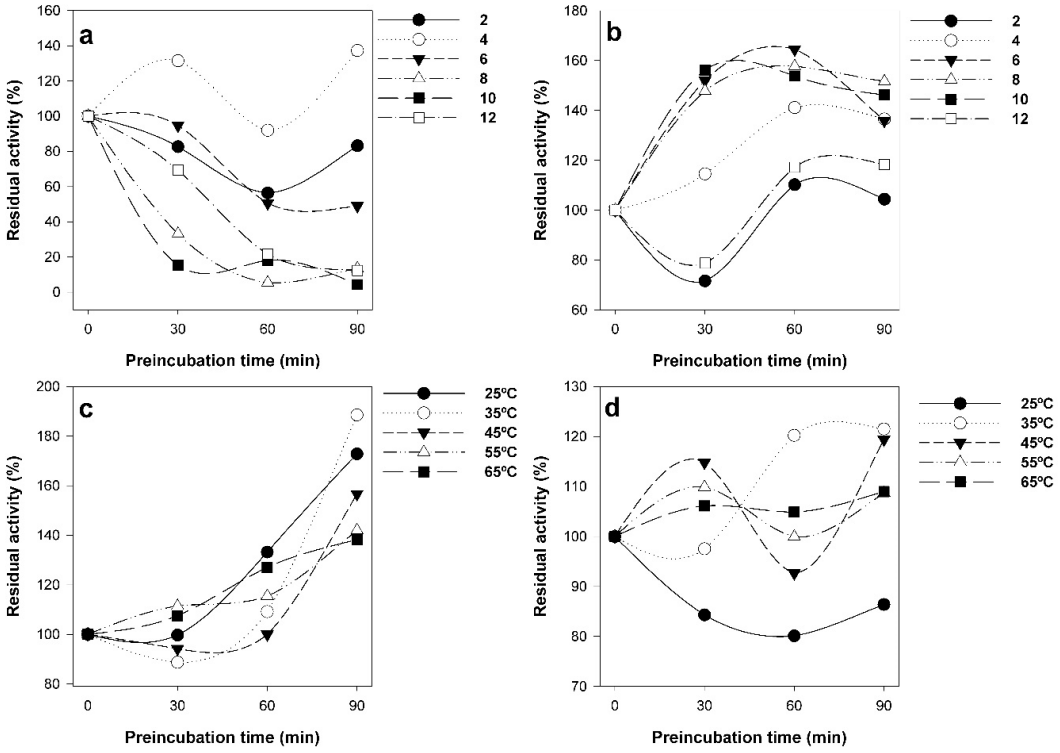

Figure 2. Stability of pH and temperature on digestive proteases of *Centropomus undecimalis* juvenile: (a) pH stability of acid proteases, (b) pH stability of alkaline proteases, (c) temperature stability of acid proteases, (b) temperature stability of alkaline proteases

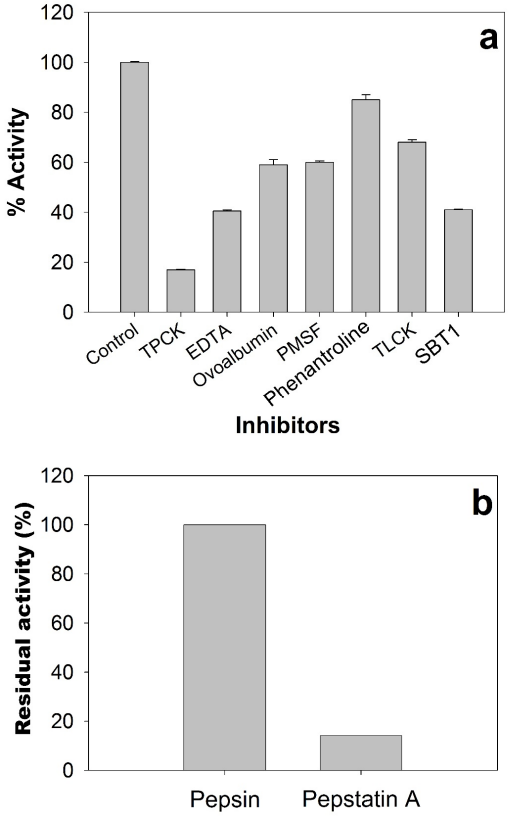

Figure 3. Effect on digestive protease activity reduction in multi-enzymatic extracts of *Centropomus undecimalis* juveniles by different inhibitors. (a) Alkaline proteases with no inhibitor (Control), tosylphenylanyl-chloromethyl ketone (TPCK), phenanthroline (Phen), ethyl-diamine tetra-acetic acid (EDTA), tosyl-lysyl-chloromethyl ketone (TLCK), ovalbumin (OVO), soybean trypsin inhibitor (SBT1), phenyl methyl sulphonyl fluoride (PMSF). (b) Acid proteases with no inhibitor (Pepsin), pepstatin A

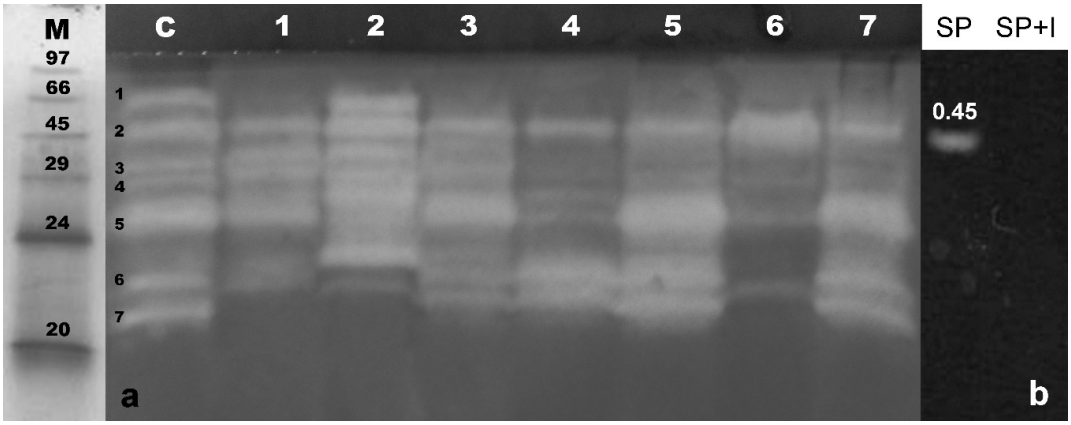

Figure 4. (a) SDS-PAGE electrophoresis analysis of alkaline digestive proteases of *Centropomus undecimalis* juveniles: M (LRMWM): phosphorylase b (97 kDa), serum bovine albumin (66 kDa), turkey egg albumin (45 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa) and trypsin soybean inhibitor (20 kDa); the control (band 1: 65.2, band 2: 45.6, band 3: 30.1, band 4: 28.7, band 5: 25.2, band 6: 22.6 and band 7: 21.3 kDa); inhibitors: Line 1: Phenyl methyl sulphonyl fluoride (PMSF); Line 2: Soybean trypsin inhibitor (SBT1); Line 3: Tosyl-phenylanyl-chloromethyl ketone (TPCK); Line 4: Tosyil-lysyl-chloromethyl ketone (TLCK); Line 5: Phenanthroline (Phen); Line 6: Ovalbumin (OVO); and Line 7: Ethyl-diamine tetra-acetic acid (EDTA). (b) effect of pepstatin A on common snook pepsin. SP: Snook pepsin, SP+I: snook pepsin + inhibitor

For the inhibition test, the enzymatic extracts of *C. undecimalis* juveniles showed that tosyl phenylalanyl chloromethyl ketone (TPCK) reduced alkaline protease activity by 17%, while tosyl lysyl chloromethyl ketone (TLCK) inhibited it by 68%. The 1-10 phenanthroline showed a greater inhibition percentage with 85%, soy trypsin inhibitor (SBT1) reduced the activity by 41%, EDTA (ethylenediaminetetraacetic acid) showed 40.5% activity reduction, PMSF (phenyl-methylsulfonyl fluoride) reduced the proteolytic activity by 60% and the ovalbumin inhibited the intestinal proteases activity by 59% (Figure 3a). Eighty-six percent of the acid proteases were affected when preincubated with pepstatin A compared with the control without an inhibitor (Figure 3b).

The data obtained by the SDS-PAGE technique showed three active bands, indicating the presence of three alkaline digestive protease groups which where inhibited partially by the inhibitors PMSF, TLCK, TPCK, Ovalbumin, SBT1, EDTA and 1-10 phenanthroline. The inhibitors TPCK, 1-10 phenanthroline, EDTA and PMSF inactivated the expression of the three proteolytic bands in common snook, whereas TLCK and ovalbumin only inhibited one band and SBT1 inhibited two active bands of alkaline proteases (Figure 4a). The inhibition of acid proteases showed only one active band, which was inhibited completely by pepstatin A. In this assay porcine pepsin was used as the control enzyme, and it was inhibited by the same inhibitor (Figure 4b).

4. Discussion

In common snook, the pepsin activity was greater (25 U mL^{-1}) than the activity exhibited by the alkaline proteases (17 units ml^{-1}), revealing that the digestion of protein is performed mostly in the stomach followed by the intestines. Similar data have been reported for *Scleropages formosus, Solea senegalensis* and *Glyptosternum maculatum* (Natalia, Hashim, Ali, & Chong 2004; Saénz et al. 2005; Xiong, Xie, Zhang, & Liu, 2011). Pepsin is the main acid protease in fish stomach, and the first protolithic enzyme requires it for breaking large polypeptide chains (Tengjaroenkul, Smith, Caceci, & Smith, 2000; Zhao, Budge, Ghaly, Brooks, & Dave, 2011); it showed an optimal pH of 2, reported earlier for *S. senegalensis*, *Scophthalmus maximus L.* and *C. carpio* L. (Saénz et al. 2005; Wang, Wang, Wang, Xue, & Sun, 2006; Al-Saraji, & Nasir, 2013). It was stable for different pH values with a minor decline at alkaline pH levels showed an increase in activity after 60 minutes of incubation and destabilized at a pH of 10. Similar results were reported by Wang, Wang, Wang, Xue, and Sun (2006), in *S. maximus*, which showed instability at a pH of 8. This enzyme can hydrolyze the protein until the foregut where the pH increases and alkaline proteases start their activity (Alarcón, Díaz, Moyano, & Abellan, 1998; Álvarez-González, 2003; Sankar, Varadarajan, Bhanu, Joy, & Philip, 2014). Castillo-Yañez, Pacheco-Aguilar, García-Carreño, & Navarrete-Del Toro (2004), indicated that in warm water species, gastric proteases are stable at neutral pH.

Alkaline proteases showed two peaks at a pH of 7 and 11. Similar results have been reported for *S. aequifasciata, T. thynnus*, *P. maculatofasciatus* and *B. splendens* (Chong, Hashim, & Bin Ali, 2002b; Essed, Fernández, Alarcón, & Moyano, 2002; Álvarez-González, 2003), suggesting that the peak at a pH of 7 could be due to the trypsin because the optimal pH ranges from 7 to 9 (Das, & Tripathi, 1991; Castillo-Yáñez, Pacheco-Aguilar, García-Carreño, & Navarrete-Del Toro, 2005) while chymotrypsin works at a pH between 9 and 11. Our results are similar to those reported by Klahan, Areechon, Yoonpundh, & Engkagul (2009), for *O. niloticus.* Alkaline proteases in snook are stable at pH values from 4 to 10 during the first 120 minutes of incubation, decreasing their activity at 180 minutes, destabilizing in the first 60 minutes at extreme pH, and showing greater activity at a pH of 8. This is similar to that reported by Álvarez-González (2003) in *P. maculatofasciatus.*

Considering the above-mentioned, Thongprajukaew, Kovitvadhi, Engkagul, & Rungruangsak-Torrissen (2010). and Xiong et al. (2011) reported an optimal temperature for acid proteases at 50-60°C in *B. splendens* and *G. maculatum* with denaturalizing after 60°C. Although, pepsin in *C. undecimalis* showed an optimal temperature at 75°C. In this sense, Korostelev, Nevalenny, and Levchenko (2005), reported that fish digestive enzymes could be adapted to the operating conditions to ensure optimal digestive functions. The tolerance to high temperatures from snook pepsin can be a biological adaptation of the enzyme because this fish inhabits warm waters ranging from 15 to 35ºC (Tucker Jr, 2003). Acid proteases were stable at temperatures from 25 to 45 ºC with greater activity at 90 minutes of exposure and decreasing after 60 minutes at 65ºC, indicating a loss in activity after 90 minutes of exposure, which was similar to the study of Castillo-Yañez, Pacheco-Aguilar, Garcia-Carreño, & Navarrete-Del Toro (2004), whom reported for Monterrey sardine *Sardinops sagax caerulea* an inverse relation where if temperature and incubation time increases, the gastric protease activity decrease.

Alkaline optimal temperatures for studied in fish ranges from 30 to 60ºC (Alarcón, Díaz, Moyano, & Abellan, 1998). The alkaline proteases in snook showed maximum proteolytic activity at 65ºC, similar to what was reported for *B. orbignyanus* and *T. thynnus* (Essed, Fernández, Alarcón, & Moyano, 2002; García-Carreño, Albuquerque-Cavalcanti, Navarrete del Toro, & Zaniboni-Filho, 2002), and temperature stability from 45 to 65ºC, showing maximum activities at temperatures of 35 and 45ºC at 90 minutes of incubation. García-Carreño, Albuquerque-Cavalcanti, Navarrete del

Toro, & Zaniboni-Filho (2002), reported temperature stability at 50°C for *B. orbignyanus*, whereas Álvarez-González (2003) found stability at 45 and 55ºC at 90 minutes of preincubation for *P. maculatofasciatus*. In this study the acid proteases showed more stability than alkaline proteases, although Essed, Fernández, Alarcón, & Moyano (2002), found greater stability for alkaline proteases in *T. thynnus* than acid proteases.

Acid protease activity was reduced by 86% when exposed to pepstatin A, such as Klomklao, Kishimura, Yabe, & Benjakul (2007) reported total inhibition for pepstatin A in Giant grenadier *Coryphaenoides pectoralis*. The active band of porcine pepsin and snook were inhibited completely by the presence of pepstatin A. Similar results were reported for *B. orbignyanus* and *P. maculatofasciatus* (García-Carreño, Albuquerque-Cavalcanti, Navarrete del Toro, & Zaniboni-Filho 2002; Álvarez-González, 2003). PMSF inhibited the total activity of digestive proteases by 60%. This is similar to the data reported for *S. aequifasciata* and *Labeo rohita* (Chong, Natalia, Hashim, & Ali, 2002a; Chakrabarti, Rathore, & Kumar, 2006). Alkaline protease inhibition was 59% with the presence of ovalbumin compared with the 5% reported by Álvarez-González (2003) for *P. maculatofasciatus*. The inhibition by PMSF and ovalbumin in snook gut indicates the presence of serine proteases that act as exclusive inhibitors (García-Carreño, Albuquerque-Cavalcanti, Navarrete del Toro, & Zaniboni-Filho, 2002; Chakrabarti, Rathore, & Kumar, 2006). Jonás, Rágyanszki, Oláh, and Boross (1983), and García-Carreño, Albuquerque-Cavalcanti, Navarrete del Toro, and Zaniboni-Filho (2002), reported that trypsin showed more activity in carnivorous fish than omnivorous and herbivorous species, where chymotrypsin activity is greater. In this study the reduction of proteolytic activity by SBT1 was 41%, similar to that reported for *S. aurata* and *D. dentex* (Alarcón, Díaz, Moyano, & Abellan, 1998) where inhibition ranged from 41 to 50% of the activity. This activity was also reduced in spotted wolffish (*Anarhichas minor*) feed with diets with SBT1 (Savoie et al. 2011). TLCK inhibited 68% of the total activity, similar to the findings for *S. formosus* (Natalia, Hashim, Ali, & Chong, 2004). TPCK, an exclusive inhibitor of chymotrypsin, reduced the activity by 17%, similar to that reported by Chakrabarti, Rathore, & Kumar (2006) in *L. rohita*. Inhibition results with SBT1 and TLCK, indicating the importance of trypsin and chymotrypsin in snook digestion, where trypsin activity is greater than chymotrypsin activity; this result is similar to that reported by Kumar, García-Carreño, Chakrabarti, Navarrete-Del Toro, & Córdova-Murueta (2007) in catla *Catla catla, L. rohita* and grass carp *Hypophthalmichthys molitrix*. EDTA inhibited enzyme activity by 40.5%; similar values were reported for spotted sand bass (40%) (Álvarez-González, 2003), suggesting the presence of metalloproteases (Chong, Natalia, Hashim, & Ali, 2002a), enzymes which are absorbed directly by enterocytes, especially essential amino acids. The 1-10 phenanthroline chelate that acts as a metalloprotease showed an inhibition of 85%, a rather high value compared with those reported by Álvarez-González (2003), which fluctuated between 20-30% of enzymatic inhibition. The sensibility differences of the digestive proteases could be because the enzymatic structures could vary among species.

The three active bands showing for the snook intestine confirm the presence of proteases trypsin, chymotrypsin and metalloproteases. The molecular weight was 25 and 35 kDa for trypsin and chymotrypsin, respectively. Rivera (2003), Rodríguez (2004), and Souza, Amaral, Albérico, Carvalho Jr, & Bezerra (2007), reported a molecular weight of (24-26.1 kDa) for trypsin in Silk snapper (*Lutjanus vivanus*), white grunt (*Haemulon plumierii*) and spotted goatfish (*Pseudupeneus maculatus*). The trypsin band was affected by TLCK; SBT1 inhibited the trypsin and chymotrypsin bands, showing that trypsin inhibitors equally affect chymotrypsin activity, which was similar to the results of Alarcón, Díaz, Moyano, & Abellan (2001), where these inhibitors inhibited the band with molecular weights of 20 and 30 kDa in yellow snapper (*Lutjanus argentiventris*) and Pacific dog snapper (*Lutjanus novemfasciatus*). Lazo, Mendoza, Holt, Aguilera, & Arnold (2007), reported trypsin with molecular weights of 23 and 68 kDa and chymotrypsin with 25 kDa in *S. ocellatus*. Meanwhile, Natalia, Hashim, Ali, & Chong (2004), found trypsin and chymotrypsin with molecular weights of 32.3 kDa and 42.1 kDa, respectively, in *S. formosus.* The greatest Rf found corresponded to the metalloproteases, which have molecular weights between 45 to 97 kDa according to the reports for *S. formosus* (Natalia, Hashim, Ali, & Chong, 2004). EDTA completely inhibited 100% of the active bands, similar to the result reported by Alarcón, Díaz, Moyano, & Abellan (1998) for *D. dentex* and *S. aurata*, revealing the dependency of these enzymes on divalent cations.

5. Conclusion

Considering the biochemical and physiological aspects of this study, it is demonstrated that digestion in *C. undecimalis* is constituted of a single acidic protease in the stomach that was fully inhibited, which is not resistant to pH and temperature changes. Instead, alkaline protease digestion is lower compared with acid protease activity and it is more stable to the variation of pH, temperature, and it is poorly resistant to inhibitors, indicating that there is a good alkaline digestive protease capacity to hydrolyse different protein sources, specially those of animal origins. Consequently, this species can be considered a marine fish with carnivorous nutritional habits.

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