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Original article

Partial characterization of digestive proteases in sheepshead, *Archosargus probatocephalus* (Spariformes: Sparidae)

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Digestive proteases were partially characterized in sheepshead juveniles, using biochemical and electrophoretic techniques. Results showed higher activity level of the stomach proteases (2.39 ± 0.02 U mg protein^{-1}) compared to the intestinal proteases (1.6 ± 0.1 U mg protein^{-1}). The activity of trypsin, chymotrypsin, leucine aminopeptidase and carboxypeptidase A was also recorded. The optimum temperature of the stomach proteases was recorded at 45 °C, while for intestinal proteases was recorded at 55 °C. Stomach proteases showed less stability to temperature changes than intestinal proteases. An optimum pH of 2 was recorded for stomach proteases with high stability under acidic conditions, while an optimum pH of 9 was recorded for intestinal proteases showing high stability under alkaline conditions. Stomach proteases were inhibited around 78% with Pepstatin A, indicating the presence of pepsin as the main protease. The stomach proteases zymogam revealed one active band with Rf of 0.49, this enzyme was completely inhibited by Pepstatin A. The intestinal proteases zymogram revealed four active proteases (51.3, 34.9, 27.8 and 21.2 kDa) that were inhibited by TLCK, which mainly represent a trypsin-like serine proteases. It can be conclude that digestion in sheepshead can be considered as a carnivorous species with an omnivorous tendency.

Keywords: Aquaculture, Inhibitors, Pepsin, Proteases, Zymogram

Se caracterizaron parcialmente las proteasas digestivas de juveniles del sargo, utilizando técnicas bioquímicas y electroforéticas. Los resultados muestran mayores niveles de actividad en las proteasas estomacales (2.39 ± 0.02 U mg proteina^{-1}) comparados con los de las proteasas intestinales (1.6 ± 0.1 U mg protein^{-1}), también se registró la actividad de tripsina, quimotripsina, leucina aminopeptidasa y carboxipeptidasa A. La temperatura óptima de las proteasas estomacales fue de 45 °C, mientras que la de las proteasas intestinales fue de 55 °C. El pH óptimo fue de 2 para las proteasas estomacales con alta estabilidad a condiciones ácidas, mientras que el pH óptimo para las proteasas intestinales fue de 9, mostrando una alta estabilidad en condiciones alcalinas. Las actividades de las proteasas estomacales fue inhibida en un 78% con Pepstatina A, lo que indica la presencia de pepsina, como principal proteasa. El zimograma de proteasas estomacales reveló una sola banda con actividad proteasa, con Rf de 0.49, completamente inhibida por Pepstatina A. El zimograma de proteasas intestinales reveló cuatro bandas (51.3, 34.9, 27.8 y 21.2 kDa). Todas las bandas se inhibieron con TLCK, lo que muestra la presencia principalmente de serina proteasas tipo tripsina. Se concluye que la digestión del sargo puede ser considerada como la de una especie carnívora con tendencia al omnivorismo.

Palabras Clave: Acuicultura, Inhibietores, Pepsina, Proteasas, Zymograma

Introduction

The sheepshead fish, *Archosargus probatocephalus* (Walbaum, 1792), is a coastal, subtropical marine fish that regularly enters brackish waters. This species is an important component of the commercial and recreational-sport fisheries along the Atlantic coast of the US and the Gulf of Mexico (Figueira, Coleman, 2010; McDonough *et al*., 2011), and has recently been considered viable for the development of mariculture in the southeast Mexican region (RNIIPA, 2012).

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Tucker (1998, 2004) describes the species with aquaculture potential since it has great adaptability to captivity, a wide range of feeding sources, adapts easily to high density conditions and has been induced to spawn, allowing the production of juveniles under laboratory conditions. However, in order to achieve commercial scale cultivation it is necessary to understand the basic nutritional aspects that allow the development of balanced foods at the lowest possible cost. Therefore, it is of great importance to study digestive physiological processes in which digestive enzymes are vital to the effective hydrolysis of nutrients during digestion (Scocco *et al*., 1997; Olsson, Holmgren, 2001). The sheepshead demonstrates an omnivorous trophic strategy, an unusual behaviour between sparids that generally tends to be carnivorous, and an ability to change from a carnivorous habit to an omnivorous or herbivorous habit during their development (Patillo *et al*., 1997). Early juvenile sheepsheads have been previously reported to develop carnivorous habits when they grow to a length of around 50 mm (GSMFC, 2006), whilst the adults are considered omnivorous since they mainly feed on algae and invertebrates (Jobling, 1995; Cutwa, Turingan, 2000; Castillo-Rivera *et al*., 2007).

Hence, studies of digestive physiology are necessary for the development of a specific diet formulation, to determine the amount and the type of ingredients to be incorporated into the diet, which depends on the enzymatic activity present in the digestive system, particularly in the case of proteases, since protein is the most important nutrient in relation to energy and amino acid requirements in fish (Lazo *et al*., 2007). In fish, the digestion process involves several enzymes that are responsible for protein hydrolysis (Eroldogan *et al*., 2008), where the activity expressed depends on several factors such as the feeding habits, food type, among other (Kuz'mina, Strel'nikova, 2008). In this way, the characterization of digestive proteases allows the determination of the optimal conditions for enzymes to reach their maximum activity as well as stability at different temperature and pH conditions (Simpson, 2000), in addition to the effect of inhibitors on protease activity (Falcon- Hidalgo *et al.*, 2011; Unajak *et al*., 2012). Likewise, characterization of digestive enzymes shows the effect of physicochemical conditions, which let us to understand the kinetic mechanism of the enzymes in the species of interest (García-Carreño *et al*., 1993; Klomklao *et al*., 2008; Álvarez-González *et al*., 2010).

Considering the above mentioned, digestive protease characterization studies have been carried out for many years in different species of marine fish such as: gilthead seabream *Sparus aurata*, Atlantic bluefin tuna *Thunnus thynnus* (Linnaeus, 1758), Asian bony tongue *Scleropages formosus* (Müller & Schlegel, 1840), Monterey sardine *Sardinops sagax* (Jenyns, 1842), Senegalese sole *Solea senegalensis* Kaup, 1858, turbot *Scophthalmus maximus* (Linnaeus, 1758), Pacific bluefin tuna *Thunnus orientalis* (Temminck & Schlegel, 1844), Albacore tuna *Thunnus alalunga* (Bonnaterre, 1788), spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869) and common snook *Centropomus undecimalis* (Bloch, 1792) (Alarcón *et al*., 2001; Essed *et al*., 2002; Natalia *et al.*, 2004; Castillo-Yañez *et al*., 2005; Sáenz -de Rodrigáñez *et al.*, 2005; Wang *et al.*, 2006; Matus-De la Parra *et al.*, 2007; Nalinanon *et al.*, 2010a; Peña *et al*., 2015; Concha-Frías *et al*., 2016). In these studies it has been shown that acidic proteases commonly present an optimal activity between pH 2 and 3 and optimal temperatures between 35 and 65 °C. On the other hand, most of the alkaline proteases maintain an optimum pH between 9 and 10, and an optimal temperature of between 45 and 65 °C. In relation to inhibitors, in some carnivorous species the activity of acid proteases is totally inhibited by Pepstatin A, which represents pepsin-like type activity. In contrast, the inhibition of alkaline proteases is variable among species, as they are influenced by the environment and feeding habits (Díaz-López *et al*., 1998; Kuz`mina *et al*., 2008; Falcon-Hidalgo *et al*., 2011).

Some reports in certain fish species indicate that, changes in specific enzyme activity vary at different ages (Chiu, Pan, 2006; Falcon-Hidalgo *et al*., 2011; Peña *et al*., 2015). Therefore, the objective of this work is to partially characterize the digestive proteases of juvenile sheepshead (*A. probatocephalus*), to provide basic information on the digestive physiology, the optimal conditions, stability and mechanisms of action of these enzymes, since in this species is still unknown, which will provide a basis for the elaboration of practical diets that allows adequate growth in culture.

Material and methods

Preparation of enzymatic extracts. Enzymatic extracts were prepared from five wild sheepshead (*Archosargus probatocephalus*, voucher ECOSC 13593, four specimens) juveniles (100 ± 10 g), captured from the lagoon of Alvarado, Veracruz, Mexico. The fish were starved for 24 hours and sacrificed by thermic shock using ice water. The weight was recorded in each organism with an Ohaus® digital balance (0.01 g, Shenzhen, China), and total length with an ichthyometer. Extraction of stomach and intestine was performed under cold conditions (4 ºC), registering the weight of each organ, which were then frozen and lyophilized. Subsequently each organ was homogenized separately in a ratio of 1:30 (weight: buffer volume) and homogenized with an IKA (MCA T18 basic Ultraturrax) tissue homogenizer. The stomachs were homogenized in 100 mmol L^{-1} glycine-HCl buffer at pH 2 and the intestines were homogenized in 30 mmol L^{-1} Tris-HCl + 12.5 mmol L^{-1} $CaCl_2$ buffer at pH 7.5, the enzymatic extracts of the specimens were processed individually. The homogenates were centrifuged at 14,000 g for 30 min at 4 °C, and the supernatants were stored at -80 °C until analysis. The concentration of soluble protein in the enzymatic extracts of stomach and intestine was determined with the technique described by Bradford (1976). All tests were performed in triplicate.

Protease activity analysis. Acidic protease activity was determined using the technique described by Anson (1938),

using bovine hemoglobin 1% as a subtrate in 100 mmol L^{-1} glycine-HCl buffer at pH 2. The alkaline protease activity was determined using the technique described by Kunitz (1947) modified by Walter (1984), using casein 1% as a substrate in 100 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} $CaCl_2$ buffer at pH 9. One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µmol of product (tyrosin liberated) per minute. The hydrolysis of haemoglobin and casein was determined at 280 nm, the molar extinction coefficient of tyrosine was used (0.005 mL $\mu g^{-1}cm^{-1}$) for both proteases. The trypsin activity was determined with the method described by Erlanger *et al*. (1961), using 1 mmol L^{-1} of Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) as a substrate in 100 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} $CaCl_2$ buffer at pH 8, one unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µmol of p-nitroaniline per minute, using a molar extinction coefficient of 8.8 mL $\mu mol^{-1}cm^{-1}$. Chymotrypsin activity was measured as described by Del Mar *et al*. (1979), a technique using 0.1 mmol L^{-1} of SAAPNA (N-succinyl-ala-ala-pro-phe p-nitroanilide) as a substrate in 100 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} $CaCl_2$ buffer at pH 7.8, one unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µmol of p-nitroanilide liberated per minute, using a molar extinction coefficient of 0.906 mL $\mu mol^{-1}cm^{-1}$. The activity of leucine-aminopeptidase was measured with the technique described by Maroux *et al*. (1973), using 1 mmol L^{-1} of leucine p-nitroanilide as substrate in 50 mmol L^{-1} monobasic sodium phosphate buffer at pH 7.2, one unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µmol of alanine p-nitroaniline liberated per minute, with molar extinction coefficient of 8.2 mL $\mu mol^{-1}cm^{-1}$. The carboxypeptidase A activity was measured with the technique described by Folk and Schirmer (1963), using 1 mmol L^{-1} of hipuryl-L-phenylalanine as a substrate in 25 mmol L^{-1} Tris-HCl, 50 mmol L^{-1} NaCl buffer at pH 7.5 solution, one unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µmol of hippuric acid per minute, using molar extinction coefficient of 0.36 mL $\mu mol^{-1}cm^{-1}$. All tests were performed in triplicate. Total activity (Units mL^{-1}) = [Δabs*reaction final volume (mL)]/[MEC*time (min)*extract volume (mL)]. Specific activity (Units mg $prot^{-1}$) = Total activity/soluble protein (mg), where Δabs represent the increase in absorbance, and MEC represents the respective molar extinction coefficient.

Effect of pH and temperature on digestive proteases. The optimum pH of the acidic and alkaline proteases was observed within the range of 2 to 12, using the universal solution of Stauffer (1989), substituted in the technique of Anson (1938) and Walter (1984) respectively. For pH stability, enzyme extracts were preincubated (25 °C) at different pH's at time intervals of 0, 30, 60 and 90 min. The optimal temperature of the acidic and alkaline proteases was determined by modifying the incubation temperature within a range of 25 to 65 °C with intervals of 10 °C, in the techniques described by Anson (1938) and Walter (1984) respectively. To determine the stability of the proteases during temperature changes, the enzyme extracts were preincubated at the different temperatures mentioned above for 0, 30, 60 and 90 min, where the time 0 min (without pre-incubation) was used as a control to determine the residual activity expressed as a percentage. All tests were performed in triplicate.

Effect of inhibitors on protease activity. The sensitivity of the protease activity was determined using specific inhibitors: 1 mmol L^{-1} of Pepstatin A inhibitor was used for the acid proteases, and its residual activity was determined with the Anson (1938) technique as previously described. For the alkaline proteases, the specific inhibitors were used as described by García-Carreño *et al*. (1993): 5 mmol L^{-1} tosyl-phenylanyl-chloromethyl ketone (TPCK), 10 mmol L^{-1} phenanthroline (PHE), 10 mmol L^{-1} ethyl-diamine tetra-acetic acid (EDTA), 10 mmol L^{-1} tosyl-lysyl-chloromethyl ketone (TLCK), 250 mmol L^{-1} soybean trypsin inhibitor (SBT1), 100 mmol L^{-1} phenyl methyl sulphonyl fluoride (PMSF) and the 250 mmol L^{-1} of the inactivator ovalbumin (OVO). The residual activity of the alkaline proteases was determined with the technique described by Kunitz (1947) and modified by Walter (1984) as previously described. In acid and alkaline activities, a control sample without inhibitor was used and the residual activity was expressed as a percentage, all tests were performed in triplicate.

Zymogram analyses. Electrophoretic analyses were performed with a Mini-PROTEAN 3 Cell (Bio-Rad) with four plates containing vertical gels (8 × 10 × 0.075 cm) with 10 ml sample capacity per plate. For the analysis of stomach acid proteases, electrophoresis was run under non-denaturing native condition (native-PAGE) using a continuous polyacrylamide gel (10%) in Tris buffer (25 mmol L^{-1}) y glycine (192 mmol L^{-1}, pH 8.3, 80 volts) according to Davis (1964). For the analysis of alkaline proteases, the plate was prepared by stacking a gel with 4% polyacrylamide (PAA) and resolving the gel with 10% PAA. Electrophoresis was run under denaturalizing conditions (SDS-PAGE), with SDS in 0.1% Tris buffer (25 mmol L^{-1}) and glycine (192 mmol L^{-1}, pH 8.3, 100 volts), according to Laemmli (1970), and adapted by García-Carreño *et al*. (1993). The gels were treated to reveal proteases isoforms according to the procedure of Díaz-López *et al*. (1998), were washed and incubated for 30 min at 5°C in a 0.5% casein solution (Tris-HCl 100 mmol L^{-1} buffer, pH 9), then gels were incubated for 90 min in the same solution composition at 37 °C, and then washed and fixed in trichloroacetic acid (12%) solution for 15 min. After areas for detection of enzyme activity had been developed, they were stained according to Weber, Osborn (1969), using a 0.1% Coomassie brilliant blue R-250 solution, while distaining was carried out in a 35:10:55 solution of methanol-acetic acid-water. Clear zones revealed the activity of proteases within a few minutes, although well-defined zones were obtained after 2-4 h.

Electrophoresis was complemented with the use of specific inhibitors previously mentioned, according to the method described by Dunn (1989). The enzymatic extracts were pre-incubated for 1 h with every inhibitor (1:1 v/v) and were applied to each SDS-PAGE (5 µl per well). A molecular weight marker was used: BM523 (molecular weight marker Bio Basic Inc; rabbit phosphorylase B 96.7 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45 kDa, carbon anhydrase 31.0 kDa, trypsin soybean inhibitor 21.5 kDa and lysozyme 14.4 kDa). The relative electromobility (Rf) was calculated for the zymograms according Igbokwe, Downe (1978). Molecular weight (MW) of each band in the SDS--Zymogram (alkaline proteases) was calculated using a linearly adjusted relationship between the Rf and log10 of the MW protein markers, using the Quality One version 4.6.5 (Hercules, CA) software program.

Statistic analysis. Data did not comply with the assumptions of normality and homoscedasticity, therefore, a nonparametric variance analysis (Kruskal-Wallis) was used to compare the residual activity between pH, and Chi^2 test from the arcsine transformation was used to analyse temperature stability and the percentage of residual activity in the inhibition tests of acidic and alkaline proteases. A non-parametric Nemenyi test was used when significant differences were detected. All tests were carried out with Statistica v 7.0 software (StatSoft, Tulsa, OK, EU).

Results

The proteolytic activity level of the digestive tract of juvenile sheepshead is shown in Tab. 1. The activity of the acid proteases (2.39 ± 0.02 U mg protein^{-1}) was higher than alkaline protease activity (1.6 ± 0.1 U mg protein^{-1}), as well as the activity of other alkaline endopeptidase enzymes such as trypsin and chymotrypsin (0.45 ± 0.02 and 0.65 ± 0.1 U mg protein^{-1}, respectively) and exopeptidases leucin aminopeptidase and carboxypeptidase A (0.007 ± 0.001 and 0.5 ± 0.1 U mg protein^{-1}, respectively).

Tab. 1. Specific activity levels of digestive proteases (mean ± SD, n = 3) in enzymatic extracts of stomach and intestine from juveniles of *Archosargus probatocephalus*.

Enzyme	Specific activity (U mg protein^{-1})
Acid proteases	2.39 ± 0.02
Alkaline proteases	1.6 ± 0.1
Trypsin	0.45 ± 0.02
Chymotrypsin	0.64 ± 0.1
Carboxypeptidase A	0.5 ± 0.1
Leucine aminopeptidase	0.007 ± 0.001

The optimum pH for acid protease activity was 2 (Fig. 1a). The stability to pH changes was greater between pH 2 and 4, compared with the stability observed at the other pH values (Fig. 1b). Residual activity of acid proteases was maintained around 100% in pH 2 and 4 until 90 min of preincubation, while the rest of the pHs decreased after 30 min of preincubation. For alkaline protease activity the optimum pH was observed to be 9 (Fig. 1c), while greater stability to changes in pH was observed at pH 10, demonstrating a significant difference ($P < 0.05$) when compared to other pHs, reaching above 130% residual activity until the time of 90 minutes of preincubation. Likewise, for pH 8, 100% activity was detected at up to 90 minutes of preincubation (Fig. 1d).

The optimum temperature of acid protease activity was 45 °C (Fig. 2a). Likewise, greater stability was recorded at 25 °C (significant difference, $P < 0.05$), with 100% activity until 90 min of preincubation. The stability of acid proteases was reduced to 90% at temperatures of 55 and 65 °C after 30 minutes of preincubation (Fig. 2b). In contrast, the optimum temperature of alkaline protease activity was 55 °C (Fig. 2c), where greater stability was recorded between 25 and 45 °C, showing an activity of 115% at up to 90 min of preincubation. Finally, the stability of alkaline protease activity was reduced by 90% at 65 °C after 30 minutes of preincubation (Fig. 2d).

The analysis with inhibitors indicates that acidic protease activity was inhibited 79% by Pepstatin A. Alkaline proteases activity showed that 30% of the total activity was inhibited by TPCK, while TLCK inhibited 75%. Ovalbumin inactive 61%, SBTI inhibited 70% and with PMSF inhibited 45%. Chelants such as PHEN and EDTA inhibited the 23% and 7%, respectively (Fig. 3). The electrophoresis under native-PAGE conditions revealed a single acid protease with relative electromobility (Rf) of 0.49, this band was completely inhibited by Pepstatin A (Fig. 4). In the SDS-PAGE electrophoresis analysis of alkaline proteases without inhibitor revealed four bands with molecular weights ranging from 21.2 to 51.3 kDa (Fig. 5). Ovalbumin inhibited the band of 51.3 kDa, and SBTI inhibited two bands (21.2 and 27.8 kDa), while TLCK inhibited all bands. By the other side, presence of PMSF, TPCK, PHEN and EDTA did not inhibit any active band.

Discussion

Our results show that *A. probatocephalus* juveniles display a higher activity of acid proteases compared to alkaline proteases. These results indicate that juveniles of this species have carnivorous habits, similar to other fish such as *S. aurata* (Alarcón *et al*., 2001), *T. thynnus* (Essed *et al*., 2002), *Scleropages formosus* (Natalia *et al*., 2004), *Scophthalmus maximus* (Linnaeus, 1758) (Wang *et al*., 2006), *Atractosteus tropicus* Gill, 1863 (Guerrero-Zárate *et al*., 2014), common dentex *Dentex dentex* (Linnaeus, 1758) (Alarcón *et al*., 1998), *S. senegalensis* (Saénz -de Rodrigáñez *et al*., 2005) and *C. undecimalis* (Concha-Frias *et al*., 2016), which exhibit high pepsin-like activity in the stomach, and a lower activity of alkaline proteases detected in the intestine, where the final part of the hydrolysis process takes place, releasing peptides and amino acids, which are subsequently absorbed by the enterocytes (Yúfera, Darias, 2007).

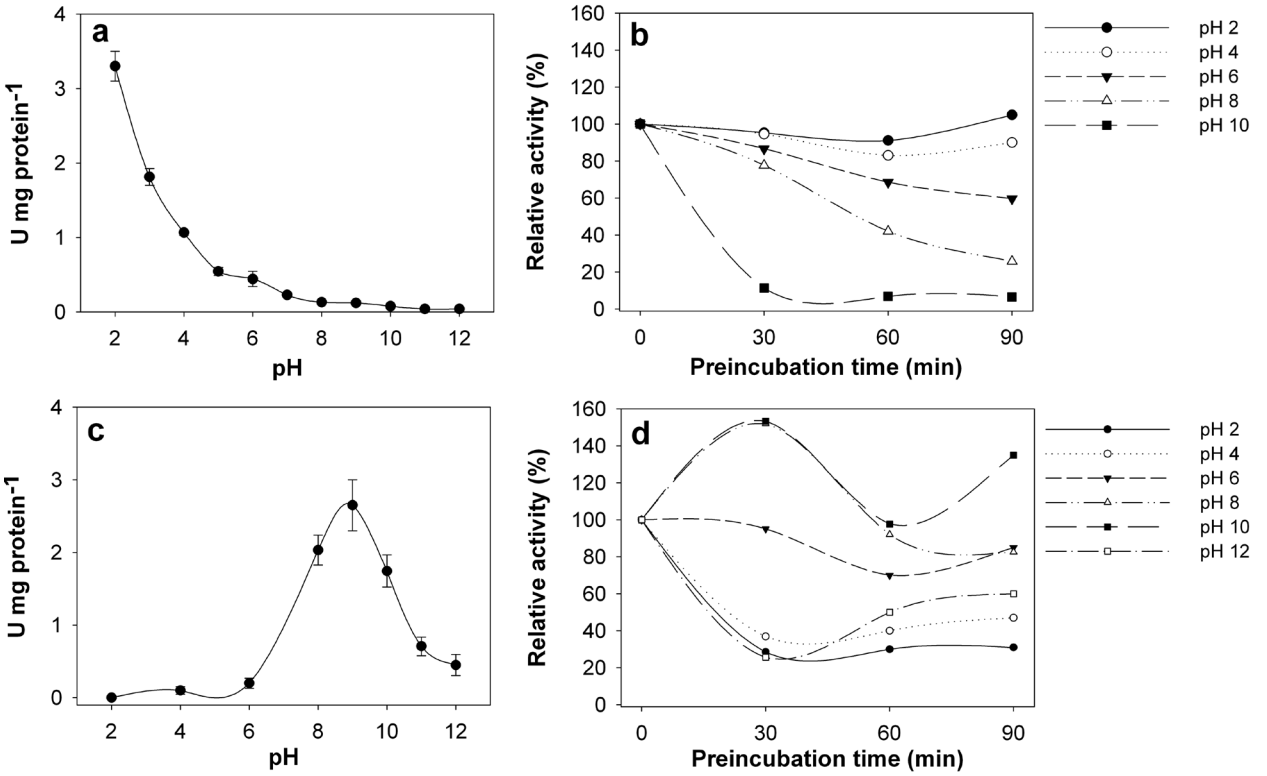

Fig. 1. pH effect on digestive proteases of juvenile sheepshead *Archosargus probatocephalus:* (a) optimal pH of acidic proteases, (b) stability of acidic proteases, (c) optimal pH of alkaline proteases, (d) stability of alkaline proteases.

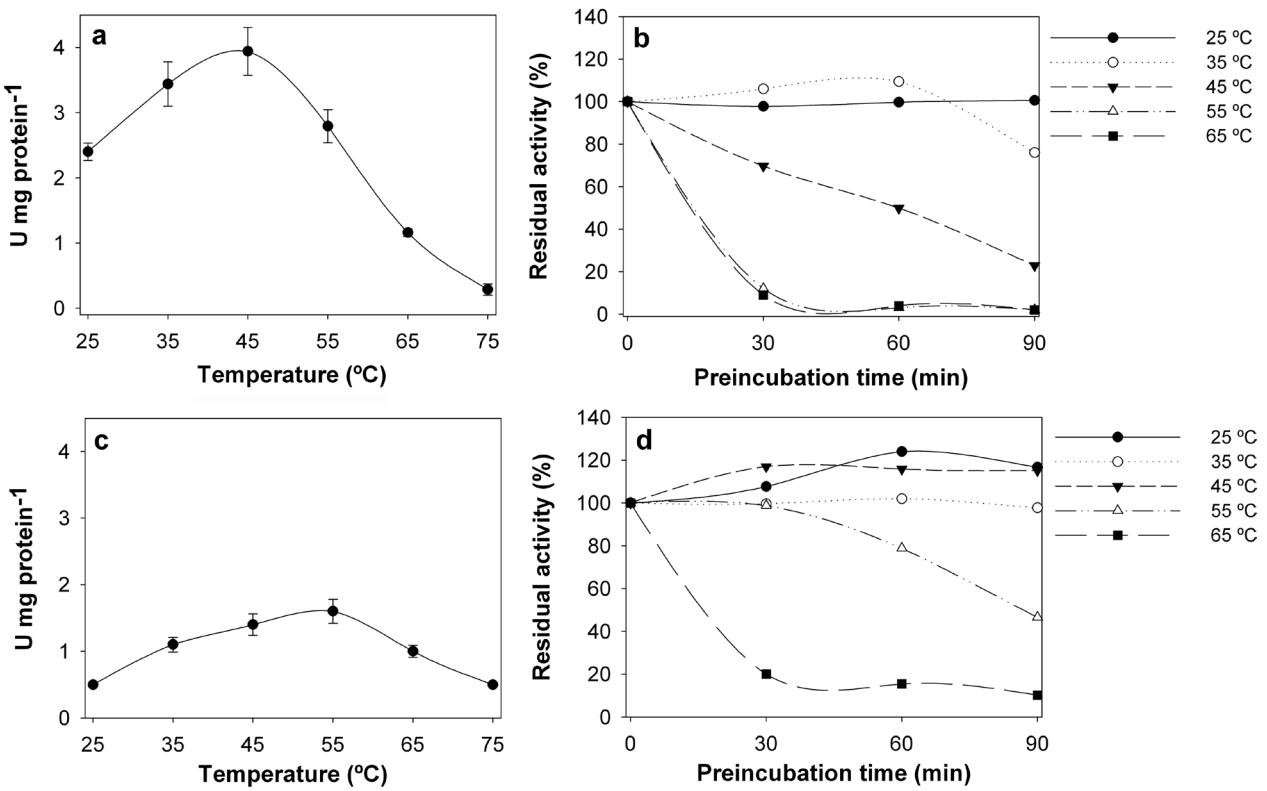

Fig. 2. Temperature effect on digestive proteases of juvenile of sheepshead *Archosargus probatocephalus*: (a) optimal temperature of acidic proteases, (b) temperature stability of acidic proteases, (c) optimal temperature of alkaline proteases, (d) temperature stability of alkaline proteases.

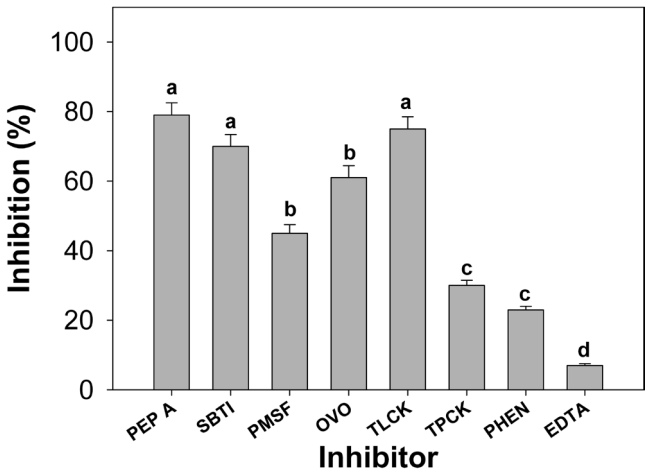

Fig. 3. Effect on the reduction of digestive protease activity in enzymatic extracts of *Archosargus probatocephalus* juveniles by different inhibitors: Pepsin inhibitor: Pepstatin A (Pep A), Serin protease inhibitors and inactivator: soybean trypsin inhibitor (SBTI), phenyl methyl sulphonyl fluoride (PMSF), and ovalbumin (OVO), Trypsin inhibitor: tosyl-lysyl-chloromethyl ketone (TLCK), Chymotrypsin inhibitor: tosyl-phenylanyl-chloromethyl ketone (TPCK), Metallo- proteases inhibitor: phenanthroline (PHEN), ethyldiamine tetra-acetic acid (EDTA). The values are expressed as percentage of inhibition against a control assay without inhibitor, the bars indicate SD values, (Nemenyi, $P < 0.05$).

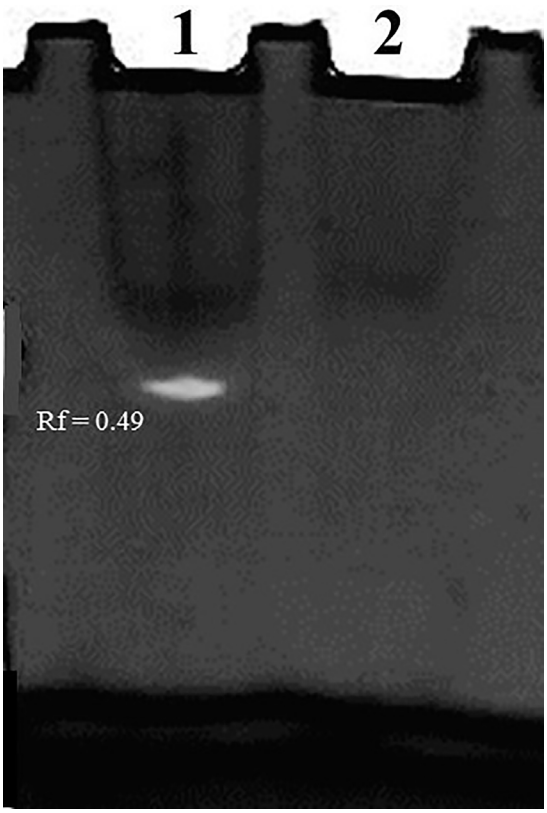

Fig. 4. Zymogram of acid proteases from enzyme stomach extracts of *Archosargus probatocephalus.* Crude enzymatic extract (1), crude enzymatic extract plus Pepstatin A (2).

In this sense, studies of the stomach content of sheepshead indicate an omnivorous trophic strategy with a tendency for carnivory, an unusual behavior among sparids, which tend to be carnivores such as *S. auratus* and *D. dentex* (Alarcón *et al*., 1998; Cutwa, Turingan, 2000; Castillo-Rivera *et al*., 2007). It is known that some species transition from a carnivorous habit to an omnivorous or herbivorous habit during their development, which is often the case in sheepshead whose diet consists predominantly of invertebrates, mainly crustaceans and plants, during adulthood (Castillo-Rivera *et al*., 2007). Thus, juveniles older than 50 mm long consume hard-shell organisms such as bivalve mollusks, brachyurans, echinoderms, barnacles, and opportunistically small fish (Jennings, 1985), while adults feed mainly on algae and invertebrates. In the same context, this species consumes crustaceans more efficiently than algae and grass (Jobling, 1995), although the abundant availability and low energy cost of location and capture of plants may favor an omnivorous strategy, rather than a strictly carnivorous strategy (Montgomery, Targett, 1992).

This capacity has been observed in other studies conducted in this species, where the ability to consume plant material can be related to the presence of morphological adaptations in the dental structure and intestine length. Thus, Castillo-Rivera *et al*. (2007) report that the species shows adaptations characteristic of the trophic morphology, exhibiting a short snout, inferior almost horizontal mouth, in addition to incisor teeth capable of cutting with a series of molars capable of crushing, which is typically observed in herbivores (Jobling, 1995; Moyle, Cech, 2000). However, the species shows a well-developed stomach in the form of a muscular sac, and the presence of pyloric caeca (on average 5.8), which apparently do not have modifications in other species of this family (Luczkovich, Stellwag, 1993) that serve to increase the surface area of digestion and absorption of certain nutrients (Buddington, Diamond, 1986). This species displays an average relative intestinal length of 129%, and is considered as an omnivorous and diurnal predator species (Matlock, García, 1983; López-López *et al*., 1991; Castillo-Rivera *et al*., 2007). Considering the above, in the present study organisms of 100 g were used, during a stage in which this species has a carnivorous tendency according to previous reports, which explains the high activity values of acidic proteases compared to alkaline proteases.

In the present study, it was observed that *A. probatocephalus* has a stomach with a defined structure, which secretes acidic proteases that require a pH of 2 for maximum activity, similar to that reported for other fishes of the Sparidae family such as; *S. aurata*, *D. dentex* (Alarcón *et al*., 1998) and sharpsnout seabream [*Diplodus puntazzo* (Walbaum 1792)] (Tramati *et al*., 2005). The pH value where an optimal activity is found is related to the pH where the active center of the enzyme presents an adequate ionic

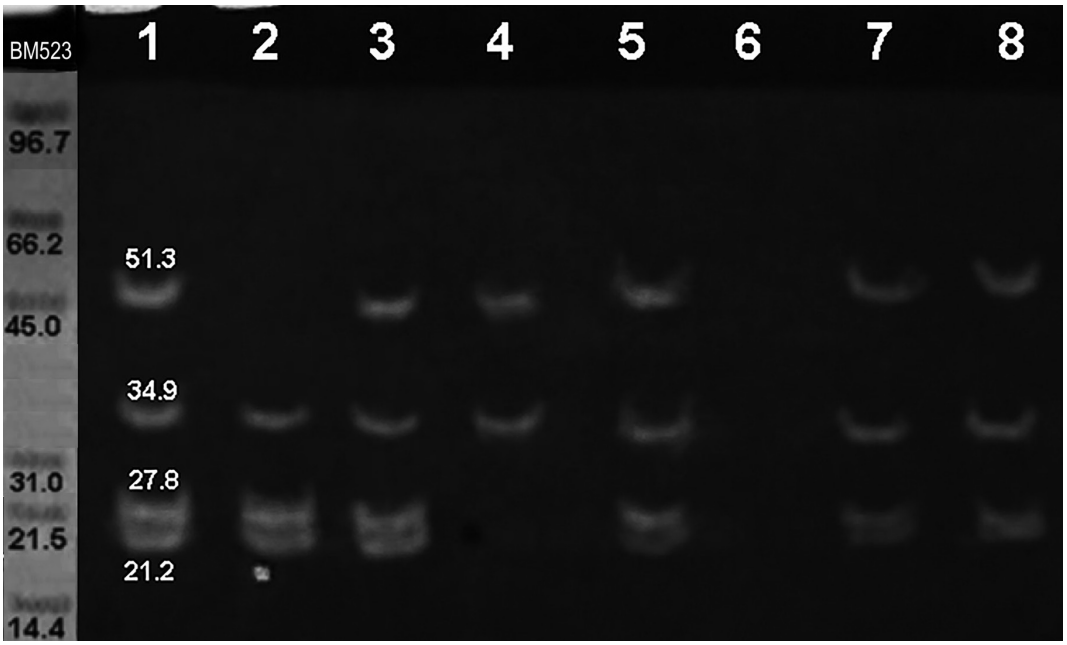

Fig. 5. SDS-PAGE electrophoresis analysis of alkaline digestive proteases of *Archosargus probatocephalus*: BM523 (molecular weight marker Bio Basic Inc; rabbit phosphorylase B 96.7 kDa, bovine serum albumin 66.2 kDa, ovalbumin 45 kDa, carbonic anhydrase 31.0 kDa soybean trypsin inhibitor 21.5 kDa and lysozyme 14.4 kDa), Control (1); inhibitors: OVO (2), PMSF (3), SBT1 (4), TPCK (5), TLCK (6), PHEN (7) and EDTA (8).

conformation to carry out the catalysis with great efficiency (Álvarez-González *et al*., 2010). In this sense, the optimal activity found for acid proteases was pH 2, which coincides with reports of other species such as *Siluru glanis* (Jonas *et al*., 1983), *S. aurata*, *D. dentex* (Alarcón *et al*., 1998), *Brycon orbignyanus* (Valenciennes, 1850) (García-Carreño *et al*., 2002), *Symphysodon aequifasciatus* Pellegrin, 1904 (Chong *et al.*, 2002), *S. formosus* (Natalia *et al*., 2004), *D. puntazzo* (Tramati *et al*., 2005), *S. senegalensis* (Sáenz -de Rodrigáñez *et al*., 2005), *S. maximus* (Wang *et al*., 2006), African coelacanth *Latimeria chalumnae* Smith, 1939 (Tanji *et al*., 2007), threadfin bream *Nemipterus* spp. (Nalinanon *et al*., 2008), *T. alalunga* and skipjack tuna *Katsuwonus pelamis* (Linnaeus, 1758) (Nalinanon *et al*., 2010a, b) and *C. undecimalis* (Concha-Frias *et al*., 2016).

On the other hand, the alkaline proteases of sheepshead show an optimum pH of 9, which is common in many species, with maximum activity occurring between pH 9 and 10, as reported in *Brycon orbignyanus* (García-Carreño *et al*., 2002), *Labeo rohita* (Hamilton, 1822) and *H. molitrix* (Kumar *et al*., 2007), *T. orientalis* (Matus-De la Parra *et al*., 2007), grass carp *Ctenopharyngodon idella* (Valenciennes, 1844) (Liu *et al*., 2008), hybrid juvenile tilapia *Oreochromis niloticus* (Linnaeus, 1758) x *Oreochromis aureus* (Steindachner, 1864) (Jun-Sheng *et al*., 2006), *D. puntazzo* (Tramati *et al*., 2005), *C. urophthalmus* (Cuenca-Soria *et al*., 2014) and *Amphilophus trimaculatus* (Günther, 1867) (Toledo--Solís *et al*., 2016). Nevertheless, species with more than one optimum pH value have been reported, such as *C. undecimalis*, showed optimal activity at pH 7 and 11 (Concha-Frías *et al*., 2016) that can be attributed to the diversity of enzymes and isoforms present in the species (Alarcón *et al*., 1998). Although it should be mentioned that sheepshead presented only one optimum pH, which indicates that the intestinal digestive proteases present in the species shows the same optimum pH value (Matus-De la Parra *et al*., 2007).

In *A. probatocephalus*, the acid proteases are stable at acid pH and their activity decreases as the environment becomes alkaline; while alkaline proteases are stable at alkaline pH and are resistant to slightly acid pH, these conditions are similar to that reported in *C. urophthalmus* (Cuenca-Soria *et al*., 2014). However, in other fish with strictly carnivorous habits that lives marine habitat, acidic proteases are stable for a short time at alkaline pH as described for *T. thynnus* (Essed *et al*., 2002) and *S. maximus* (Wang *et al*., 2006). This has also been reported for freshwater carnivorous species such as *A. tropicus* (Guerrero-Zárate *et al*., 2014) and marine omnivorous species such as *S. senegalensis* (Sáenz -de Rodrigáñez *et al*., 2005). The fact that acidic proteases are resistant to alkaline pH allows acidic proteases to continue acting in the degradation of the food when get inside the intestine, until the media is alkalized, where alkaline proteases end the digestion procces. However, in *A. probatocephalus*, acid protease activity is lost at neutral and alkaline pH, this process is not reflected, where the loss of activity is compensated by the hydrolysis of alkaline proteases that are active under acidic conditions and at the same time is compensated by the presence of the pyloric caeca, a large intestine and strong sphincters, which regulate the food transit, joined to the action of digestive hormones (such as cholecystokinin)

that stimulate peristaltic movements, increasing the contact surface in the enzyme-substrate relationship (Salvensen, 1989; Simpson, 2000).

Accordantly, alkaline digestive proteases may act since food enters to the intestine, even at acid pH. It is important to mention that, in general alkaline proteases are more stable at different pH levels, due to the presence of protease diversity (trypsin, chymotrypsin, aminopeptidase and carboxypeptidases) as demonstrated in the zymogram, when four bands, which increase the possibility of hydrolyzing food proteins across a wide pH range during variation (Alarcón *et al*., 1998). In contrast, most fish species present two or three pepsins with an optimum haemoglobin digestion between 2 and 4 (Klomklao, 2008). In this sense, Klomklao *et al*. (2007) reported the presence of pepsin A and pepsin B from giant grenadier [*Albatrossia pectoralis* (Gilbert, 1892)], Chiu, Pan (2006) report two pepsin isoforms (PI and PII), in juvenile and adult of Japanese eel (*Anguilla japonica* Temminck & Schlegel, 1846) and Peña *et al*. (2015, 2017) report two pepsin isoforms in spotted rose snappers (*L. guttatus*). Therefore, isoforms of one-enzyme types present small functional differences that increase the effects of changes to structural configuration or denaturation and consequently generates a partial or total loss of the activity (Nalinanon *et al*., 2010b).

On the other hand, the optimum temperature in *A. probatocephalus* alkaline proteases is 10 °C higher at the optimum temperature of the acid proteases and at the same time shows more thermostability. In general, this difference between the optimal temperatures of alkaline and acid proteases has been described in species such as *T. thynnus* (Essed *et al*., 2002), *B. orbignyanus* (García-Carreño *et al*., 2002) and *C. trimaculatum* (Toledo-Solís *et al*., 2016). The optimum temperature of acid proteases was observed as 45 °C, being similar to that reported for *S. aurata* (Alarcón *et al*., 1998) and *T. orientalis* (Matus-De la Parra *et al*., 2007), which shows a decrease in activity at temperatures from 55 °C, an effect related to the denaturation of pepsin (Nalinanon *et al*., 2008).

In contrast, alkaline proteases exhibit optimum activity at a temperature of 55 °C, which is similar to that found in *S. aurata, D. dentex* (Alarcón *et al*., 1998), spotted goatfish *Pseudupeneus maculatus* (Bloch, 1793) (Souza *et al*., 2007), parrotfish *Sparisoma sp.*, traira *Hoplias malabaricus* (Bloch, 1794) (Alencar *et al*., 2003) and *O. niloticus* (Bezerra *et al*., 2005), which show an optimum temperature for alkaline proteases of between 50 and 55 °C, and presents more thermostability, while temperatures from 65 °C only affect 80% of residual activity. However, the activity fall could be compensated by the increasing retention time of food in the digestive tract to perform proteins hydrolysis at lower temperatures than optimal (Alarcón *et al*., 1998; Uscanga *et al*., 2010). It should be mentioned that optimal temperature values and thermal stability of protease activities are only operational parameters of the enzymes, rather than results of physiological importance; which can be associated with the configuration of enzymes as well as the habitat, environment and genetic aspects of the species (Nalinanon *et al*., 2008).

The identification of the proteases that constitute acid digestion in sheepshead was evaluated with the use of Pepstatin A inhibitor, which showed a 79% inhibition, a similar value to freshwater or omnivorous species with a carnivory tendency which has previously been reported in *C. urophthalmus* (Cuenca-Soria *et al*., 2014) and *C. trimaculatum* (Toledo-Solís *et al*., 2016) and lower than the percentage of inhibition found exclusively in carnivorous species such as *S. maximus* (Wang *et al*., 2006), mandarin fish (*Siniperca chuatsi*) (Zhou *et al*., 2008), skipjack tuna *Katsuwonus pelamis* (Nalinanon *et al*., 2010b) and *C. undecimalis* (Concha-Frias *et al*., 2016). Therefore, hydrolysis in acid conditions is in first term performed by presence of pepsin A type, with the possible presence of other pepsin isoforms as observed in species such as Japanese seabass *Lateolabrax japonicus* (Cuvier, 1828) (Cao *et al*., 2011), tropical gar *A. tropicus* (Guerrero-Zárate *et al*., 2014) and the sparids gilthead seabream *S.aurata* and common dentex *D. dentex* (Alarcón *et al*., 1998), among others already mentioned, where diverse types of pepsins were detected, which gives a high capacity to hydrolyze proteins in the stomach. The lower inhibition of pepsin in the stomach of *A. probatocephalus* could be related to the presence of other types of pepsin, since a high residual activity was observed at pH 4 and showed a 79% inhibition with Pepstatin A inhibitor. This was not revealed with the electrophoresis under native-PAGE conditions owing to a single active band with acid protease activity was found. Similar results were reported by Alvarez-González *et al*. (2010) in spotted sand bass *Paralabrax maculatofasciatus* (Steindachner, 1868). The band whith acid protease activity registered in the electrophoresis under native-PAGE conditions shows Rf of 0.49, which compared to values recorded in other fish species such spotted sand bass *P. maculatofasciatus* with a Rf = 0.75 (Alvarez-González *et al*., 2010), spotted rose snappers *L. guttatus* with a Rfs of 0.71 and 0.77 (Peña *et al*., 2017) and the sparids gilthead seabream *S. aurata* and common dentex *D. dentex* of Rfs 0.81 and 0.66 (Alarcon *et al*., 1998), and similar was reported in tropical gar *A. tropicus* (Rfs 0.35 and 0.71).

On the other hand, the seven types of specific inhibitors for the alkaline proteases had a variable effect on the enzymatic activity. Of these, the group of serine proteases was inhibited by PMFS (45%), OVO (61%) and SBTI (70%), these high inhibition values reveal the high activity of serine protease type enzymes in the intestine of sheepshead. This pattern of serine protease inhibition is similar to that reported in *S. aequifasciata* (Chong *et al*., 2002), *T. thynnus* (Essed *et al*., 2002), *C. idella* (Liu *et al*., 2008), *C. trimaculatum* (Toledo-Solís *et al*., 2015) and *C. undecimalis* (Concha-Frias *et al*., 2016). On the other hand, the group of metalloproteases exhibited a low inhibition PHEN (23%), a similar value to that reported by Dimes, Haard (1994) in coho salmon [*Oncorhynchus kisutch* (Walbaum, 1792)] with EDTA (7%). In contrast, the TPCK inhibited chymotrypsin-like activity by 30%, similar to reports by Alarcón *et al*. (1998) in *D. dentex* and Chakrabarti *et al*. (2006) in *L. rohita*, whereas the TLCK

inhibited trypsin-like activity by 75%, indicate a greater proportion of trypsin compared to chymotrypsin, which is similar to previous observations in *B. orbiginyanus* (García--Carreño *et al*., 2002) and *S. formosus* (Natalia *et al*., 2004). Moreover, these data suggest that the inclusion of certain vegetable ingredients in balanced foods without leading to a subsequent reduction in digestive enzymatic activity and the digestibility of food (Essed *et al*., 2002). In this sense, the zymogram of alkaline proteases showed four bands with activity, which is lower than the number detected for other sparids such as *S. aurata* and *D. dentex*, where five and eight active proteases with caseinolitic activity were reported (Alarcón *et al*., 1998). Concurrently, other fish species have been reported with higher numbers of alkaline proteases, such as *A. tropicus* with the presence of five bands (Guerrero-Zárate *et al*., 2014) and *C. undecimalis* with seven isoforms (Concha-Frías *et al*., 2016).

The four bands registered confirm the predominating presence of the proteases trypsin, chymotrypsin and metallo-proteases, with molecular weights of 21.2 kDa, 27.8 kDa and 51.3 kDa, respectively. TLCK inhibited all bands recorded for trypsin, which was expected since TLCK has been identified as a trypsin inhibitor (García-Carreño, 1992), an observation previously reported by Lazo *et al*. (2007), whom indicated a molecular weight of 23 to 68 kDa for trypsin in red drum *Sciaenops ocellatus*. It is likely that the band with a molecular weight of 34.9 kDa was treated with chymotrypsin, a similar value to that reported by Souza *et al*. (2007) in *P. maculatus*. The inhibitors of serine proteases such as OVO inhibited the 51.3 kDa band, SBT1 inhibited the 27.8 and 21.2 kDa bands, yet PMSF did not inhibit any band, showing a high resistance of the intestinal digestive proteases of *A. probatocephalus* to inhibitors of trypsin-like or chymotrypsin-like enzymes, which is in agreement with previous reports in other studies of several species with omnivorous habits (Simpson, 2000; Castillo-Yáñez *et al*., 2005, 2006, 2009; Ben *et al*., 2011). The metallo-proteases generally have molecular weights between 45 kDa and 97 kDa (Natalia *et al*., 2004), and are typically inhibited by the phenanthroline and chelator EDTA; however, in the present study EDTA did not inhibit any band which could indicate that trypsin and chymotrypsin do not require metal ions for their activation, as previously indicated by Cuenca-Soria *et al*. (2014).

In *A. probatocephalus* the presence of pepsin, serine proteases such as trypsin and chymotrypsin, and metalloproteases such as aminopeptidase and carboxipeptidase A suggest a protein digestion mechanism, similar to other fish and are involved in initiating hydrolysis, which also requires the activity of endopeptidases, followed by the action of exopeptidases, that are limited to degrading short peptide chains, releasing terminal aminoacids as indicated by Chong *et al*. (2002) and López-Ramírez *et al*. (2010). The digestive capacity in sheepshead is carried out by two possible factors: the presence of one pepsin isoform, as well as the characteristics of the intestinal digestive proteases, including their high resistance to pH conditions, temperature and inhibitors, representing food habits of carnivores with omnivore tendencies during this life stage.

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