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Partial characterization of digestive proteases in juveniles of *Microphis brachyurus* (short-tailed pipefish) (Syngnathiformes: Syngnathidae)

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Short-tailed pipe fish (*Microphis brachyurus*) is a freshwater organism with high economic potential for the aquarium hobby, so it is necessary to implement methods to promote its culture through studies of digestive physiology. General activities of acid and alkaline proteases were evaluated, as well as the effect of pH, temperature and inhibitors. The optimal pH of stomach proteases was 2, while the optimal pH of intestinal proteases was 10. Optimal temperature for the acidic proteases was 35 °C, while for alkaline proteases it was 45 °C. Thermal stability showed high resistance at 35 °C for both acid and alkaline proteases (above 100% residual activity). Acid proteases are resistant at pH 2 (50% of residual activity), meanwhile alkaline proteases were highly resistant at pH 10 (90% of residual activity). Acid proteases were inhibited by 80% with pepstatin A and alkaline proteases were inhibited with TLCK and TPCK for trypsin (75%) and chymotrypsin (80%), respectively. Finally, metallo-proteases were 75% partially inhibited some serine proteases by 75% with EDTA. In conclusion, *M. brachyurus* has a good digestive capacity, since they can degrade a wide variety of proteins due to their greater proteolytic activity.

Keywords: Enzyme activity, Sygnathidae, pH, Protease inhibitors, Temperature.

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El pez pipa (*Microphis brachyurus*) es un organismo dulceacuícola con alto potencial económico para la acuarofilia; sin embargo, es necesario implementar su cultivo a través de estudios de fisiología digestiva. Se evaluó el efecto del pH, temperatura e inhibidores sobre las actividades enzimáticas de proteasas ácidas y alcalinas. El pH óptimo de proteasas estomacales es de 2, mientras que el de proteases intestinales es de 10. La temperatura óptima de proteasas ácidas es de 35 °C y las alcalinas de 45 °C. La estabilidad térmica para proteasas ácidas y alcalinas es a los 35 °C (más de 100% de actividad residual). La estabilidad a los diferentes pH de las proteasas ácidas es en 2 (50 % de la actividad residual), mientras que para las proteasas alcalinas es en 10 (90 % de la actividad residual). Las proteasas ácidas fueron inhibidas en 80% con pepstatina A y las proteasas alcalinas fueron altamente inhibidas con TLCK para tripsina (75%) y TPCK quimitripsina (80%). Finalmente, las metaloproteasas fueron inactivadas con EDTA en 70%. En conclusión, *M. brachyurus* tiene una buena capacidad digestiva al degradar una amplia variedad de proteinas debido a su alta actividad proteolítica.

Palabras clave: Actividad enzimática, Inhibidores de proteasas, pH, Sygnathidae, Temperatura.

INTRODUCTION

The short-tailed pipefish, *Microphis brachyurus* (Bleeker, 1854), is a subtropical estuarine fish, distributed along the Gulf of Mexico. It belongs to the Syngnathidae family, which includes sea dragons, seahorses, and pipefish. The main characteristic of syngnathids is male parental care, as eggs are glued by females either to the ventral male structures or deposited into the male brood pouch (Wilson *et al*., 2001). In Mexico, populations of native fishes are under pressure due to anthropogenic alteration of habitat and introduction of exotic species (Jelks *et al*., 2008). The subspecies, *M. brachyurus lineatus*, has been considered as a species of concern since 1991 in the USA by the National Oceanic and Atmospheric Administration (NOAA, 2004). Natural populations of *M. brachyurus* are also affected in Mexico by unregulated fishing as this species is traded as an aquarium fish without formal records as occurs with other syngnathid species around the world (Rosa *et al*., 2011). Approximately 37 species of pipefish have been reported to inhabit both freshwater and saltwater systems (Nelson *et al*., 2016), including *M. brachyurus, which* often faces inadequate culture practices in the average home aquarium as it is maintained in tanks with conventional freshwater tropical fish.

However, survival of *M. brachyurus* in captivity is compromised by conventional fish diets (flakes, pellets, and frozen *Artemia*), whereas the species naturally preys on live items, mainly crustaceans and fish larvae small enough to be ingested by the characteristic reduced mouth of the Syngnathidae family (Miller *et al*., 2005).

In recent years, there has been a growing interest in developing technologies with native species for their culture at commercial scale. However, the knowledge for *M. brachyurus* production is unknown, particularly in relation to their digestive physiology. Currently, research efforts are aimed at determining the conditions that increase the

survival and viability of culture production, as well as to characterize digestive physiology, in order to better plan and feed design. In this last case, the studies are based on the detailed knowledge of the digestive physiology in the different stages of life by means of the determination of the digestive enzymatic activities and characterization of proteases, as it is known that the of activity of some enzymes acts as a good indicator of nutritional, so that the data obtained may be relevant to establish an optimal artificial feeds and to reduce production costs in hatcheries (Ueberschär, 1995). Recently, several studies have been conducted to relate the appearance of digestive enzymes with physiological and nutritional features in various species such as *Atractosteus tropicus* Gill, 1863, *Cichlasoma urophthalmus* (=*Mayaheros urophthalmus* (Günther, 1862)), *Cichlasoma trimaculatum* (=*Amphilophus trimaculatus* (Günther, 1867)), *Lutjanus guttatus* (Steindachner, 1869), *Chelon labrosus* (Risso, 1827), *Centropomus undecimalis* (Bloch, 1792), *Cichlasoma beani* (=*Mayaheros beani* (Jordan, 1889)), and *Archosargus probatocephalus* (Walbaum, 1792) (Cuenca-Soria *et al*., 2014; Guerrero-Zárate *et al*., 2014; Peña *et al*., 2015; Toledo-Solís *et al*., 2015; Concha-Frias *et al*., 2016; Pujante *et al*., 2017; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018). In this way, this work aims to characterize the digestive proteases in juveniles of *Microphis brachyurus*.

MATERIAL AND METHODS

Sample Preparation. For this study, 132 pipefish juveniles with a weight 0.25 g±0.3 SD and length 89.6 mm±0.5 SD, were captured in the Jamapa River (19°05'40.42"N, 96°08'23.40"W). After collection, they were transported in an insulated water tank containing water at 26°C and 8 g L^{-1} salinity (recorded at collection) to the wet laboratory located in Tepic, Nayarit. Fish were subsequently sacrificed with an overdose of tricaine methanesulfonate (MS-222, Sigma-Aldrich, Saint Louis, MI, USA). The total weight and length of each fish was recorded and subsequently, the stomach and intestine were extracted individually on a frozen plate. Subsequently, the organs were lyophilized and preserved at -20 °C until processing. Dried organs were sent to the Biochemistry Laboratory in the División Académica de Ciencias Biológicas at Universidad Juárez Autónoma de Tabasco. Stomachs were homogenized in a proportion of 200 mg mL^{-1} in 100 mmol L^{-1} of glycine-HCl buffer at pH 2, while Tris-HCl buffer 12.5 mmol L^{-1} + 30 mmol L^{-1} $CaCl_2$ L^{-1} at pH 7.5 was used for the intestines, in the same proportion respectively. The homogenate was centrifuged at 16,000 x g for 30 min at 4 °C; the multienzyme extract thus obtained was stored at -80 °C for further analysis. The concentration of soluble protein was determined using the Bradford (1975) technique with the bovine serum albumin as the standard.

Biochemical Analyses. The total activity of the acid proteases was determined using 1% hemoglobin in 100 mmol L^{-1} glycine-HCl buffer at pH 2 following the method of Walter (1984). The activity of alkaline proteases was evaluated following the method of Anson (1938), as modified by Kunitz (1947), using 1% casein in 100 mmol L^{-1} Tris-HCl + 10 mmol L^{-1} $CaCl_2$ buffer at pH 9. One unit of activity was defined as 1 μg of tyrosine liberated per minute with a Molar Extinction Coefficient (MEC) of 0.005 mL^{-1} μg^{-1} cm^{-1}. To determine the MEC of tyrosine, a standard curve was made with

different concentrations of tyrosine (from 0 to 300 mg mL^{-1}). All trials were performed in triplicate.

Effects of pH and Temperature on Digestive Enzyme Activity. The optimum pH of the acid and alkaline proteases was evaluated using a universal buffer Stauffer (1989) with a pH range of 2–12 following the technique of Walter (1984) and Anson (1938), previously described to determine protease activity. The optimum temperature of the acid and alkaline proteases was established by enzyme assays at different temperatures (35, 45, 55, 65 °C). The effects of pH and temperature on the stability of acid and alkaline proteases were determined by pre-incubating the multienzyme extracts for 0, 30, 60 and 90 min with a buffer at the assay temperature (Stauffer, 1989). For the determination of the stability under different conditions of pH and temperature the pre-incubation time 0 min value was used as a control to determine the residual activity expressed as a percentage of residual activity comparing with the extracts that were incubated at different pH and temperatures.

Zymograms and Effects of Inhibitors. 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 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 0.1% SDS 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). They 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 at 37 °C, and then washed and fixed in trichloroacetic acid (12%) solution for 15 min. Areas of enzyme activity were developed according to Weber, Osborn (1969), using a 0.1% Coomassie brilliant blue R-250 solution to stain the gels, while further distaining was carried out in a 35:10:55 solution of methanol-acetic acid-water. Clear zones revealed the activity of proteases within few minutes, although well-defined zones were obtained after 2–4 h. The inhibitors were used for the alkaline proteases: phenyl-methanesulphonyl fluoride 100 mmol L^{-1} (PMSF), N-q-tosyl-lysine-chloromethyl ketone hydrochloride 10 mmol L^{-1} (TLCK), soybean trypsin inhibitor 250 mmol L^{-1} (SBT1), N-q-tosyl-L-phenylalanine chloromethyl ketone 10 mmol L^{-1} (TPCK), 1,10-phenanthroline 10 mmol L^{-1} (Phen), trypsin inhibitor, type II-T: turkey egg ovoalbumine 250 mmol L^{-1} (Ovo) and ethylene diamine tetra acetic acid 10 mmol L^{-1} (EDTA). Extracts were incubated in the presence of each inhibitor for 1 h (1:1 v/v) at pH 7.5, the pH value of intestine extracts, and at pH 2 for stomach extracts during sample preparation. In both tests a control without inhibitor was used as 100% of total activity and the residual activity was expressed as a percentage of reduction of the activity with the different inhibitors (Dunn, 1989). They were pre-incubated in a 1:1 ratio (enzyme: inhibitor) for 1 h. The molecular weight marker SDS-PAGE standards unstained Low Range (Biorad cat. 1610304) and the program Quantity One 1-D Analysis Software de Bio-Rad® were used to calculate the molecular weight of each active bands.

Statistical Analysis. All the enzymatic activity assays were carried out in triplicate of the same homogenate and were evaluated by means of nonparametric statistical analysis of Kruskal-Wallis test and a posteriori Nemenyi test as the data were not normally distributed. Statistical tests were conducted with the Statistica software v. 7.5 (Statsoft, Tulsa, OK, USA) using a significance value of 0.05.

RESULTS

The optimum pH for the activity of acid proteases was 2 (Fig. 1A) and for alkaline proteases activity the pH optimum was 10 (Fig. 1B). After 30 min preincubation, the remaining enzyme activity for acid proteases was 50% pH 2, while the remaining activities were around 15%–20%, at pH 4–7 after 90 min pre-incubation (Fig. 1C). The maximum pH stability of alkaline proteases occurred after 30 minutes of preincubation at pH 10, showing a decrease in its residual activity until reaching 90% after 90 minutes of preincubation (Fig. 1D).

The optimum temperature for acidic proteases was 35 °C (Fig. 2A) and optimum temperature of alkaline protease activity was 45 °C (Fig. 2B). The stability of the acidic proteases presented thermal variations above 100% at 35 °C (Fig. 2C). Instead, maximal stability of alkaline protease was observed at 35 °C, which decreased after 90 minutes

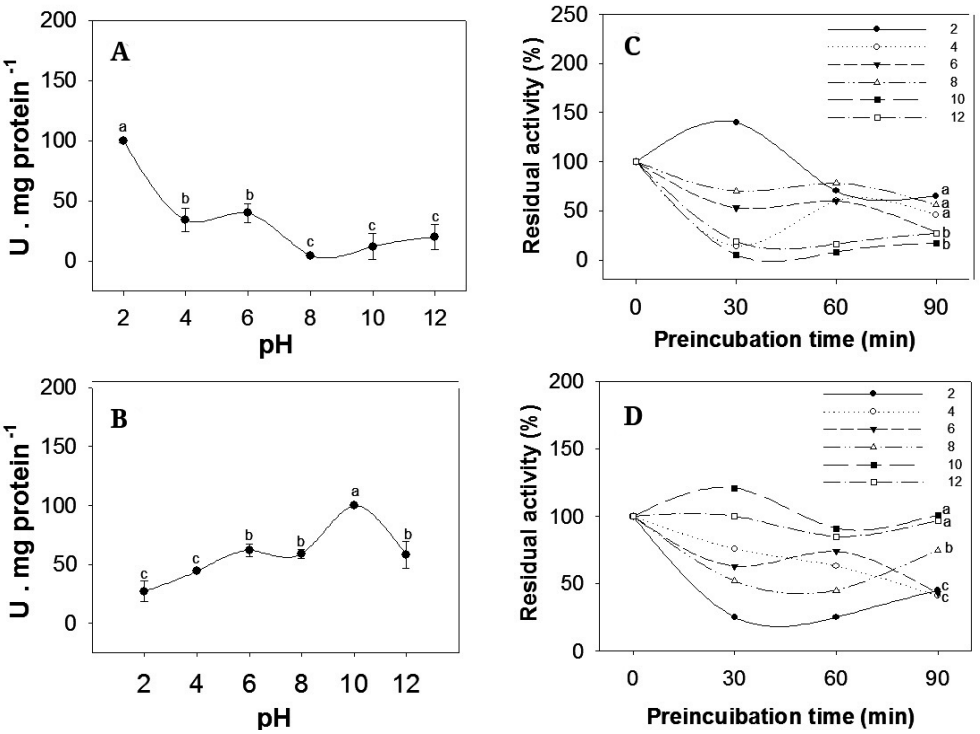

FIGURE 1 | Effect of pH on the enzymatic activity of digestive proteases of short-tailed pipefish (*Microphis brachyurus*) juveniles for the determination of: **A.** optimal pH of acidic proteases; **B.** optimal pH of alkaline proteases; **C.** pH stability of acidic proteases; and **D.** pH stability of alkaline proteases (mean ± SD, n = 3). Mean values denoted with different letters are statistically different, $p < 0.05$.

of preincubation, followed by the temperature of 45 °C where the activity remained stable, while temperatures of 55 and 65 °C decreased the activity to 40–55% at 90 min preincubation (Fig. 2D).

Analysis of the effects of inhibitors indicated that acid protease was inhibited by 80% by pepstatin A, a specific inhibitor of pepsin type A enzymes. The activity of alkaline proteases showed an inhibition of 75% of activity by TLCK, a specific inhibitor of trypsin, inhibition of 80% of activity by TPCK, a specific inhibitor of chymotrypsin; activity inhibition of 58% by SBT1 and 35% by ovalbumin, which inhibit serine proteases, a reduction of 55% and 75% of activity by the metalo-protease inhibitors Phenanthroline and EDTA, respectively, and inhibition of 40% of activity by PMSF, a general inhibitor of serine proteases (Fig. 3).

The analysis of SDS-PAGE zymograms for alkaline proteases revealed six active bands in control without inhibitor (87.9, 64.8, 59.7, 52.6, 38.1 and 35.2 kDa). TPCK inhibited four active bands (87.9, 59.7, 52.6 and 35.2 kDa), Phenanthroline and EDTA inhibited five active bands (87.9, 64.8, 59.7, 52.6 and 35.2 kDa), TLCK inhibited four active bands (87.9, 64.8, 59.7, 52.6 kDa), Ovalbumin inhibited four active bands (64.8, 59.7, 52.6, and 35.2 kDa), while SBT1 inhibited three active bands (64.8, 59.7 and 35.2 kDa) and PMSF inhibited two active bands (64.8 and 59.7 kDa respectively) (Fig. 4).

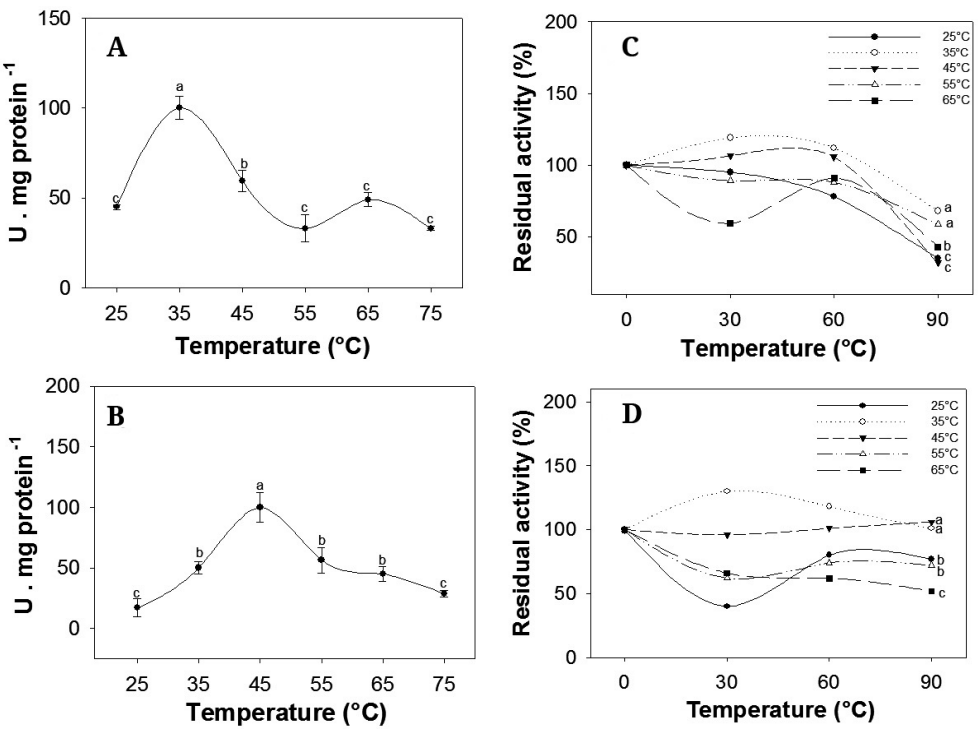

FIGURE 2 | Effect of temperature on digestive proteases of juvenile of short-tailed pipefish (*Microphis brachyurus*): **A.** optimum temperature of acidic proteases; **B.** thermal stability of acidic proteases; **C.** optimum temperature of alkaline proteases; and **D.** thermal stability of alkaline proteases (mean ± SD, n = 3). Mean values denoted with different letters are statistically different, $p < 0.05$.

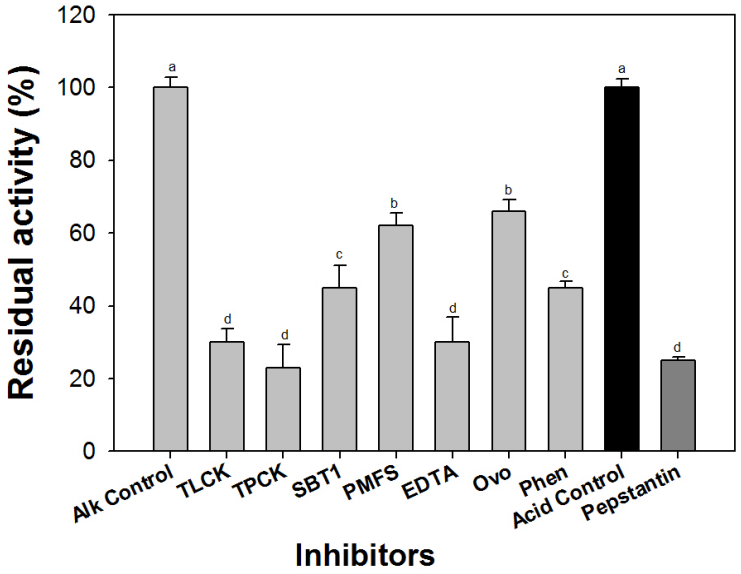

FIGURE 3 | Residual activity (%) of digestive proteases using several inhibitors on multienzyme extracts of short-tailed pipefish (*Microphis brachyurus*) juveniles. Alkaline proteases with no inhibitor (Alk control), tosylphenylanylchloromethyl ketone (TPCK), phenanthroline (PHEN), ethyl-diamine tetra-acetic acid (EDTA), tosyllysyl- chloromethyl ketone (TLCK), ovalbumin (OVO), soybean trypsin inhibitor (SBT1), phenyl methyl sulphonyl fluoride (PMSF), acidic proteases with no inhibitor (Acid control), pepstatin A (mean ± SD, n = 3). Columns with different letters represent significant differences ($p < 0.05$).

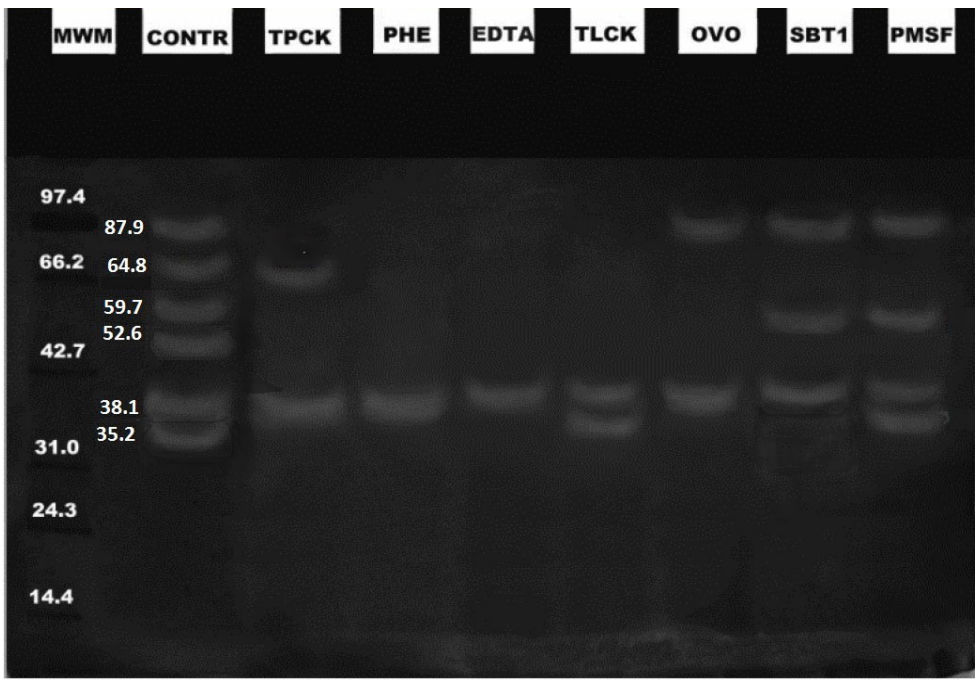

FIGURE 4 | SDS-PAGE zymogram of alkaline digestive proteases of short-tailed pipefish (*Microphis brachyurus*) juveniles: Molecular weight marker (MWM), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbon anhydrase (31.0 kDa) and lysozyme (14.4 kDa); control (without inhibitor); inhibitors were the same as in Fig. 3.

DISCUSSION

The results obtained in the enzymatic characterization of juveniles of short-tailed pipefish (*Microphis brachyurus*) showed that this species exhibits a relatively high activity of alkaline digestive proteases compared with other fish species such as *Scleropages formosus* (Müller, Schlegel, 1840) (Natalia *et al*., 2004), turbot *Scophthalmus maximus* (Linnaeus, 1758) (Wang *et al*., 2006), Mayan cichlid *Cichlasoma uropthalmus* (Cuenca-Soria *et al*., 2014), *Cichlasoma trimaculatum* (=*Amphilophus trimaculatus*) (Toledo-Solís *et al*., 2017), and *Cichlasoma beani* (=*Mayaheros beani*) (Mártinez-Cárdenas *et al*., 2017). The latter two species have carnivorous habits characterized by high acid protease activities (>1500 Unit Enzymatic Activity (UEA) mg of protein^{-1}) in the stomach, which is the most important organ for the acid digestion of proteins, as well as a lower alkaline digestive activity in the intestine (<250 UEA mg protein^{-1}) that is in charge of the hydrolysis of large peptides to release small peptides and amino acids to be absorbed by enterocytes (García-Carreño *et al*., 2002).

In the present study, acid proteases reached their maximum activity at pH 2, which is consistent with findings of the majority of previous studies for species such as *Solea senegalensis* Kaup, 1858 (Yúfera, Darías, 2007), *Symphysodon aequifasciatus* Pellegrin, 1904 (Chong *et al*., 2002), *Diplodus puntazzo* (Walbaum, 1792) (Tramati *et al*., 2005), *Latimeria chalumnae* Smith, 1939 (Tanji *et al*., 2007), *Nemipterus* spp. (Nalinanon *et al*., 2008), and *Acipenser naccarii* Bonaparte, 1836 (Furné *et al*., 2005). However, *Oncorhynchus mykiss* (Walbaum, 1792) and *Petenia splendida* Günther, 1862 have higher optimum pH values (5 and 4 respectively) for acid and alkaline enzymes, that is uncommon for acid digestion (Furné *et al*., 2005; Uscanga-Martínez *et al*., 2011) and different to that found in *M. brachyurus*. For proteases, pH is a chemical key factor at which activity can be maximized, as allowing the active center of the enzymes to join the anchor site (enzyme-substrate binding) to perform a highly efficient catalysis (Álvarez-González, 2003).

In *M. brachyurus*, alkaline digestive proteases showed an optimum pH at 10, which has been recorded for other marine and freshwater fish species such as *Brycon orbignyanus* (Valenciennes, 1850) (García-Carreño *et al*., 2002), *Symphysodon aequifasciatus* (Chong *et al*., 2002); *D. puntazzo* (Tramati *et al*., 2005), *Oreochromis niloticus* (Linnaeus, 1758) x *O. aureus* (Steindachner, 1864) (Jun-sheng *et al*., 2006), *Catla catla* (=*Labeo catla* (Hamilton, 1822)), *Labeo rohita* (Hamilton, 1822), and *Hypophthalmichthys molitrix* (Valenciennes, 1844) (Kumar *et al*., 2007); *Thunnus orientalis* (Temminck, Schlegel, 1844) (de la Parra *et al*., 2007), *Ctenopharyngodon idella* (Valenciennes, 1844) (Liu *et al*., 2008); *Cichlasoma uropthalmus* (Cuenca-Soria *et al*., 2014), *Cichlasoma trimaculatum* (=*Amphilophus trimaculatus*) (Toledo-Solís *et al*., 2017), *Cichlasoma beani* (=*Mayaheros beani*) (Mártinez-Cárdenas *et al*., 2017), and *Archosargus probatocephalus* (Merino-Contreras *et al*., 2018). However, some studies have reported different pH optimum; even two pH values of maximum activity have been recorded as in *Palabrax maculatofasciatus* (Steindachner, 1868) (Álvarez-González, 2003) and *C. undecimalis* (Concha-Frias *et al*., 2016). This is possible because the multienzymatic extract could be composed of different digestive proteases with similar specificity or by different isoforms of the same enzyme, with different optimum pH (Alarcón *et al*., 1998). Finding of a well-defined optimum pH in *M. brachyurus* suggests a major contribution of enzymes that share the same pH

optimum (de la Parra *et al*., 2007).

On the other hand, digestive acid proteases in *M. brachyurus* are stable at acid pH (2) and their activity decreases with increasing pH values. In contrast, alkaline proteases are stable in alkaline pH and are resistant to slightly acidic conditions, similar to that reported in the freshwater cichlid *P. splendida* (Uscanga-Martínez *et al*., 2011). In contrast, the acidic (stomach) proteases of marine fish are stable for a very short time at alkaline pH conditions, as reported for *T. thynnus* (Linnaeus, 1758) (Essed *et al*., 2002) and *S. maximus* (Wang *et al*., 2006). This has also been found in omnivore marine species such as *S. senegalensis* (Sáenz de Rodrigáñez *et al*., 2005) and carnivorous freshwater species such as *A. tropicus* (Guerrero-Zárate *et al*., 2014). This could hardly occur in *M. brachyurus* because acid proteases lose activity at a neutral or alkaline pH. However, this loss of activity is compensated, by the hydrolysis of alkaline proteases at an acidic pH, which is also compensated by a long intestine and strong sphincters that allow longer gut residence of the food, in addition to the joint action of digestive hormones such as cholecystokinin that causes peristalsis, increasing surface contact with the enzymatic substrate (Álvarez-González, 2003).

Worth mentioning alkaline proteases may be more stable at different pH values, because they comprised a greater number of enzymes (trypsin, chymotrypsin, L-aminopeptidase, carboxypeptidase A, elastases, among others), which increases the possibility of hydrolyzing food proteins despite the pH variation (Alarcón *et al*., 1998). Instead, acid proteases are often more susceptible to pH changes, since they only have an enzyme corresponding to pepsin, which when subjected to different pH values, undergoes changes in its structural configuration or denaturation that leads to partial or total loss of activity (Nalinanon *et al*., 2010).

Optimum temperature for digestive proteases of *M. brachyurus* has typical values (35 °C for acidic proteases and 45 °C for alkaline proteases) that are similar for other fish species. Thus, variations in activity due to temperature depend on the genetic composition and habitat conditions. In this aspect, the optimum temperature of the alkaline proteases is typically 10 °C higher than the optimum temperature of the acid proteases and also is more thermostable (Álvarez-González, 2003). Usually, the difference between the optimum temperatures of alkaline and acid proteases has been described in carnivorous fish species such as *T. thynnus* (Essed *et al*., 2002), *B. orbignyanus* (García-Carreño *et al*., 2002) and *P. maculatofasciatus* (Álvarez-González, 2003). The optimum temperature of acid proteases in *M. brachyurus* was 35 °C, similar to that described in the hybrid *O. niloticus* x *O. aureus* (Jun-sheng *et al*., 2006), but higher than that found for *S. senegalensis* (Sáenz de Rodrigáñez *et al*., 2005), *T. orientalis* (de la Parra *et al*., 2007), *C. trimaculatum* (=*Amphilophus trimaculatus*) (Toledo-Solís *et al*., 2015), and *A. probatocephalus* (Merino-Contreras *et al*., 2018), with optimum temperature range 35 to 55 °C. The activity of acid proteases is stable up to 45 °C, resulting in values >100% for some of the incubation times, but over 60% of the activity is lost above 55 °C. This could be due to denaturation of pepsin at temperatures over 55 °C, in agreement with results described by Furné *et al.* (2005). In contrast, alkaline proteases have an optimal temperature of 45 °C, which is less than reported for the *Caranx hippos* (Linnaeus, 1766), *Pseudupeneus maculatus* (Bloch, 1793), *Sparisoma* sp., *Hoplias malabaricus* (Bloch, 1794) (Alencar *et al*., 2003), and *O. niloticus* (Bezerra *et al*., 2005), species that show an optimum temperature ranging from 50 to 55 °C. The high thermostability of the

alkaline proteases in *M. brachyurus* is similar to that reported for *A. tropicus* (Guerrero-Zárate *et al*., 2014), *C. trimaculatum* (=*Amphilophus trimaculatus*) (Toledo-Solís *et al*., 2015), and *C. undecimalis* (Concha-Frias *et al*., 2016). It is noteworthy that optimum temperature and thermal stability of the digestive proteases are only operational parameters of enzymes, rather than physiologically relevant results, since activities may vary with enzyme configuration, habitat, environment and genetic aspects of the species (Alarcón *et al*., 1998; Furné *et al*., 2005; Tanji *et al*., 2007). This deficiency in activity is compensated by a longer retention of food in the digestive system to perform the hydrolysis of proteins at lower temperatures than the optimum (Furné *et al*., 2005; Lazo *et al*., 2007; Nalinanon *et al*., 2010; Uscanga *et al*., 2010).

The seven types of specific inhibitors reduced the activity of alkaline proteases by at least 53%. Of these inhibitors, the group of serine proteases was inhibited by 40% by Phenyl methyl sulphonyl fluoride (PMSF), and by 35% and 58% with ovalbumin and soybean trypsin inhibitor (SBT1), respectively. This pattern in which serine proteases were more markedly affected than the rest of the enzymes was similar to findings in *S. aequifasciatus* (Chong *et al*., 2002), *T. thynnus* (Essed *et al*., 2002), *C. idella* (Liu *et al*., 2008), *C. trimaculatum* (=*Amphilophus trimaculatus*) (Toledo-Solís *et al*., 2015), and *C. beani* (=*Mayaheros beani*) (Mártinez-Cárdenas *et al*., 2017). The other kind of digestive enzyme group subject to high inhibition was the metalloproteases with 55% inhibition by Phenanthroline and 75% by ethyl-diamine tetra-acetic acid (EDTA), which are specific inhibitors and inactivators of these enzymes, which are dependent on cofactors such as Ca and Mg. This indicates that through alkaline digestion, *M. brachyurus* has the ability to hydrolyze proteins from the carboxyl and amino terminal ends of the peptide chain.

It is important to note that the presence of inhibitors in protein sources should be considered in diet formulation to determine the composition of ingredients that do not contain inhibitors of metalloproteases, aspartic or serine proteases. Our results also showed that TPCK inhibited 80% of chymotrypsin activity and TLCK inhibited 75% of trypsin activity. This result was similar to that found in *B. orbignyanus* (García-Carreño *et al*., 2002) and *S. formosus* (Natalia *et al*., 2004), in which greater inhibition of trypsin than of chymotrypsin by TLCK was documented. Thus, it is confirmed the determination of the types of hydrolases that comprise the digestive proteases of a species using specific inhibitors allows identification of dietary ingredients that may reduce the activity of specific enzymes and thus limit feed digestibility (Essed *et al*., 2002).

Specific inhibitors of alkaline proteases was performed on SDS-PAGE, six active bands were detected in the control well, differentiating two groups of enzymes: the first group of four active bands with molecular weights between 87.9, 64.8, 59.7 and 52.6 kDa, and the second group of two active bands with molecular weights of 38.1 and 35.2 kDa. The number of active bands found in *M. brachyurus* was similar to that reported in marine species such as *P. maculatofasciatus* (Álvarez-González, 2003), and freshwater species such as *P. splendida* (Uscanga-Martínez *et al*., 2011), *A. tropicus* (Guerrero-Zárate *et al*., 2014), *C. undecimalis* (Concha-Frias *et al*., 2016), and *C. beani* (=*Mayaheros beani*) (Mártinez-Cárdenas *et al*., 2017). In other omnivorous species such as the South American pilchard *Sardinops sagax caerulea* (=*Sardinops sagax* (Jenyns, 1842)) (Castillo-Yáñez *et al*., 2005) and round sardinella *Sardinella aurita* Valenciennes, 1847 (Ben Khaled *et al*., 2011), trypsin showed active bands with different molecular weights ranging

between 35 and 38.8 kDa, while purified chymotrypsin from *S. sagax caerulea* had a molecular weight of 35.5 kDa (Castillo-Yañez *et al*., 2009). The inhibitors SBT1 and PMSF, specific for serine protease, presented the highest inhibition as they eliminated three active bands in *M. brachyurus*, which could be trypsin-like or chymotrypsin-like enzymes (Simpson, 2000; Zhou *et al*., 2008; Castillo-Yañez *et al*., 2009; Ben Khaled *et al*., 2011; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018). On the other hand, the specific inhibitors for metalloproteases PHE and EDTA inhibited two active bands in the first group of enzymes. Therefore, future studies should be conducted with purified enzymes to determine the specific type of enzymes present in *M. brachyurus*.

In conclusion, juvenile of *M. brachyurus* have a high stability that alkaline digestive proteases that are shown at different pH and temperature values, so we consider that there are other types of digestive enzymes (trypsin-like, chymotrypsin-like, among others), which may be different to those observed in other species. Likewise, the alkaline proteases of pipefish are more resistant to inhibitors such as SBT1 and PMSF than other fish species, so it is probable that several protein ingredients of different origin can be used for develop a balanced diet, at least in the juvenile period; however, it is necessary to complement this research with in vitro and in vivo studies to develop a basal diet in the culture of *M. brachyurus*.

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AUTHOR'S CONTRIBUTION

Leonardo Martínez-Cárdenas: Conceptualization, Data curation, Investigation, Methodology, Writing-original draft, Writing-review & editing.

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Carlos A. Álvarez-González: Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing-original draft, Writing-review & editing.

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Jesús T. Ponce-Palafox: Conceptualization, Formal analysis, Writing-original draft, Writing-review & editing.

ETHICAL STATEMENT

Animals were handled in compliance with the Norma Oficial Mexicana NOM-062-ZOO-1999 from Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, the Mexican standards for good welfare practices of laboratory animals.

COMPETING INTERESTS

The authors declare no competing interests.

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