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Research Article

Partial characterization of digestive proteases in Pacific red snapper *Lutjanus peru* Nichols & Murphy, 1922 (Perciformes: Lutjanidae)

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ABSTRACT. Pacific red snapper (*Lutjanus peru*) is an important commercial species in Mexico with great aquaculture potential; however, digestive physiology is still unknown. Therefore, the objective of the present work was to characterize the digestive proteases of *L. peru* juvenile using biochemical and electrophoretic techniques. Results showed a higher acid protease activity than the alkaline proteases, trypsin, chymotrypsin, and leucine aminopeptidase (LAP). The optimum temperature for acid proteases was between 30 to 40°C. Trypsin activity showed two maximum peaks of temperature (30 and 50°C), while alkaline proteases, chymotrypsin, and LAP had optimum temperatures of 50, 50 to 60, and 40°C, respectively. Moreover, the optimum pH of acid proteases was between 2 and 3. Also, alkaline proteases, trypsin, chymotrypsin showed pH optimums at pH 6, 9, and 5, respectively, although LAP showed two optimum pH values at 6 and 9. Acid protease zymogram showed three isoforms, totally inhibited by pepstatin A. Alkaline protease zymogram revealed six bands (125.4, 67.2, 57.9, 48.6, 29.8, and 26.9 kDa), which were inhibited by specific serine-proteases and metalloproteases inhibitors. In conclusion, the main digestion in *L. peru* depends on stomach proteases, which are characteristic of carnivorous fish, followed by intestinal digestion supported mainly by chymotrypsin.

Keywords: *Lutjanus peru*; proteases; enzymes; inhibitors; physiology; characterization; aquaculture

INTRODUCTION

Lutjanidae family comprises more than 112 species distributed on the Indian Ocean and the tropical and subtropical regions of the western Pacific Ocean (Chu et al. 2013). The fishery of Pacific red snapper *Lutjanus peru* (Nichols & Murphy, 1922) is one of the most important along the Pacific coast of Mexico due to its capture volume and commercial value (Rojas-Herrera et al. 2003). Furthermore, *L. peru* is listed as a native marine fish grown in Mexico with technological advances in reproduction, larviculture, and grow-out techniques (Dávila-Camacho et al. 2019). However, scarce information is available about nutrition or diges-

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tive physiology. Only one study focused on organogenesis of the digestive system has been reported (Peña et al. 2016), which serves as support information to understand nutritional aspects (Tufan et al. 2008).

Digestive proteases characterization allows the determination of the optimal conditions of physicochemical parameters, kinetic mechanism of the enzymes, and identifying specific enzyme classes. Proteins are the most important ingredients concerning energy and amino acid requirements in fishes (Lazo et al. 2007). Therefore, studies concerning protein digestive physiology are necessary to develop a species-specific diet and determine the amount and type of ingredients that could be included. In this way, the appearance of digestive enzymes has been related to various physiological characteristics (feeding habits) and nutritional characteristics (requirements) in many species of marine fish, such as spotted rose snapper *Lutjanus guttatus* (Steindachner, 1869), thicklip grey mullet *Chelon labrosus* (Risso, 1827), common snook *Centropomus undecimalis* (Bloch, 1792) and sheepshead *Archosargus probatocephalus* (Walbaum, 1792) (Peña et al. 2015, Concha-Frias et al. 2016, Pujante et al. 2017, Merino-Contreras et al. 2018), as well as some freshwater fish, such as tropical gar *Atractosteus tropicus* Gill, 1863, Mayan cichlid *Mayaheros urophthalmus* (Günther, 1862), tree-spot cichlid *Cichlasoma trimaculatum* (Günther, 1867), green cichlid *Cichlasoma beani* (=*Mayaheros beani*) (Jordan, 1889) and short-tailed pipefish *Microphis brachyurus* (Bleeker, 1854) (Cuenca-Soria et al. 2014, Guerrero-Zárate et al. 2014, Toledo-Solís et al. 2015, Martínez-Cárdenas et al. 2020). Thus, the objective of the present work was to partially characterize the digestive proteases in the *L. peru* using biochemical and electrophoretic techniques.

MATERIALS AND METHODS

Fish and culture conditions

Juveniles *Lutjanus peru* (89.2 ± 30.6 g wet weight and 16.4 ± 1.5 cm total length) were obtained from the Pilot Marine Fish Hatchery of the Research Center in Food and Development Unit Mazatlán, according to Ibarra-Castro & Alvarez-Lajonchère (2011). Fish were fed three times a day at apparent satiety with commercial diet Skretting feeds (marine mix; 46% protein and 12% lipids) until sacrifice and dissection.

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

Preparation of enzymatic extracts

Enzymatic extracts were prepared from nine fish, starved by 24 h before being euthanized by cold thermal shock. The individual organ weight was recorded with an Ohaus® digital balance (0.01 g, Shenzhen, China). Stomachs and intestines were individually dissected under cold conditions (4ºC), and then organs were frozen and lyophilized. Subsequently, two homogenates were performed (stomachs and intestines separately) in a ratio of 1:10 (weight: saline solution 0.9% NaCl) with an Ultra Turrax (IKA T18 basic, Wilmington, USA), according to Matus de la Parra et al. (2007). The homogenates were centrifuged at 14000 *g* for 15 min at 4°C, and the supernatants were stored at -80°C until analysis.

Protease activity analysis

Acid protease activity was determined using the technique described by Anson (1938), using bovine hemoglobin 1% as substrate at pH 2. The alkaline protease activity was determined using the technique described by Walter (1984), using casein 1% as substrate. Trypsin activity was determined with the method described by Erlanger et al. (1961), using Nα-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) as substrate. The chymotrypsin activity was measured by Del Mar et al. (1979) technique using SAAPNA (N-succinyl-ala-ala-pro-phep-nitroanilide) substrate. Leucine-aminopeptidase (LAP) was measured according to Maroux et al. (1973), using L-leucine *p*-nitroanilide as substrate. The concentration of soluble protein in the enzymatic extracts was determined with the technique described by Bradford (1976).

One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µM of product released per minute. Tyrosine amount liberated from hemoglobin and casein hydrolysis was determined at 280 nm, while the amount of *p*-nitroanilide liberated from BAPNA, SAAPNA, and L-leucine-*p*-nitroanilide was determined at 410 nm. Total activity (units mL^{-1}) = [Δabs × final reaction volume (mL)] / [MEC × time (min) × extract volume (mL)]. Specific activity (units mg^{-1} protein) = [total activity / soluble protein (mg)], where Δabs represent the increase in absorbance, and MEC represents the molar extinction coefficient for tyrosine or p-nitroanilide (0.005 and 0.008 mL μM^{-1} cm, respectively).

Optimal temperature and pH of digestive proteases

The optimum temperature of the acidic and alkaline proteases, trypsin, chymotrypsin, and LAP, was determined in a range between 10 to 60°C with intervals of 10°C, using the techniques described in the

previous section for each enzyme type. The acid proteases' optimum pH was determined in the range of 1 to 5, while alkaline proteases, trypsin, chymotrypsin, and LAP were determined in the pH range of 5 to 10.

Electrophoretic analyses

For the analysis of stomach acid proteases, electrophoresis was run under non-denaturing native conditions (Native-PAGE) using a continuous acrylamide gel (10%) in Tris (25 mM) and glycine buffers (192 mM, pH 8.3, 80 volts) according to Davis (1964). Gels were revealed for proteases isoforms according to the procedure of Díaz-López et al. (1998). The gels were removed from the cell and soaked in 100 mM Tris-HCl to lower the pH to 2.0 to allow them to become enzymatically active. After 15 min, the gel was submerged for 15 min at 25°C in a solution containing 0.25% hemoglobin (100 mM glycine-HCl buffer, pH 2.0). The gels were washed with distilled water and then fixed in trichloroacetic acid (12%) solution for 15 min. The electrophoretic analysis of alkaline proteases was conducted 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 mM) and glycine (192 mM, pH 8.3, 100 volts), according to Laemmli (1970) and adapted by García-Carreño et al. (1993). After alkaline SDS-PAGE electrophoresis, the gels were washed and incubated for 60 min at 4°C in a 0.5% casein solution (Tris-HCl 100 mM buffer, pH 9). The gels were then incubated for 60 min in this solution at 37°C and then washed and fixed as previously described. According to Weber & Osborn (1969), the staining procedure was performed using a 0.1% coomassie brilliant blue R-250 solution, while distaining was carried out in a 35:10:55 methanol-acetic acid-water solution.

Additional characterization was complemented using specific inhibitors, according to the method described by Dunn (1989). The intestine and stomach extracts were pre-incubated for 1 h with the following inhibitors: 1 mM of pepstatin A inhibitor was used for the acid proteases, while alkaline proteases inhibitors were: 10 mM tosyl-phenylalanyl-chloromethyl ketone (TPCK), 10 mM phenanthroline (PHEN), 10 mM ethyl-diamine tetra-acetic acid (EDTA), 10 mM tosyl-lysyl-chloromethyl ketone (TLCK), 250 mM ovalbumin (OVO), 250 mM soybean trypsin inhibitor (SBT1) and 100 mM phenylmethylsulphonyl fluoride (PMSF). A molecular weight marker was applied to each SDS-PAGE (5 µL per well); marker: protein leader composed of 14 recombinant proteins ranging from 10 kDa to 200 kDa. (Thermo Scientific, cat. 26614). The relative electromobility (Rf) was calculated for all zymograms (Igbokwe & Downe 1978). The molecular weight (MW) of each band in the SDS-zymograms (alkaline protease) 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.

Statistics

The total enzymatic activity (U mL^{-1}) and the specific activity (U mg^{-1} protein) data of the different proteases were submitted to normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene test). Relative activity (%) data at different pH and temperatures were arcsin (x1/2)-transformed previous to submit to normality and homoscedasticity. When the assumptions complied, a one-way analysis of variance (ANOVA) was applied; when differences were found, Tukey's HSD test was used ($P \leq 0.05$). All the statistical analyses were performed using Statistica 7.0 software for Windows (StatSoft, USA).

RESULTS

The enzymatic activity (U mL^{-1} and U mg^{-1} protein) values of acid proteases, alkaline proteases, trypsin, chymotrypsin, and leucine aminopeptidase from the digestive tract of *Lutjanus peru* juvenile are shown in Table 1. The activity of the acid proteases was higher than alkaline protease activity, as well as trypsin, chymotrypsin (49.61 ± 3.65 U mg^{-1} protein), and LAP.

The optimum temperature of activity for acid protease varies between 30 and 40°C (Fig. 1a). On the other hand, the optimum temperature of the alkaline protease activity is 50°C, trypsin activity presents two optimums at 30 and 50°C. The optimum of chymotrypsin activity is at 50 and 60°C, and the optimum of leucine aminopeptidase activity is at 40°C (Fig. 1b).

The optimum pH of acid protease activity is at 2 with 90% relative activity at pH 3 (Fig. 2a). On the other hand, the optimum pH of the alkaline protease activity is at 6, and trypsin optimum is at pH 9, the chymotrypsin optimum is at pH 5, while LAP activity presents optimums at pH 6 and 9 (Fig. 2b).

Acid protease zymogram without inhibitor revealed three bands with acid activity, with Rf of 0.19, 0.63, and 0.77, where the inclusion of pepstatin A inhibits the three bands (Fig. 3). The SDS-PAGE for alkaline protease analysis without inhibitor revealed six bands (125.4, 67.2, 57.9, 48.6, 29.8, and 26.9 kDa). Ovalbumin and SBT1 inhibited five bands (67.2, 57.9, 48.6, 29.8 and 26.9 kDa), TLCK inhibited three bands (57.9, 48.6 and 26.9 kDa), EDTA inhibited two bands (67.2 and 57.9 kDa), PHEN inhibited the 57.9 kDa

Table 1. Digestive enzyme activity (mean ± standard deviation, n = 9) from the enzymatic extracts in stomach and intestine of *Lutjanus peru* juveniles. LAP: leucine aminopeptidase. Superscripts within each column indicate a significant difference ($P < 0.05$) by Tukey's test.

Protease type	U mL^{-1}	U mg^{-1} protein
Acid proteases	27900.0 ± 886.9^{a}	6821.5 ± 216.9^{a}
Alkaline proteases	123.7 ± 8.1^{d}	5.4 ± 0.2^{d}
Trypsin	84.5 ± 7.0^{e}	3.8 ± 0.3^{e}
Chymotrypsin	1092.4 ± 80.4^{b}	49.6 ± 3.7^{b}
LAP	354.2 ± 29.4^{c}	16.1 ± 1.3^{c}

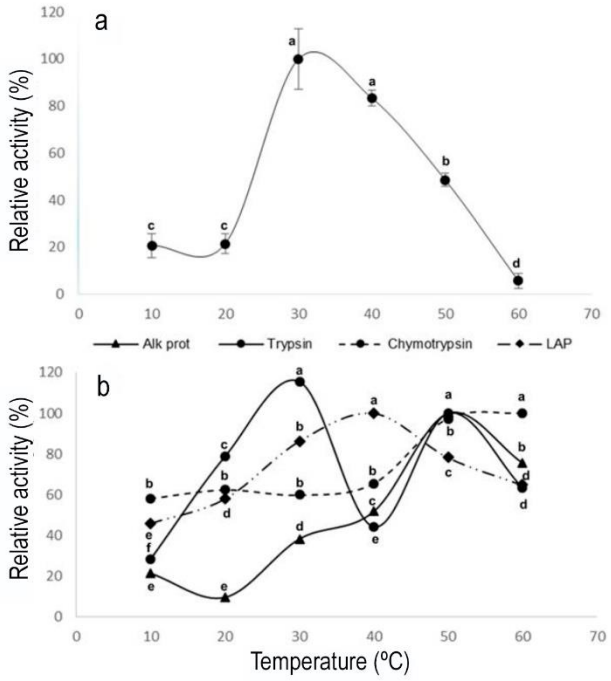

Figure 1. Effect of temperature (°C) on relative activity (%) of digestive enzymes of *Lutjanus peru*. a) Pepsin, b) total alkaline proteases (Alk prot), trypsin, chymotrypsin, leucine aminopeptidase (LAP) from the intestine pool. Superscripts within each enzyme type are significantly ($P < 0.05$) different by Tukey's test.

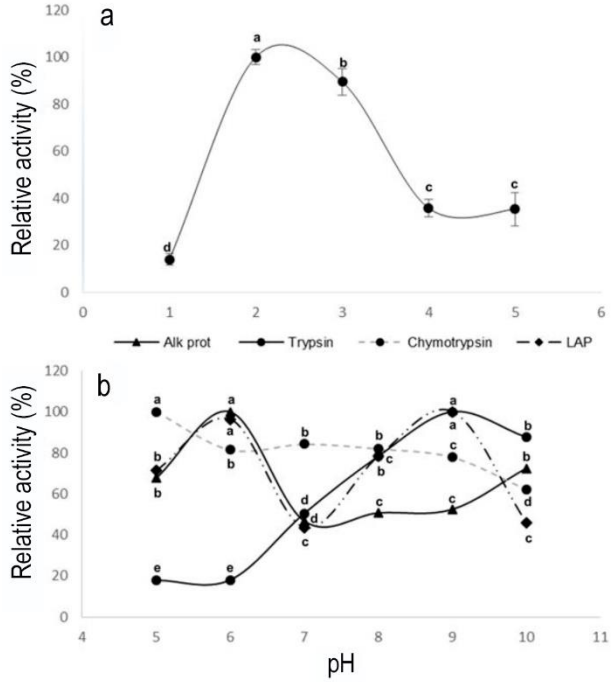

Figure 2. Effect of pH on relative activity (%) of digestive enzymes of *Lutjanus peru* digestive enzymes. a) Pepsin, b) total alkaline proteases (Alk prot), trypsin, chymotrypsin, leucine aminopeptidase (LAP) from the intestine pool. Superscripts within each enzyme type are significantly ($P < 0.05$) different by Tukey's test.

band, while PMFS inhibited all bands, and finally, TPCK did not inhibit any band (Fig. 4).

DISCUSSION

The high activity of acid proteases found in *Lutjanus peru* juveniles concerning alkaline proteases highlights the carnivorous tendency of the species, as reported in other Lutjanidae species such as yellow snapper *L. argentiventris* (Peters, 1869), Pacific dog snapper *L. novemfasciatus* (Gill, 1862) and *L. guttatus* (Alarcón et al. 2001, Peña et al. 2015).

The optimal activity found for acid proteases was between 2 and 3 from three bands with acid activity (Rf of 0.19, 0.63, and 077), all identified as aspartic peptidases, such as pepsin-like. Most fish species with stomachs have two to four isoforms of pepsins which show an optimum activity between pH 2.0 to 3.5 (Klomklao 2008, Zhao et al. 2011). Previous studies in *L. gutattus* showed pH pepsin optimum between 2 and 3, with two bands (Rf of 0.72 and 0.79) (Peña et al. 2015, 2017). Some fish species shows a single pepsin band, such as *C. undecimalis* (Concha-Frías et al. 2016), fat snook C*entropomus parallelus* (Poey, 1860) (Jesús-Ramírez et al. 2017), albacore *Thunnus alalunga* (Bonnaterre, 1788) (Nalinanon et al. 2010) and *A.*

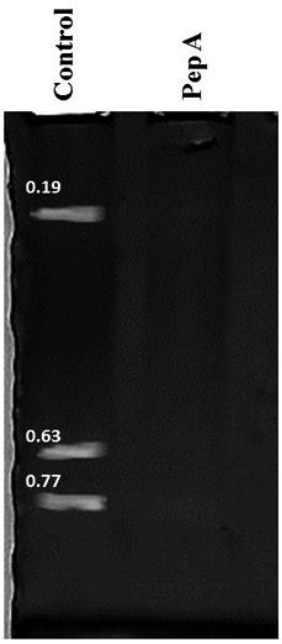

Figure 3. Native-PAGE of acid proteases of *Lutjanus peru* showing proteolytic activity at pH 2.0 present in stomach extract (Control) and stomach extract mixed with pepstatin A (Pep A).

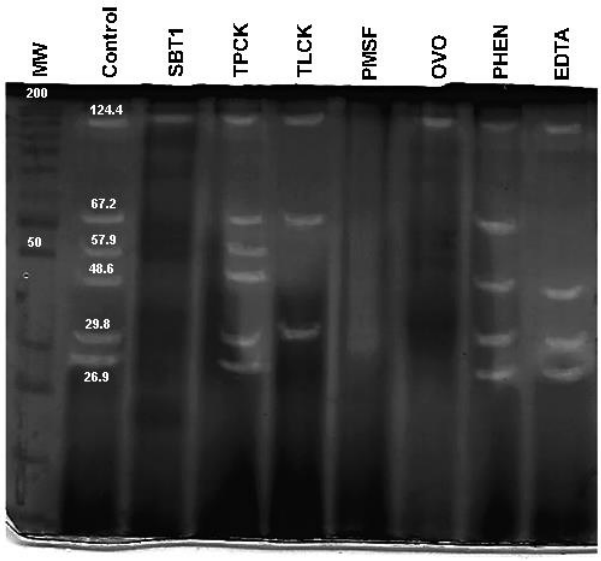

Figure 4. SDS-PAGE of alkaline digestive proteases of *Lutjanus peru* using different inhibitors. Alkaline proteases with no inhibitor (Control), tosyl-phenylanyl-chloromethyl ketone (TPCK), phenanthroline (PHEN), ethyl-diamine tetra-acetic acid (EDTA), tosyl-lysyl-chloromethyl ketone (TLCK), ovalbumin (OVO), soybean trypsin inhibitor (SBT1), phenylmethyl sulphonyl fluoride (PMSF): marker of molecular weights (MW; line 1): Thermo Scientific, protein leader composed by 14 recombinant protein ranging from 10 kDa to 200 kDa (Cat# 26614). Control (line 2); inhibitors: SBT1 (line 3), TPCK (line 4), TLCK (line 5), PMSF (line 6), OVO (line 7), PHEN (line 8) and EDTA (line 9).

probatocephalus (Merino-Contreras et al. 2018). Other species show pepsin isoforms such as European eel *Anguilla anguilla* (Linnaeus, 1758) with three isoforms (Wu et al. 2009) and yellowfin seabream *Acanthopagrus latus* (Houttuyn, 1782) and mandarin fish *Siniperca chuatsi* (Basilewsky, 1855) that shows four pepsin isoforms (Zhou et al. 2007, 2008). Moreover, 30% of the pepsin relative activity is detected at pH 4 and 5 (Fig. 2a), ensuring its functionality even during the pH-shift process from an acidic pH in the stomach to an alkaline pH in the pyloric caeca and anterior intestinal region.

Accordingly, alkaline protease activity of *L. peru* presented the maximum value at pH 6, which has not been previously reported in lutjanid species, while the pH range of chymotrypsin activity in mammals and most aquatic organisms is 7.5-9.0 (Zhou et al. 2011). On the contrary, the optimum pH of trypsin was found at pH 9, according to many other fish trypsin, ranging between 7 to 11 and stable at alkaline pH (7 to 9) (Jesús-de la Cruz et al. 2018). However, optimum pH found in total alkaline proteases seems to be more influenced by the chymotrypsin activity and LAP rather than trypsin-like enzymes, supported by pH optimums found in chymotrypsin (pH 5) and LAP (pH 6 and 9) (Fig. 2b). At the same time, higher activities were found for chymotrypsin and LAP than trypsin (Table 1). This pattern of activity ratio has been more frequently registered for freshwater species with omnivorous habits (Cuenca-Soria et al. 2014, Toledo-Solís et al. 2015). The high chymotrypsin activity could represent an advantage concerning diet formulation. Low-quality protein sources (low in essential amino acids) could be well digested by this species, where preferentially cleave polypeptide chains at the carboxyl sides of the bulky hydrophobic amino acid (phenylalanine, tyrosine, tryptophan, and leucine) (El Hadj Ali et al. 2010).

LAP activity showed two peaks (pH 6 and 9), suggesting the presence of isoenzymes of LAP in the intestinal epithelium of *L. peru*. Scarce information about studies on biochemical characterization of leucine aminopeptidase in fishes is available; however, a report in bluefin tuna *Thunnus orientalis* (Temminck & Schlegel, 1844) shows a slight difference in optimal pH (8 to 9) between intestinal sections, which suggest that isoenzymes are present (Matus de la Parra et al. 2007). In red sea bream *Pagrus major* (Temminck & Schlegel, 1843) optimal pH for LAP was 7.5 with a slight decrease of its activity at a pH of 6.5 (Wu et al. 2008). Therefore, optimum pH found for total alkaline proteases, chymotrypsin and LAP, at acid pH (5 and 6)

in *L. peru*, could be an adaptation to a short alkaline digestive phase. The pH switch from acid to alkaline pH environment is inefficient, at least in the first phase of alkaline digestion (in pyloric caeca and proximal intestine), where digestion is carried out with slightly acid and neutral pH (pH 5 to 7).

The optimum temperature of acid proteases in *L. peru* was found at 30 and 40°C, while reports in *L. guttatus* registered optimum temperature for pepsin activity at 45°C during the juvenile stage (Peña et al. 2015). Consequently, the broad range of temperature optimum found in *L. peru* pepsin is related to differences in structural properties among the three isoforms found in the present study (Fig. 3), which confers to the species the possibility to maximize pepsin activity under different environmental conditions, related to wide range temperature in eastern Pacific (20-29°C), where this specie is distributed (Coria-Monter et al. 2019).

The optimum temperature alkaline proteases in *L. peru* were found at 50°C, generated by the balance from the pancreatic and luminal origin (endo- and exopeptidases). In particular, trypsin-like activities showed two peaks (30 and 50°C) that highlight the presence of two isoforms, as found in the alkaline zymogram, where TLCK inhibits three bands (Fig. 4). Reports in *L. guttatus* showed optimum temperatures for trypsin between 50 and 60°C (Peña et al. 2015), while brown stripe red snapper *Lutjanus vitta* (Quoy & Gaimard, 1824) shows a peak for trypsin activity at 60°C (Khantaphant & Benjakul 2010). Therefore, the high activity of trypsin isoforms at 30°C gives digestive advantages at the environmental condition as previously mentioned comparing with the other Lutjanidae species. In the same context, chymotrypsin shows optimum activity at 50 and 60°C; however, this enzyme shows high relative activities (60%) at 10°C, conferring high capacity to protein hydrolysis even in low temperatures. LAP optimum activity was detected at 40°C, close to optimum activity (45°C) reported for LAP in *T. orientalis* (Matus de la Parra et al. 2007). As a cytosolic and membrane-bound enzyme, LAP possesses high physiological importance in digestion by small peptides incorporation into de intestinal enterocytes by pinocytosis (David-Ruales et al. 2018). This process could be of high importance in species with small intestine longitude, such as *L. peru*.

In the present study, various general and specific inhibitors were used to determine the subclass of peptidases present in both pyloric caeca and intestine of *L. peru* juveniles. PMSF, SBT1, and turkey ovomucoid (OVO) were used for serine proteases; for trypsin and chymotrypsin detection, specific inhibitors, TLCK and TPCK, were used, respectively; EDTA and PHE were used as chelating deactivators to identify metalloproteases. In addition, a zymogram of alkaline proteases in *L. peru* showed six bands, which is similar for *L. guttatus*, where five to nine active alkaline bands were detected during juvenile ontogeny of the species (Peña et al. 2017).

General inhibitors for serine proteases such as OVO and SBT1 inhibited five bands from 67.2 to 26.9 kDa. In contrast, PMSF inhibits all bands, showing a high sensibility for the intestinal digestive serine proteases (trypsin-like or chymotrypsin-like enzymes), which is in agreement with other studies of several species (Simpson 2000, Castillo-Yáñez et al. 2006, 2009, Ben-Khaled et al. 2011). TLCK inhibited three bands related to the trypsin-like activity. Otherwise, metalloproteases inhibitors PHEN and EDTA inhibited a single band (57.9 kDa) and two bands (57.9 and 67.2 kDa), respectively. Metalloproteases include aminopeptidases and carboxypeptidases, which are associated with the intestinal brush-border hydrolysis, playing an important role in final protein digestion (Alarcón et al. 1998), which has been detected in *L. guttatus* (Peña et al. 2017) and freshwater carnivorous and herbivorous fishes (Dimes & Haard 1994, Chong et al. 2002, Guerrero-Zárate et al. 2014). More studies of characterization are needed to elucidate the metal cofactor which constitutes LAP of *L. peru* because other reports indicate that many, but not all LAP are metalloenzymes with requirements of a different divalent metal ion such as Mn^{2+}, Co^{2+}, instead of Zn^{2+}, which are essential for digestive enzyme activity (Kim & Lipscomb 1993). In mammals and fish pancreas, two Zn^{2+}-dependent carboxypeptidases are reported (Hajjou et al. 1995, Kishimura et al. 2006), while Atlantic cod *Gadus morhua* (Linnaeus, 1758) presents a collagenolytic serine protease that belongs to Zn^{2+} metalloproteinase. This enzyme displays both trypsin-like and chymotrypsin-like activities with high digestive capacity (Haard 1994, Kristjansson et al. 1995).

CONCLUSIONS

The characterization of digestive enzymes of *L. peru* showed that the stomach is the main digestive organ with the presence of three pepsin-like isoforms that shows higher proteolytic activity than intestinal proteases. Chymotrypsin and LAP showed higher activities at acid and neutral conditions and displayed higher activity than trypsin-like enzymes. Our findings indicate that this species has feeding habits of a carnivorous fish, but with a tendency to omnivorism, which would allow us to evaluate alternative by-products ingredients to formulate balanced foods to achieve its culture.

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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.

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