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Comparative characterization of digestive proteases in redhead cichlid (*Vieja melanurus*) and twoband cichlid (*Vieja bifasciata*) (Percoidei: Cichlidae)

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In the Southeast of Mexico, there are many native cichlids with commercial interest such as redhead cichlid (*Vieja melanurus*) and twoband cichlid (*V. bifasciata*), which have a great local demand and excellent meat quality. However, it is necessary to implement their culture based on nutrition studies and digestive biochemistry. This study's objective was to characterize these two cichlids' digestive proteases (pH, temperature, and inhibitors) through biochemistry techniques. Results showed that *V. melanurus* and *V. bifasciata* have a digestive capacity analogous to other omnivore fishes, where the optimal pH values of stomach proteases (4 and 2, respectively) and intestinal proteases (6 and 12, respectively), the optimal temperature of acid (35°C and 55°C, respectively) and alkaline proteases (45°C and 55°C, respectively) are quite similar. Both species presented high thermal and pH stabilities. Inhibition showed that *V. melanurus* is more sensitive to specific inhibitors for alkaline proteases than *V. bifasciata*. In conclusion, *V. bisfasciata* and *V. melanurus* have different digestive protease patterns. Both species can hydrolyze different protein ingredients to formulate a specific diet. Nevertheless, *V. bifasciata* is more resistant to the presence of inhibitors, which allow it to include vegetable proteins in its diet.

Keywords: Inhibitors, pH, Proteases, Stability, Temperature.

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En el sureste de México, existen muchas especies de cíclidos nativos de interés comercial como el cíclido rojo (*Vieja melanurus*) y el cíclido de dos bandas (*V. bifasciata*), los cuales tienen una gran demanda local y tienen una excelente calidad de carne; sin embargo, es necesario implementar su cultivo con base en estudios de nutrición y bioquímica digestiva. El objetivo de este estudio fue caracterizar las proteasas digestivas (pH, temperatura e inhibidores) de estos dos cíclidos nativos mediante técnicas bioquímicas. Los resultados mostraron que V. melanurus y *V. bifasciata* tienen una capacidad digestiva similar a otros peces omnívoros, donde los valores óptimos de pH de proteasas estomacales (4 y 2, respectivamente) e intestinales (6 y 12, respectivamente), la temperatura óptima de proteasas ácidas (35°C y 55°C, respectivamente) y alcalinas (45°C y 55°C, respectivamente) son muy parecidas. Ambas especies presentaron alta estabilidad térmica y de pH. La inhibición mostró que *V. melanurus* es más sensible a inhibidores específicos de proteasas alcalinas que *V. bifasciata*. En conclusión, *V. bisfasciata* y *V. melanurus* tienen diferentes patrones de proteasas digestivas, pero ambas especies pueden hidrolizar diversos ingredientes proteicos para formular dietas específicas; sin embargo, *V. bifasciata* es más resistente a la presencia de inhibidores, lo que permitiría incluir proteínas vegetales en su dieta.

Palabras clave: Estabilidad, Inhibidores, pH, Proteasas, Temperatura.

INTRODUCTION

The fish culture in Mexico has been supported from the beginning by the culture of introduced fish such as rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), grass carp *Ctenopharyngodon idella* (Valenciennes, 1844), and Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758), which has imitated the development of technology for the culture of native fish species. As a scientific and technological discipline, aquaculture has had a relatively recent development and research, particularly with native fish species, to understand many fundamental aspects such as biology, ecology, and physiology (Dávila-Camacho *et al*., 2019). However, in Southeast Mexico, there are some native cichlid species with great commercial importance such as bay snook *Petenia splendida* Günther, 1862, Mayan cichlid *Mayaheros urophthalmus* (Günther, 1862), redhead cichlid *Vieja melanurus* (Günther, 1862), and twoband cichlid *Vieja bifasciata* (Steindachner, 1864) (Pérez-Sánchez, Páramo-Delgadillo, 2008). All these species have a high demand in the local market, proper growth, and excellent meat quality (Uscanga-Martínez *et al*., 2011). *Vieja melanurus* and *V. bifasciata* have a neotropical distribution and can be found in shallow waters such as lakes, lagoons, and swampy areas and on the banks of rivers and flood zones of the state of Tabasco (lower part of Usumacinta), in Guatemala (to the north in the Petén area) and Belize. Their feeding habit is omnivorous, mainly based on insects, larvae of smaller fish, and aquatic plants. However, although larviculture is currently achieved using *Artemia* nauplii and commercial trout feed, it is unknown if these foods are suitable for this stage since necessary studies have not been carried out on the digestive physiology in *V. bifasciata* and *V. melanurus*. Thus, there has been a

growing interest in developing technologies with native species for their incorporation and culture for commercial purposes. Research efforts aim to determine the conditions that increase the survival and viability of crop production and characterize digestive physiology to develop a better plan feeding schedule. In the last case, the studies are based on the detailed knowledge of the digestive physiology in different life stages by determining the digestive enzymatic activities and characterization of proteases. This aspect has been verified that the level of activity of some enzymes acts as a good indicator of nutritional status, so that the data obtained may be relevant to establish an optimal artificial feed to be used in its culture and reduce production costs in hatcheries (Ueberschär, 1993). Recently, several studies have been conducted to relate the appearance of digestive enzymes with physiological and nutritional aspects in various species such as the Adriatic sturgeon *Acipenser naccarii* Bonaparte, 1836, California halibut *Paralichthys californicus* (Ayres, 1859), sardine *Sardinella aurita* Valenciennes, 1847, manjuarí *Atractosteus tristoechus* (Bloch & Schneider, 1801), pike perch *Sander lucioperca* (Linnaeus, 1758), *Mayaheros urophthalmus*, *Petenia splendida*, tropical gar *Atractosteus tropicus* Gill, 1863, three spot cichlid *Cichlasoma trimaculatum* (= *Amphilophus trimaculatus* (Günther, 1867), thicklip grey mullet *Chelon labrosus* (Risso, 1827), common snook *Centropomus undecimalis* (Bloch, 1792), green cichlid *Cichlasoma beani* (= *Mayaheros beani* (Jordan, 1889)), sheepshead *Archosargus probatocephalus* (Walbaum, 1792), longfin yellowtail *Seriola rivoliana* Valenciennes, 1833, and short-tailed pipefish *Microphis brachyurus* (Bleeker, 1854) (Furnè *et al*., 2005; Álvarez-González *et al*., 2005; Comabella *et al*., 2006; Hamza *et al*., 2007; Khaled *et al*., 2011; López-Ramírez *et al*., 2011; Uscanga-Martínez *et al*., 2011; Guerrero-Zarate *et al*., 2014; Toledo-Solís *et al*., 2015; Pujante *et al*., 2016; Concha-Frias *et al*., 2016; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018; Teles *et al*., 2019; Martínez-Cárdenas *et al*., 2020). In this way, this work aims to characterize the digestive proteases determining the optimum and stability of pH and temperature and the effect of general and specific inhibitors in juveniles of *V. bifasciata* and *V. melanurus* using the biochemical technique.

MATERIAL AND METHODS

Obtaining and processing samples. For digestive protease characterization trials 100 juveniles (50 fish per species, 1–2 g wet weight and 5–8 cm of total length) of *Vieja bifasciata* (voucher ECOSC 14764, five specimens), and *V. melanurus* (voucher ECOSC 14765, five specimens) were captured in the Sánchez Magallanes Lagoon (average temperature of 32.1 ± 0.5 °C and >5 mg L^{-1} dissolved oxygen) and transported to the Laboratorio de Acuicultura Tropical of the Universidad Juárez Autónoma de Tabasco and placed in three 70 L capacity tanks with constant aeration. Fish were fed an apparent satiation three times per day (8:00, 14:00 and 20:00 h) with trout diet (Silver Cup, 45% protein and 16% lipid) for a period of 30 days. All organisms per species were used for digestive enzymatic characterization, which were previously starved for 48 h, and then, fish were sacrificed by freezing in ice-cold water. Afterward, they were weighed before, and after evisceration, the stomach and intestine were removed separately, which were homogenized with a tissue homogenizer (ULTRA TURRAX® IKA T18 Basic). The extracts were prepared in a 100 mmol L^{-1} glycine buffer solution pH 2 for the stomach

extracts, and tris buffer 100 mmol L^{-1} $CaCl_2$ 10 mmol L^{-1} at pH 9 for the intestine extracts in a 5: 1 ratio (5 mL of buffer per g of tissue) at 4°C, the obtained mixture was placed in Eppendorf tubes (1 mL per tube) and centrifuged at 14000 rpm at 4°C. The supernatant was extracted, and the pH required for each extract was adjusted then stored in Eppendorf tubes at -80°C until further enzymatic analysis.

Digestive proteases evaluation. The concentration of soluble protein in the multienzymatic extracts was determined following Bradford's (1976) technique, using bovine serum albumin (600 mg mL^{-1}) as the standard protein. For the activity of acid protease, the technique of Anson (1938) was applied using as substrate hemoglobin (1%), and with the following modifications: 1 mL of hemoglobin al (1%) in buffer 100 mmol L^{-1} glycine-HCl at pH 2.0 was added 50 µL of multienzyme extract. The extract was incubated for 2 hours at 37°C, and the reaction was stopped by the addition of 0.5 mL of trichloroacetic acid (20% TCA). After standing the reaction mixture (15 to 30 min) at 4°C, it was centrifuged at 16000 *g* for 5 min. The amount of tyrosine released (280 nm) was measured by uv/visible spectrophotometer in the supernatant. One activity unit was defined as the amount of enzyme that catalyzes the formation of 1 µg of tyrosine per minute. Tyrosine molar extinction coefficient was determined using different tyrosine concentrations (from 0 to 300 µg mL^{-1}). All tests were performed in triplicate.

The determination of alkaline protease activity was performed by the method of Kunitz (1947) modified by Walter (1984) using 1% casein as the substrate in a buffer 100 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} $CaCl_2$ at pH 9. The reaction was stopped with 20% trichloroacetic acid (TCA), and the amount of tyrosine released was determined according to the protocol described in the previous section.

Effect of pH on the activity and stability of proteases. The effect of pH on acid and alkaline protease activities in the enzymatic extracts of juveniles of *Vieja bifasciata* and *V. melanurus* were incubated with hemoglobin (1%) diluted with universal buffer Stauffer (1989), with a pH range from 2 to 12, following the procedure of activity determination enzyme described above for this type of proteases. In the case of alkaline proteases, casein (1%) buffered with the same buffer and using the same pH values was used as the substrate. All these tests were performed in triplicate.

The effect of pH on the stability of the acid and alkaline protease activity was determined by preincubated the extracts at different pH (from 2 to 12) with times of 0, 30, 60 and 90 min, then their activity was measured at normal pH (2 for acidic proteases and 9 for alkaline proteases) following the techniques described above. The results were shown in relation to the residual activity at regular intervals with respect to a control without preincubating. For all the stability tests, the values of zero reaction times were taken, such as 100% of the residual activity that allowed observing the enzyme's fluctuations from that moment.

Effect of temperature on the activity and stability of proteases. To determine the optimal temperature and the influence of temperature on the stability of the acid and alkaline proteases of the digestive proteases, the extracts of the juveniles were incubated in the substrates of hemoglobin (1%) and casein (1%), with the techniques

described above for activities of acidic proteases (Anson, 1938) and alkaline (Kunitz, 1947 modified by Walter, 1984), respectively at a temperature range from 25 to 75°C. Different incubation times were used for pH and temperature stability for this type of proteases at temperatures from 25 to 65°C with preincubation times of 0, 30, 60, and 90 min for each temperature. All these tests were performed in triplicate.

Enzymatic inhibition in digestive proteases. The characterization of alkaline proteases was obtained also applying the method of Dunn (1989), using different types of inhibitors: Tosyl-L-lysyl-chloromethane hydrochloride 10 mmol L^{-1} (TLCK), N-p-Tosyl-L-phenylalanine chloromethyl ketone 10 mmol L^{-1} (TPCK), Soybean trypsin inhibitor 250 mmol L^{-1} (SBT1), Phenylmethylsulfonyl fluoride 100 mmol L^{-1} (PMSF), Ethylenediaminetetraacetic acid 10 mmol L^{-1} (EDTA), Ovalbumin 250 mmol L^{-1} (OVO), and Phenanthroline 10 mmol L^{-1} (PHEN), mixing 20 µL of multienzyme extract and preincubated with 20 µL of each inhibitor for 1 hour at 37°C. After the preincubation, the technique described for the determination of alkaline proteases was applied; the result of the tests was compared with a control without inhibitors, to obtain the residual activity.

Statistical 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 analyze 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

Optimum pH and stability of acidic and alkaline proteases. The optimal pH of the proteases in the stomach extracts was 4 for *Vieja bifasciata*, this being the highest peak of relative activity, which was decreasing constantly, while *V. melanurus* obtained an optimum pH of 2, which represents the maximum activity and starting from this peak activity decreased gradually (Fig. 1A). The optimum pH obtained for alkaline proteases was 6 for *V. melanurus*, which presented different oscillations in its activity, with pH 6 being the highest peak of activity, while for *V. bifasciata* it presented oscillations in its alkaline activity, with a peak of maximum activity at pH 12 (Fig. 1B).

The acid digestive proteases of *Vieja melanurus* presented a peak of maximum activity around 110%, during 90 min of preincubation with a pH 2.0; same that decreased its activity at 60 min, presenting again an increase at 90 min of preincubation, while the other activities at different pHs maintained percentages below 50% (Fig. 2A). Meanwhile, *V. bifasciata* obtained a percentage of activity of the acid proteases of 105% during 30 min of preincubation at pH 4.0, which was decreasing at 60 min and increased slightly at 90 min of preincubation, being the activity of pH 4, which it stood out above 100% of relative activity compared to other evaluated pH's (Fig. 2B). The maximum residual activity of digestive alkaline proteases for *V. melanurus* was obtained at pH

10, which was increased from 30 min to 90 min of preincubation, where it presented its peak of maximum activity (approximately 125%) (Fig. 2C). Finally, the activity of alkaline proteases in *V. bifasciata* increase of approximately 120% at a pH of 10, during 30 min of preincubation itself, which decreased after 60 and 90 min, while the other activities remained below 110% of relative activity (Fig. 2D).

Optimum temperature and stability in acidic and alkaline proteases. The optimal temperature in the stomach enzymatic extract was detected at 55°C for *Vieja bifasciata* which increased from 45°C, presenting its maximum activity peak at 55°C and decreased at 65°C, while for *V. melanurus* it showed a peak of maximum activity at 35°C and gradually decreased to 65°C (Fig. 3A). Regarding the optimal temperature of the alkaline proteases for *V. melanurus*, an increase in temperature occurred from 35°C to 45°C, this being the maximum activity peak and subsequently, its activity decreased until reaching 65°C, while for *V. bifasciata*, it showed oscillations in the increase in activity until reaching 55°C as the temperature with the highest activity of the alkaline proteases (Fig. 3B).

Concerning the temperature stability in acidic conditions for *Vieja melanurus*, it was observed that at 45°C the temperature is maintained over 100% of the relative activity (approximately 110) during 60 min of preincubation, increasing to 90 min, while at 25°C the protease activity also remains quite stable (Fig. 4A); however, for *V. bifasciata* it presents an increase in activity at 55°C for 60 min, showing the highest residual activity (110%), descending drastically at 90 min, however, the temperature of 65°C showed oscillations at 30, 60 and 90 min, while the temperature of 35°C showed a gradual decrease (Fig. 4B). For the stability of temperature in alkaline proteases of *V. melanurus*, an increase of the relative activity was shown at 45°C, presenting the maximum peak (approximately 105%) at 90 min of preincubation, at 35°C the protease activity also remains quite stable with a peak of activity at 30 min of preincubation (Fig. 4C), as well as *V. bifasciata*, there was an increase at 55°C with a preincubation time of 30 min, this being the maximum activity around

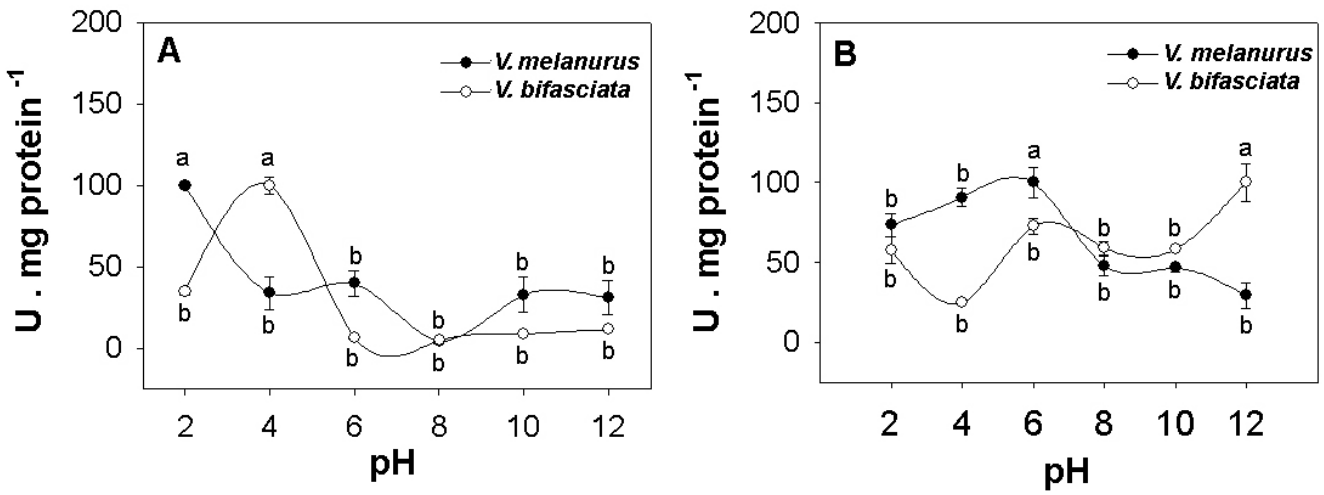

FIGURE 1 | Effect of optimal pH (mean ± SD, n = 3) on **A**. acid proteases and **B**. alkaline proteases of *Vieja melanurus* and *V. bifasciata*. Significant differences (P< 0.05) between pH values are shown by letters.

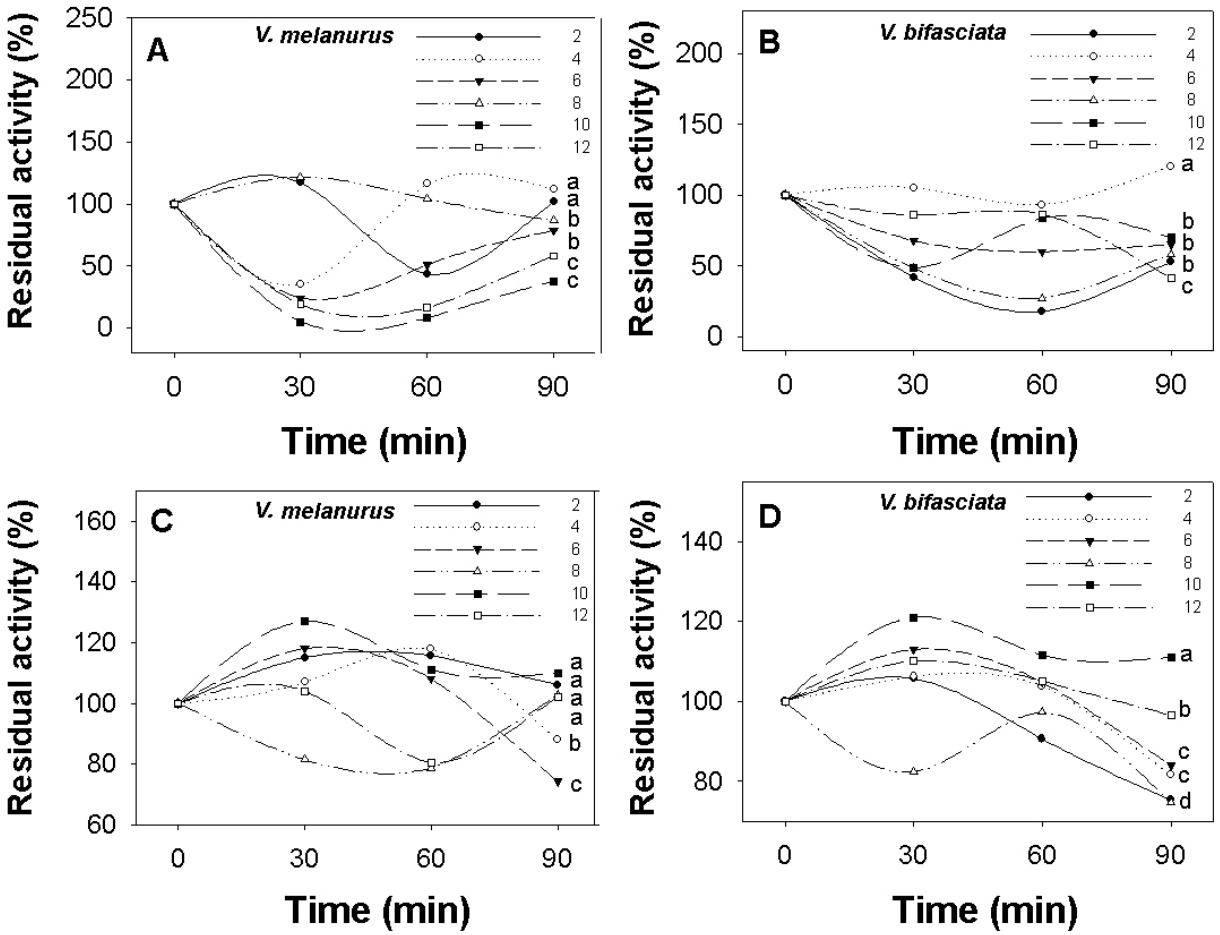

FIGURE 2 | pH stability of acid digestive protease for **A**. *Vieja melanurus* and **B**. *V. bifasciata*; and alkaline digestive protease for **C**. *V. melanurus* and **D**. *V. bifasciata* (mean ± SD, n = 3). Significant differences (P< 0.05) between pH values residual activity are shown by letters.

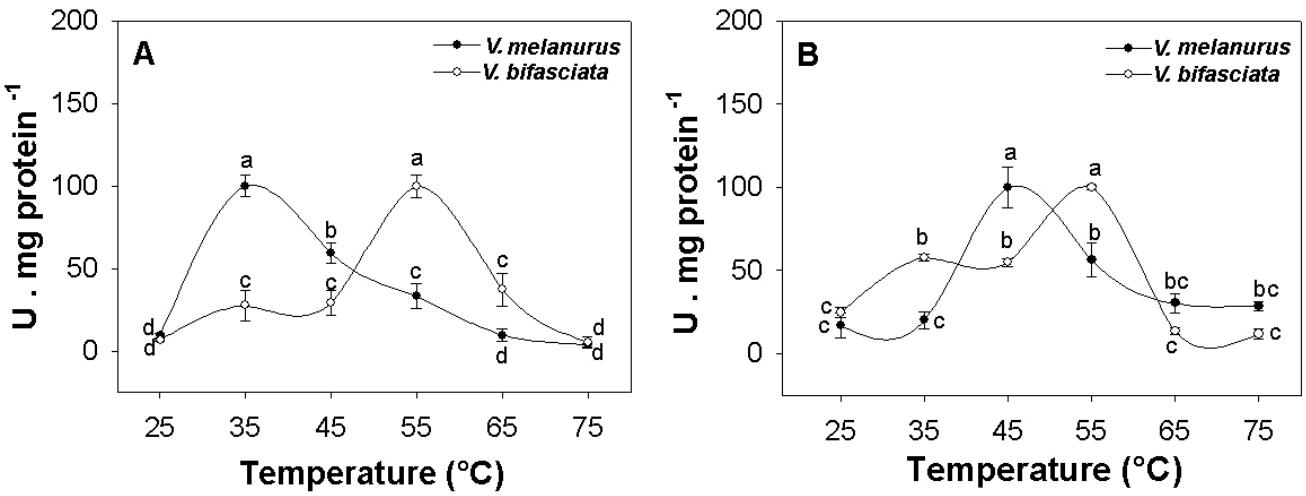

FIGURE 3 | Effect of optimal temperature (mean ± SD, n = 3) on **A**. acid proteases and **B**. alkaline proteases of *Vieja melanurus* and *V. bifasciata*. Significant differences (P< 0.05) between pH values are shown by letters.

120% relative activity, with a drastic decrease in activity at 90°C, at 25 and 35°C the protease activity also remains fairly stable until 60 min of preincubation, after which the activity at 35°C declines (Fig. 4D). The rest of the temperatures recorded residual activities that remain around 100%, in case of 55 and 65°C they remain below 50% of the residual activities.

Effect of the inhibitors on acid and alkaline enzymes. The residual activity of alkaline proteases showed that for trypsin TLCK was 24% for *Vieja melanurus* and 50% for *V. bifasciata*, in the case of chymotrypsin the residual activity was 27% and 53% using TPCK as a specific inhibitor for both species, meanwhile with SBT1 the residual activity was 7% and 55% respectively; PMSF showed 1.3% and 42%, on case of metalloproteases the EDTA left 10% and 52%, while with ovalbumin left 2.7% and 46% of activity residual, finally with phenanthroline (PHEN) the residual activity was 12% and 20% for *V. melanurus* and *V. bifasciata*, respectively (Fig. 5).

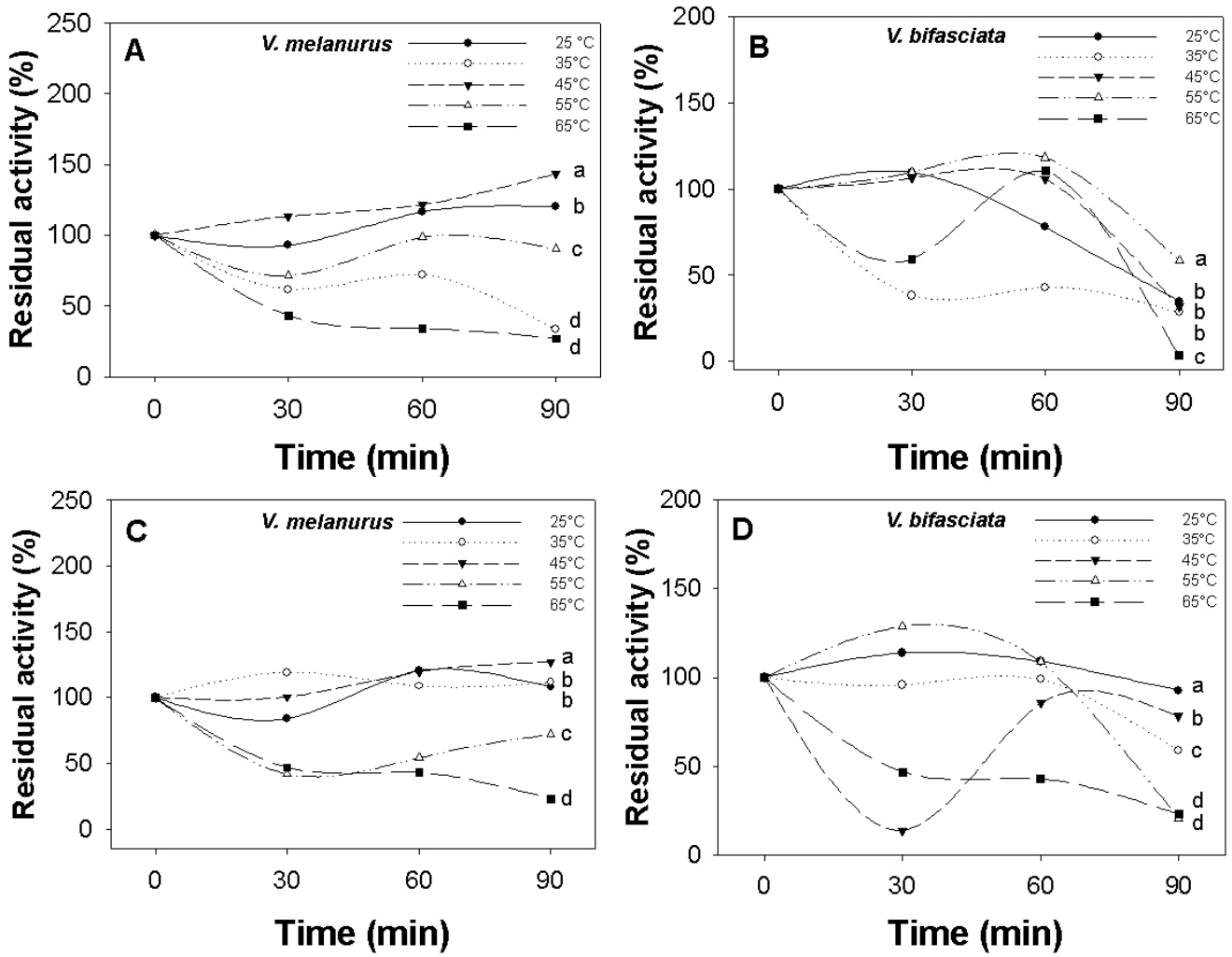

FIGURE 4 | Temperature stability of acid digestive protease for **A**. *Vieja melanurus* and **B**. *V. bifasciata*; and alkaline digestive protease for **C**. *V. melanurus* and **D**. *V. bifasciata* (mean ± SD, n = 3). Significant differences (P< 0.05) between pH values residual activity are shown by letters.

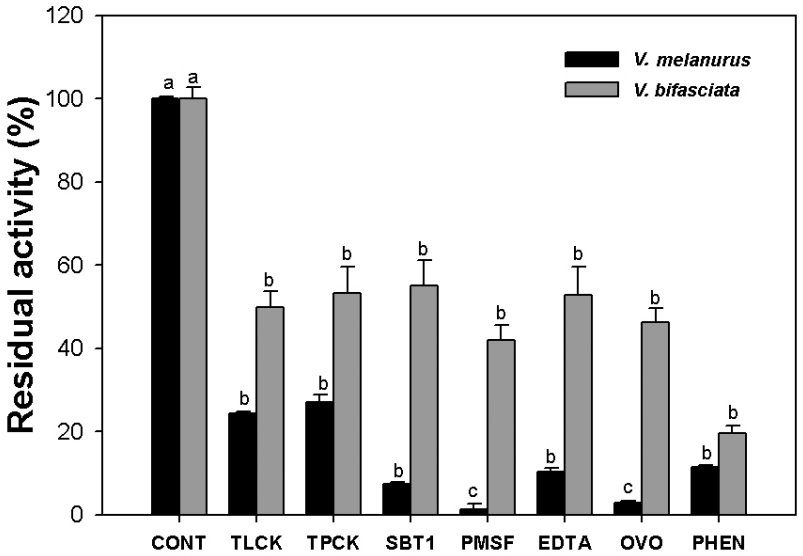

FIGURE 5 | Effect of inhibitors on alkaline digestive proteases of *Vieja melanurus* and *V. bifasciata*: Alkaline control (alkaline proteases without inhibitor), TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone), PHEN (phenanthroline), EDTA (ethylenediaminetetraacetic acid), TLCK (Tosyl-L-lysyl-chloromethane hydrochloride), OVO (ovalbumin), SBT1 (soybean trypsin inhibitor), PMSF (phenylmethylsulfonyl fluoride) (mean ± SD, n = 3) significant differences ($P < 0.05$) between inhibitors values are shown by letters. Different letter between bars indicates statistical differences.

DISCUSSION

The maximum pH value of the stomach extracts found in *Vieja melanurus* was located within the parameters reported by other studies using hemoglobin in the acid hydrolysis (Rodrigáñez *et al*., 2005; Kumar *et al*., 2007; Xiong *et al*., 2011; Guerrero-Zárate *et al*., 2014; Toledo-Solís *et al*., 2015; Pujante *et al*., 2016; Concha-Frias *et al*., 2016; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018; Teles *et al*., 2019; Martínez-Cárdenas *et al*., 2020). Some studies reported the maximum pH values for the stomach between 2.0–3.0; however, they differ with *V. bifasciata*, which obtained a pH value of 4, although it is still an acidic condition not previously reported. Considering the above-mentioned, Uscanga-Martínez *et al.* (2011) recorded a stomach pH close to neutral at pH 5 in *P. splendida*, which suggests that pH 4 is within the acidity parameters suitable for the enzymatic activity of *V. bifasciata*.

In the case of alkaline proteases, the maximum value in enzymatic activity at pH was reported in a single peak at pH 6 for *V. melanurus* that is different from that found in other species. However, there are investigations with the blue disc *Symphysodon aequifasciatus* Pellegrin, 1904 and gilthead seabream *Sparus aurata* Linnaeus, 1758 (Chong *et al*., 2002; Deguara *et al*., 2003) in which two activity peaks were found in the alkaline part, lower than pH 8.0. In this aspect, Concha-Frias *et al.* (2016) obtained a peak of activity at pH 7 in *C. undecimalis* juveniles, which is consistent with these authors; this may be since *V. melanurus* requires an acid, almost neutral environment to activate alkaline proteases. However for *V. bifasciata*, two maximum pH peaks of 6 and 12, which is outside the

range of values reported in other marine and freshwater species that generally lies between pH 8.0–11.0, such as those found in bluefin tuna *Thunnus thynnus* (Linnaeus, 1758), mahi languish fish *Scleropages formosus* (Müller & Schlegel, 1840), Senegalese sole *Solea senegalensis* Kaup, 1858, *P. splendida*, *A. tropicus*, *C. trimaculatum*, *C. labrosus*, *C. undecimalis*, *C. beani*, *A. probatocephalus* and *M. brachyurus* (Eshel *et al*., 1993; Natalia *et al*., 2004; Rodrigáñez *et al*., 2005; Uscanga-Martínez *et al*., 2011; Guerrero-Zárate *et al*., 2014; Toledo-Solís *et al*., 2015; Pujante *et al*., 2016; Concha-Frias *et al*., 2016; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018; Martínez-Cárdenas *et al*., 2020), this could be explained as the residual activities recorded depend not only on a protein but on a group of proteins that interact with each other and can get to be activated at different pHs and temperatures even in alkaline conditions and this will depend on the type of enzyme and the substrate in which it is interacting with other proteins that result in different residual activities.

The stability tests of the pH on the activity in the stomach enzymatic extracts of *V. melanurus* showed a remarkable ability to maintain its activity above 100% of the activity at acidic conditions (pH 4). For *V. bifasciata* the residual activity was above 100% at a pH of 4 during the 30 min of preincubation, keeping enzymatic activity constant because it is in the preferential activity range. As already mentioned, as the pH towards the alkaline part was increased, the enzymatic activity declined, which agrees with those reported in *A. tropicus*, *C. trimaculatum*, *C. labrosus*, *C. undecimalis*, *C. beani*, *A. probatocephalus*, and *M. brachyrus* (Guerrero-Zárate *et al*., 2014; Toledo-Solís *et al*., 2015; Pujante *et al*., 2016; Concha-Frias *et al*., 2016; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018; Martínez-Cárdenas *et al*., 2020). This situation shows that enzymes have higher activity in acidic conditions when the pH is within the range of 2–4, due to the secretion of HCl from the stomach glands, which promotes acid digestion, while peristaltic movements are the cause of the movement of the chyme to the anterior intestine for alkaline digestion (Moyano *et al*., 1996; Díaz-López *et al*., 1998).

On the other hand, the pH stability for alkaline protease was 6 for *V. melanurus*, and pH 12 for *V. bifasciata.* The last one corresponds to the normal range of pH for alkaline proteases in marine and freshwater (Natalia *et al*., 2004; Rodrigáñez *et al*., 2005; Uscanga-Martínez *et al*., 2011; Guerrero-Zárate *et al*., 2014; Toledo-Solís *et al*., 2015; Pujante *et al*., 2016; Concha-Frias *et al*., 2016; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018; Martínez-Cárdenas *et al*., 2020). However, these pH values presented variations of incubation times for *V. melanurus* were 90 min and for *V. bifasciata* 30 min, which is an indication that even though both species handle the same alkaline pH values. These variations in pH for the activation time of alkaline proteases vary by species, food habit or environmental variations (Solovyev *et al*., 2015).

The maximum temperatures recorded in the stomach enzymatic extracts for *V. melanurus* and *V. bifasciata* were 35 °C and 55 °C, respectively; the results for *V. melanurus* are within the ranges of 40–55 °C, established both in marine and sweet aquaculture species (Alarcón *et al*., 1998; Rodrigáñez *et al*., 2005; de la Parra *et al*., 2007; Uscanga-Martínez *et al*., 2011; Toledo-Solís *et al*., 2015; Pujante *et al*., 2016; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018; Teles *et al*., 2019; Martínez-Cárdenas *et al*., 2020); however, *V. bifasciata* recorded a maximum digestive activity at 55 °C in the stomach, which suggests that differences in temperature vary to carry out the

denaturation of proteins by stomach enzymes, which occurs at a temperature range from 35°C to 65°C, these are determined by different features linked to the molecular structure of the proteins (amino acid sequence, folding, number and position of disulfide bonds, the structure of the active site, etc.); however, these studies have do not been done in this research. On the other hand, the temperature in alkaline proteases was obtained at 45°C for *V. melanurus* and 55°C for *V. bifasciata*, which are within the same range (Jónás *et al*., 1983; Xiong *et al*., 2011; Villalba-Villalba *et al*., 2011), where they reported maximum activity at temperatures of 45–55°C. In contrast, temperatures of 35°C and 55°C for acidic conditions have been reported in both marine and freshwater species (Toledo-Solís *et al*., 2015; Pujante *et al*., 2016; Martínez-Cárdenas *et al*., 2017; Merino-Contreras *et al*., 2018; Martínez-Cárdenas *et al*., 2020).

Regarding the stability of the proteases, the enzymatic activities of the stomach and intestine for *V. melanurus* was 45°C and for *V. bifasciata* 55°C on 100% of the relative activity, both during 60 min of preincubation, these temperatures remaining stable for both species, these data are different from those reported by Guerrero-Zárate *et al*. (2014), where they mention stable temperatures below 45°C since most fish are ectotherms. Since fish cannot regulate their temperature, they depend entirely on the environment, being temperatures of 30–32°C that predominate for tropical fish species. Of course, the digestive enzymes cannot respond to such temperatures (above 45 °C), as in the case of *P. splendida* (Uscanga-Martínez *et al*., 2011), *C. trimaculatum* (Toledo-Solís *et al*., 2015); *C. beani* (Martínez-Cárdenas *et al*., 2017); *A. probatocephalus* (Merino-Contreras e*t al*., 2018) with recorded intervals of 35°C and 55°C. In the case of *C. undecimalis* (Concha-Frias *et al*., 2016), alkaline proteases tolerate and even work better at 65°C. In such cases, digestive physiology compensates the high temperatures required for the enzymes (35–65°C), increasing the retention time of food, by closing the cardiac and pyloric sphincters, in addition to the effect that gastric hormones (gastrin and CCK) cause by increasing the peristaltic movements of the stomach and intestine, respectively (Kurokawa *et al*., 2003; Cahu *et al*., 2004).

Inhibition of alkaline proteases in both species was high; however, the most affected species is *V. melanurus*, with the lowest residual activity for all inhibitors and inactivators. These high inhibition values correspond to omnivorous fish species, which has been reported for other neotropical cichlids by Toledo-Solís *et al*. (2015) in *C. trimaculatum* and Martínez-Cárdenas *et al*. (2017) in *C. beani.* Both authors found similar inhibition percentages for trypsin and chymotrypsin with TLCK, TPCK, and SBT1. Different results on the effect of the protease inhibitors have been reported in other fish species such as the carp *Catla catla* (= *Labeo catla* (Hamilton, 1822)), roho labeo *Labeo rohita* (Hamilton, 1822), silver carp *Hypophthalmichthys molitrix* (Valenciennes, 1844), *P. splendida*, sailfin catfish *Pterygoplichthys disjunctivus* (Weber, 1991), *A. tropicus*, *C. labrosus* and *A. probatocephalus* (Kumar *et al*., 2007; Chakrabarti, Rathore, 2010; Uscanga-Martínez *et al*., 2011; Villalba-Villalba *et al*., 2011; Guerrero-Zárate *et al*., 2014; Pujante *et al*., 2016; Merino-Contreras *et al*., 2018). All these differences depend on each species' feeding habits. For example, in the black carp *Mylopharyngodon piceus* (Richardson, 1846), the main alkaline proteases were trypsin and chymotrypsin, and eighth protease isoforms were detected, which have been related to its herbivorous feeding habit (Liu, Li, 2008).

In *Vieja melanurus* and *V. bifasciata*, results indicate that metalloproteases are essential

digestive enzymes that intervene mainly in protein hydrolysis, releasing amino acids that are absorbed by the enterocytes and their role in the resolution of inflammatory processes (Chadzinska *et al*., 2008). So, serine proteases' luminal digestive activity (trypsin and chymotrypsin) seems to be second but still relevant in the intestinal digestive process. Both species have similar digestive protease capacities, which is caused by they sharing the same habitat and even feeding habits (Pease *et al*., 2018), gives them the advantage of digesting the various foods that the environment provides, in addition to the possibility of adapting to the consumption of balanced foods that include, not only ingredients of animal origin, but plant origin. However, their inclusion must be carefully evaluated to ensure that these vegetable meals do not contain high antinutrient concentrations when incorporated into the formulation (Alarcón *et al*., 2001).

In conclusion, both species showed typical capacities of digestive enzymes observed for other neotropical freshwater species with high thermal and pH stability and high sensitivity to specific inhibitors. However, *V. bifasciata* has a higher resistance to the presence of inhibitors, so the development of a food based on different protein ingredients of animal or vegetable origin should be tested using *in vitro* and *in vivo* techniques. The digestive enzymes are of particular interest since the rate of digestion in the intestinal system limits the amount of nutrients that can be contributed to the bloodstream and, therefore, influences the entire organism's growth due to the great importance of protein fraction in fish nutrition. The levels of secretion of this enzyme are related to food intake and stomach filling, so a period of fasting or poor feeding results in a decrease in activity. On the other hand, other alkaline proteases such as chymotrypsin may even be a better indicator of nutritional status in some species. Therefore, both species present thermostable enzymatic activities that could be taken as a reference to evaluate optimal protein ingredients for the development of specific diets for each species and minimize production costs in both species' culture.

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ETHICAL STATEMENT

Biological material is registered at the Colección de Peces de ECOSUR, Unidad San Cristóbal de Las Casas (ECOSC) number INE-SEMARNAP (CHI.PE.010.0497).

COMPETING INTERESTS

The authors declare no competing interests.

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