Partial characterization, quantification and optimum activity of trypsin and lipase from the sciaenids *Cynoscion othonopterus*, *Cynoscion parvipinnis* and *Cynoscion xanthulus*

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Abstract: Trypsin and pancreatic lipase promote the digestion of proteins and lipids, respectively, when they are secreted into the anterior intestine; however, since the pancreas is a diffuse tissue in fish, the characterization and quantification of pancreatic enzymes is uncommon. The objective of this study was to partially characterize and compare the enzymatic activities of lipase and trypsin within the gastrointestinal tract of *Cynoscion parvipinnis*, *Cynoscion othonopterus* and *Cynoscion xanthulus*, to contribute to the knowledge of the digestive physiology of these important commercial sciaenids and to reveal whether they have potential for biotechnological applications. The presence of lipase and trypsin was confirmed by zymography and the molecular weights of both enzymes were determined by electrophoresis. For lipase, molecular weights of 65.8 and 69.5 kDa were determined for *C. othonopterus* and *C. xanthulus*, respectively. For *C. parvipinnis*, two lipases of 61.5 and 36.0 kDa were determined. In all three species the largest lipase activity was observed in the anterior intestine, followed by pyloric caeca, with optimum activity observed at pH 8.0 and at temperatures ranging between 40 and 45°C. Molecular weights of trypsin were 24.4, 23.6 and 23.7 kDa in *C. othonopterus, C. parvipinnis*, and *C. xanthulus*, respectively. The optimum pH of activity ranged between 7.0 and 9.0 and optimum temperature between 55 and 65°C for all species. These enzymes meet certain criteria that make them potential candidates for some industrial applications, such as the food industry and the production of detergents.

Keywords: *Sciaenids*; digestive enzymes; trypsin; lipase; partial characterization; optimum activity

INTRODUCTION

Proteins and lipids are important macronutrients in the diet of carnivorous marine finfish. Their adequate utilization and incorporation into tissues largely depends on the presence of digestive enzymes, which break them down into simpler components for absorption to proceed. Proteases and lipases are two key enzyme groups that catabolize dietary proteins and lipids, respectively [1]. Both types of enzymes are secreted by the pancreas or pancreatic-like tissue and belong to the αβ-hydrolase family. They all bear a catalytic triad, i.e., a set of three coordinated amino acids in their structure, composed of histidine (base), aspartate (acidic residue) and serine (nucleophile). However, glutamate may also be present in lipases as an acidic residue [2,3]. Trypsin is an alkaline endopeptidase that cleaves peptide bonds at the carboxyl end of arginine or lysine residues [4], and it is an im-

portant protease because it activates other digestive enzymes [5]. In turn, pancreatic lipase is quantitatively one of the most important lipolytic enzymes. It cleaves fatty acids at positions 1 and 3 of glycerol moieties, resulting in free fatty acids and monoacylglycerides [1]. In order to effectively anchor to dietary lipids, pancreatic lipase needs the presence of pancreatic colipase, a coenzyme that is also secreted by the pancreas and activated by trypsin. For this reason, pancreatic lipase is also known as a colipase-dependent lipase [1,6-8]. In addition, carboxyl ester lipase, another type of lipase with greater substrate specificity, is capable of cleaving mono-, di- and triacylglycerols, as well as phospholipids, lysophospholipids, ceramides, cholesteryl, aryl and wax esters, and vitamin A and E esters. Because it requires the presence of bile salts to hydrolyze triacylglycerols or phospholipids with medium or long-chain fatty acids, this enzyme is also known

as bile salt-dependent lipase [9,10]. Both lipases have been described in marine finfish [11-20].

Characterizing digestive enzymes is a crucial step towards a better understanding of the utilization of dietary proteins and lipids by finfish and contributes to the development of formulated feeds for cultured species. Furthermore, for finfish species subjected to commercial fishing, the viscera, which are often discarded, are a potential source of digestive enzymes with industrial applications, such as the manufacture of cleaning products, food products, and laundry detergents. They may also have potential applications in the agrochemical, pharmaceutical, bioremediation and leather industries [15,21-24].

Examples of finfish subjected to commercial fisheries in the Gulf of California, Mexico, and whose viscera are usually discarded but may otherwise be used as a source of digestive enzymes for industrial purposes, are the Gulf corvina *Cynoscion othonopterus*, the shortfin corvina *C. parvipinnis* and the orangemouth corvina *C. xanthulus*. All three species belong to the family Sciaenidae. The Gulf corvina is, in terms of volume of capture, the most important species, with 5389 metric tons obtained in 2016 [25]. Although capture data specific to the shortfin and orangemouth corvinas are not available, they are both important and appreciated locally as food and game fishes. Interestingly, *C. othonopterus* and *C. parvipinnis* appear to have good potential as candidate species for aquaculture [26-28]. Characterizing their digestive enzymes would increase our knowledge of their digestive capacities and help formulate balanced feeds for captive rearing, but perhaps more importantly, it may contribute to the transformation of fishery by-product wastes into a source of valuable products with wide biotechnological potential. Therefore, the objective of the present study was to partially characterize, quantify and determine the optimum activities of trypsin and lipase of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus*.

MATERIALS AND METHODS

Experimental organisms and biological indices

A total of 15 wild *C. othonopterus*, 23 *C. xanthulus* and 33 *C. parvipinnis* were caught with seine nets on two separate occasions during the month of October 2017 in the Gulf of California, around the area of Kino Bay (latitude 28°48'59.99"N, longitude 111°55'59.99"W), Sonora, Mexico. On each occasion, the organisms were immediately transported in an ice-filled cooler (≈ 4°C) to the Department of Scientific and Technological Research of the University of Sonora (DICTUS); upon arrival, individuals were identified using the FAO's Western Central Pacific species identification guide for fish [29]. Then, fish were measured, weighed and dissected. The gastrointestinal tract (GIT), liver and gonads were individually weighed to estimate the following indices: viscerosomatic index (VSI, %)=(viscera weight/total weight)×100; hepatosomatic index (HSI, %)=(liver weight/total weight)×100; and gonadosomatic index (GSI, %)=(gonad weight/total weight)×100. After weighing, tissues were stored and labelled in individual resealable bags at -82°C until further analyses.

Enzyme extracts

The GIT of fish was separated into four sections: anterior, middle and posterior intestine, and pyloric caeca. Approximately 0.5 g of tissue from each section was individually homogenized (Ultra Turrax, T18 basic, IKAWorks Inc., Wilmington, NC, USA) at 4°C in 2.5 mL of 50 mM Tris-HCl buffer solution (pH 7.5) and 0.2 mL benzamidine-HCl 8.7 mM for the lipase extracts, and in 2.5 mL of 50 mM Tris-HCl buffer solution (pH 8.2) with 20 mM $CaCl_2$ for the trypsin extracts. The crude extracts were then centrifuged (Heraeus Fresco 21, Thermo Fisher Scientific, Dreieich, Germany) at 21000 × g and 4°C for 30 min, the precipitate was discarded and the supernatant was recovered and centrifuged three times until the samples were clear. After centrifugation, the supernatants containing the enzymes were semipurified and concentrated using 30 kDa and 10 kDa centrifugal filters (Amicon°Ultra-4, Centrifugal filter device, Millipore Corp., Billerica, MA, USA) for lipase and trypsin, respectively, at 3600 × g and 4°C for 30 min. The protein content of the semipurified extract was analyzed in 100 μL of sample from each extract via combustion by the Dumas method (N factor=6.25; method 968.06) with a Dumas Nitrogen Analyzer (Model NDA 702, VELP Scientifica, Usmate, Italy). The remaining extract was stored at -20°C until further analyses.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To establish the molecular weights of trypsins from the three sciaenids, samples were prepared by mixing equal volumes of the crude extract with a solution of 95% 2X Laemmli Sample Buffer (Bio-Rad®, Hercules, CA, USA) and 5% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and then placed in a 95°C water bath for 2 min [30]. After cooling to room temperature (25°C), 20 μL aliquots were loaded into 12% polyacrylamide gels and resolved at 115 V for 2.5 h at 10°C in a four-gel vertical electrophoresis system (Mini-Protean Tetra Cell, Bio-Rad®, Hercules, CA, USA). Gels were fixed in a solution of 50% trichloro-acetic acid (TCA) for 18 h, stained for 1 h at 37°C in a solution of 50% TCA with 0.1% Coomassie brilliant blue R-250 and destained by repeatedly washing with 7% acetic acid. Trypsin (T8003) from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA) was used as the reference molecular marker and as a quantitative internal standard to establish the enzyme concentration by densitometry. The molecular weight was estimated by comparison with an internal molecular weight standard (Precision Plus Protein Standard Dual Color, Bio-Rad®, Hercules, CA, USA) with protein markers of 10 to 250 kDa. Gels were documented in a calibrated densitometer (Model GS-900, Bio-Rad®, Hercules, CA, USA) for identification and quantification of the bands using the ImageLab 5.0 software (Bio-Rad®, Hercules, CA, USA). In the case of lipases, the crude extracts were further purified using a solution of 30% polyethylene glycol (PEG-6000) to get a 1:1 dilution. After vortexing for 2 min, samples were kept in constant agitation for 30 min at 4°C. Next, they were centrifuged at $21000 \times g$ for 30 min at 4°C. The supernatants were carefully recovered and 20 μL aliquots were loaded into 10% polyacrylamide gels and run under the same conditions previously mentioned. Gels were stained for 18 h by gentle agitation in QC Colloidal Coomassie stain (Bio-Rad®, Hercules, CA, USA), and then destained by rinsing in distilled water. Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) was used as an internal marker and as a quantitative internal standard to establish the enzyme concentration by densitometry.

Zymography

A specific zymographic assay was used to identify and confirm the band belonging to each enzyme in the gel. Trypsin was detected by developing an activity band when hydrolysis of casein took place [31]. After electrophoresis, the gel was rinsed in distilled water and submerged in 50 mL of 2% casein in 50 mM Tris-HCl buffer at 5°C for 30 min, followed by incubation at 37°C for 90 min for digestion of the protein substrate, and rinsing with distilled water. A solution of 40% ethanol:10% acetic acid:0.1% Coomassie brilliant blue R-250 was used for staining the gel overnight. Trypsin was visualized by the contrast of a clear white band in a dark blue background. A band with trypsin (T8003, Sigma-Aldrich, St. Louis, MO, USA) from bovine pancreas was used to confirm the effectiveness of the reaction of the enzyme against casein.

Detection of lipase in a zymogram was done by preparing a chromogenic substrate with 3.0 g of acacia gum dissolved in 30 mL of 0.2 M phosphate buffer solution (pH 8.0) and brought to a final volume of 50 mL. Then, 20 mL of olive oil and 10 g of crushed ice were incorporated and homogenized for 5 min, followed by the addition of 1% agar. The emulsion was then warmed to 50°C. Separately, 10 mg of Victoria blue B were dissolved in 1 mL of 70% ethanol, incorporated into the emulsion and maintained at 50°C [32]. The chromogenic substrate was poured over the gel and incubated at 37°C for 12 h, washed with distilled water and stained with QC Colloidal Coomassie for 18 h. Activity of lipase against olive oil triglycerides was observed as a dark blue band in the zymogram gel.

Assessment of enzymatic activities

Lipase

Activity of lipase within the different sections of the GIT of the three sciaenids was determined in triplicate samples, each from a different experimental organism, with triplicate blanks; each triplicate sample and its blank were run in duplicates. The methodology used was the enzymatic assay of lipase of the Enzyme Commission 3.1.1.3, with minor modifications. Tissue homogenates were prepared using three volumes of 200 mM Tris-HCl buffer solution (pH 7.7) and centrifuging

three times at 21000 × g and 4°C for 30 min, until the samples were clear. The supernatant was recovered and used to prepare the reaction mix with 1 mL 200 mM Tris-HCl buffer solution (pH 7.7), 2.5 mL tridistilled water, 1 mL of crude extract from either the anterior intestine (AI), middle intestine (MI), posterior intestine (PI) or pyloric caeca (PC), and 3 mL of olive oil. The samples were then mixed vigorously and incubated in a shaker at 35°C at 120 rpm for 30 min. The reaction was stopped by adding 3 mL of 95% ethanol, followed by the addition of 80 μL of 0.1% phenolphthalein and titration with a solution of 50 mM NaOH to a light pink color. In all blanks, the enzyme crude extract was added after the incubation period. One unit of activity of lipase (U) per mL of crude extract was defined as the amount of enzyme that hydrolyzed 1.0 microequivalent of fatty acid from a triglyceride in 1 h at pH 7.7 and 35°C, using the equation: activity (U mL^{-1})=[(mL NaOH used) (molarity of NaOH) (1000) (2) (dilution factor)]/(volume of enzyme used in mL). In this equation, 1000 is the conversion factor from milliequivalent to microequivalent, and 2 is the time conversion factor from 30 min to 1 h (unit definition).

Enzymatic activity was higher in anterior intestine as compared to the other sections of the GIT in all three species. Therefore, this section was exclusively used to estimate the optimum temperature and pH for enzymatic activity. The analyses were run in triplicate samples, and each triplicate sample and its blank were run in duplicates, following previously described procedures [33] with minor modifications. Briefly, 100 μL of 100 mM sodium cholate hydrate, 1.9 mL of 50 mM Tris-HCl (pH 7.2) buffer solution and 10 μL of the enzyme extract (omitted in the blank tubes) were placed in 10-mL glass tubes and mixed thoroughly; then 20 μL of 200 mM β-naphthyl-acetate were added and incubated for 30 min at 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 and 70°C, followed by the addition of 20 μL of 100 mM fast blue BB salt and an additional incubation period of 5 min. Next, 200 μL of 0.72 N TCA, 10 μL of enzyme extract in the blank tubes and 2.71 mL of ethanol:ethyl acetate were added to stop the reaction; the tubes were vortexed and absorbance was read at 540 nm. The Δ absorbance (extract absorbance - blank absorbance) was used to estimate the units of absorbance (Δ absorbance ÷ constant (0.01)). Then the units of lipase min^{-1} (units of absorbance ÷ incubation time) were used to estimate lipase activity,

expressed as units of lipase mL^{-1} (units of lipase min^{-1} ÷ extract volume). In the case of the pH, the reactions were run using a pH range from 6 to 10. The buffer solutions were adjusted according to the pH evaluated; a 50 mM citrate-phosphate buffer was used for pH 5.0 and 6.0, a 50 mM Tris-HCl buffer for pH 7.0 to 9.0 and a 50 mM glycine-NaOH buffer for pH 10. The temperature for incubation was also adjusted to 40°C for *C. parvipinnis* and *C. xanthulus* and to 45°C for *C. othonopterus* because, at these values, lipase activity was observed to be optimal in the previous test.

Trypsin

For each of the three sciaenids, the activity of trypsin within AI, MI, PI and PC was determined in triplicate samples, each from a different experimental organism, with triplicate blanks; each triplicate sample and its blank were also run in duplicates. A substrate prepared with 100 mM of Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) diluted in 1 mL of dimethyl sulfoxide (DMSO) was brought to 100 mL with 50 mM Tris-HCl buffer and 10 mM CaCl$_2$ (pH 8.2). A total of 1.25 mL of the substrate was pipetted into a microcentrifuge tube and 20 μL of the enzyme extract were added. Samples were then incubated in a water bath at 35°C for 30 min. After incubation, the reaction was stopped by adding 0.25 mL of 30% acetic acid and the absorbance was read at 410 nm. For the blanks, 20 μL of tridistilled water were added instead of the enzyme extract. The activity of trypsin was estimated with the following formula: trypsin activity (U mL^{-1})=(Δ absorbance ÷ total reaction volume) ÷ (coefficient of extinction molar × incubation time × extract volume) [34]. Trypsin concentration was estimated from the absorbance readings of the samples. A calibration curve with different concentrations of trypsin from bovine pancreas (Sigma-Aldrich, St. Louis, MO, USA) was performed; the determined linear regression equation was y=0.1455x+0.0073 with R^2=0.9786, where the coefficient of extinction molar (CEM=0.1455) and the slope (m=0.0073) were used in the following formula: trypsin concentration (mg mL^{-1})=Δ absorbance - m ÷ CEM.

As observed in lipase, the enzymatic activity of trypsin was higher in the anterior intestine in all three species. Thus, this section was exclusively used to estimate the optimum temperature and pH for enzymatic activity [34]. The analyses were also run in triplicate

samples, and each triplicate sample and its blank were run in duplicates, adjusting the incubation temperature to 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 and 90°C, whereas the pH was evaluated substituting the buffer in the substrate solution according to the pH evaluated; a 100 mM citrate-NaOH buffer was used for pH 6.0, a 100 mM Tris-HCl buffer for pH 7.0 to 9.0 and a 100 mM glycine-NaOH buffer for pH 10. The temperature for incubation was also adjusted to 65°C for *C. parvipinnis* and *C. xanthulus* and to 60°C for *C. othonopterus* because, at these values, trypsin activity was observed to be optimal in the previous test.

Statistical analysis

Biological indices, molecular weights and concentrations of lipase and trypsin determined by densitometry were analyzed by descriptive statistics, whereas enzymatic activity within the different sections of the GIT at different temperatures and at different pH were subjected to one-way analysis of variance (ANOVA) using a significance level of $P \leq 0.05$. Tukey's HSD test was used as the mean separation procedure when significant differences were observed. All statistical analyses were performed using the Statistical Analysis System (SAS Institute Inc., 2013, Software Release 9.4, Cary, NC, USA) software package.

RESULTS

Fish biological indices

The total weight and length of the three sciaenids in this study ranged from 862-1129.4 g, and from 46.5-51 cm, respectively, confirming these experimental organisms had a commercial size. The gonads were not fully developed and were small in all species, 0.9-4.0 g, therefore, the GSI were also low, 0.08-0.53%. The HSI ranged from 1.2-1.6%, whereas the VSI indicated that viscera represent close to 7.3-9.5% of the body weight of the sciaenids (Table 1).

SDS-PAGE analyses of trypsin and lipase

The mean molecular weights±standard error of the mean (SEM) of trypsins of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* were 24.4±0.2, 23.6±0.1 and 23.7±0.1 kDa (Fig. 1), respectively. Zymography of

Table 1. Biological parameters and indices of wild *Cynoscion othonopterus*, *Cynoscion parvipinnis* and *Cynoscion xanthulus*.

Biological indices	*C. othonopterus*	*C. parvipinnis*	*C. xanthulus*
Total weight (g)	1129.4±53.9	862.0±49.2	879.4±45.6
Total length (cm)	51.0±0.8	46.5±1.0	47.3±0.9
Liver weight (g)	14.2±1.3	10.0±0.6	12.7±1.3
Gonad weight (g)	0.9±0.3	4.0±0.9	2.0±0.5
Viscera weight (g)	82.1±3.9	65.8±4.6	70.7±4.5
HSI (%)	1.2±0.1	1.4±0.1	1.6±0.1
GSI (%)	0.08±0.02	0.53±0.11	0.24±0.05
VSI (%)	7.3±0.2	9.5±0.6	9.1±0.7

Total weight and length values are means±SEM of 33 *C. parvipinnis*, 15 *C. othonopterus* and 23 *C. xanthulus*. Organs, viscera and indices values are means±SEM of 15 fish from each species.
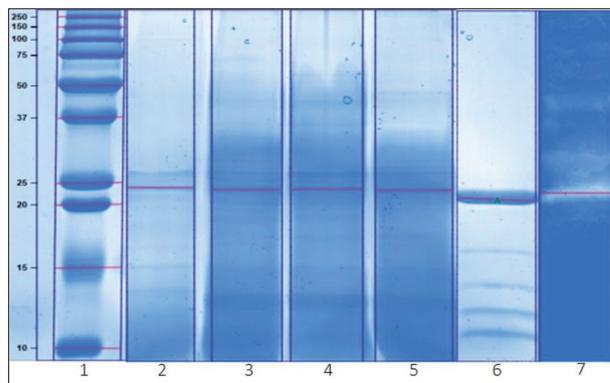

Fig. 1. *Cynoscion xanthulus* trypsin. SDS-PAGE gel: Lane 1 – Molecular weight standard, 2 – Anterior intestine, 3 – Middle intestine, 4 – Posterior intestine, 5 – Pyloric caeca, 6 – Bovine trypsin (A: quantitative standard); Zymogram: 7 – Anterior intestine.

the crude extract showed a clear band on a dark blue background (lane 7 in Fig. 1) produced by the activity of this protease against casein, which matched exactly the band observed on the SDS-PAGE gel. Lipases of *C. othonopterus* and *C. xanthulus* had molecular weights of 65.8±0.3 (Fig. 2A) and 69.5±0.8 kDa (Fig. 2B), respectively. Two bands with lipase activity were detected for *C. parvipinnis*, one of 61.5±0.2 kDa and another one of 36.0±0.3 kDa (Fig. 2C). Lipase bands were evident from the reaction of the enzyme against its substrate and were observed as dark blue bands in the zymograms.

The concentrations of trypsins and lipases were analyzed by densitometry on the SDS-PAGE gels. Mean concentration values±SEM for trypsins and li-
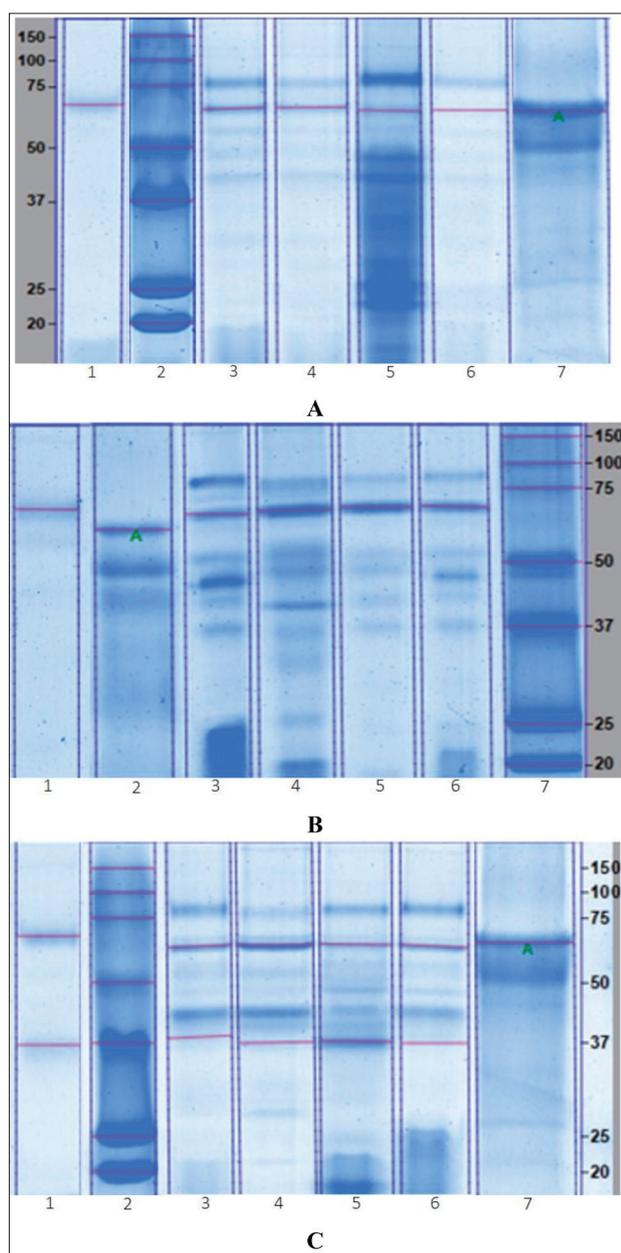
Fig. 2. Lipases. **A:** *C. othonopterus*. Zymogram: Lane 1 – Anterior intestine; SDS-PAGE gel: 2 – Molecular weight standard, 3 – Anterior intestine, 4 – Middle intestine, 5 – Posterior intestine, 6 – Pyloric caeca, 7 – Bovine serum albumin (A: quantitative standard). **B:** *C. xanthulus*. Zymogram: Lane 1 – Anterior intestine; SDS-PAGE gel: 2 – Bovine serum albumin (A: quantitative standard), 3 – Anterior intestine, 4 – Middle intestine, 5 – Posterior intestine, 6 – Pyloric caeca, 7 – Molecular weight standard. **C:** *C. parvipinnis*. Zymogram: Lane 1 – Anterior intestine; SDS-PAGE gel: 2 – Molecular weight standard, 3 – Anterior intestine, 4 – Middle intestine, 5 – Posterior intestine, 6 – Pyloric caeca, 7 – Bovine serum albumin (A: quantitative standard).

pases observed in the three sciaenids are presented in Table 2. For trypsin, the concentration ranged from 6.0-12.8 mg g^{-1} of tissue, and in *C. othonopterus* and *C. parvipinnis*, the AI and PC appeared to have the largest concentrations of the enzyme. Lipase concentration estimated by densitometry ranged from 16.3-50.0 mg g^{-1} of tissue, with no noticeable differences between the analyzed sections of the GIT, except that in all sciaenids the concentration in PI appeared to be lower.

Trypsin and lipase activities

Trypsin activity was significantly higher in AI of *C. othonopterus* (65.1 U mL^{-1}, *P*=0.0352), in *C. parvipinnis* it was significantly higher in AI and PC (66.5 and 30.8 U mL^{-1}, respectively; *P*=0.0286), whereas in *C. xanthulus*, no significant differences (*P*=0.2757) in activity were evident between the four sections of the GIT, but numerically, more activity (108.8 U mL^{-1}) was registered in the AI (Table 3). These observations were consistent with the concentrations of trypsin estimated through the calibration curve with trypsin from bovine pancreas; the IA showed significantly higher activity in *C. othonopterus* (0.94 mg mL^{-1}), in *C. parvipinnis* it was significantly higher in the AI and PC (0.96 and 0.42 mg mL^{-1}, respectively), and in *C. xanthulus*, no significant differences in activity were observed, ranging from 1.61 mg mL^{-1} in the AI, to 0.38 mg mL^{-1} in the PI (Table 4). Optimum temperature for trypsin activity (Table 5) was 60°C (426.3 U mL^{-1}) in *C. othonopterus*, though it was not significantly different from the activity observed at 55 or 65°C (307.1 or 318.5 U mL^{-1}, respectively). In *C. parvipinnis* and *C. xanthulus*, trypsin activity was significantly higher (*P*<0.0001) at 65°C (699.4 and 160.7 U mL^{-1}, respectively). Optimum pH for trypsin activity in *C. othonopterus* was 8.0 (797.9 U mL^{-1}), but it was not significantly different from pH 7.0 or 9.0 (493.4 or 527.9 U mL^{-1}, respectively). In *C. parvipinnis* activity was significantly higher (*P*<0.0001) at pH 8.0 (845.2 U mL^{-1}), but not statistically different from pH 7.0 (653.3 U mL^{-1}). Finally, in *C. xanthulus* the optimum pH for trypsin activity was 8.0 (782.8 U mL^{-1}), which was significantly higher (*P*<0.0001) than the rest of the pH values tested (Table 6).

Lipase activity on the other hand, was significantly higher in the AI and PC of *C. parvipinnis* (133.3 and

Table 2. Concentration (mg g^{-1} of tissue) of trypsin and lipase within the different sections of the GIT of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* determined by densitometry in SDS-PAGE gels.

GIT section	Trypsin concentration (mg g^{-1} of tissue)			Lipase concentration (mg g^{-1} of tissue)		
	C. othonopterus	*C. parvipinnis*	*C. xanthulus*	*C. othonopterus*	*C. parvipinnis*	*C. xanthulus*
AI	12.8±0.9	8.1±2.7	7.0±1.4	35.8±2.2	35.8±3.8	45.4±3.2
MI	9.4±0.7	7.7±2.9	6.0±0.4	50.0±2.2	37.2±5.9	42.3±4.2
PI	9.2±1.1	7.3±2.4	7.0±0.2	28.3±0.2	16.3±6.2	25.1±2.8
PC	11.8±0.5	11.3±1.8	7.0±0.4	42.6±2.8	35.3±4.4	39.9±5.0

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; GIT: gastrointestinal tract; AI: anterior intestine; MI: middle intestine; PI: posterior intestine; PC: pyloric caeca. Values are means±SEM of three fish per species.

Table 3. Activity (U mL^{-1}) of trypsin and lipase within the different sections of the GIT of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus*.

GIT section	Trypsin activity (U mL^{-1})			Lipase activity (U mL^{-1})		
	C. othonopterus	*C. parvipinnis*	*C. xanthulus*	*C. othonopterus*	*C. parvipinnis*	*C. xanthulus*
AI	65.1[a] ±19.9	66.5[a]±21.3	108.8±52.3	101.0[a]±9.6	133.3[a]±19.6	113.6[a]±15.5
PC	17.3[b]±5.8	30.8[ab]±4.4	57.1±12.5	81.3[ab]±7.0	86.0[ab]±15.1	89.0[ab]±13.0
MI	24.6[b]±7.0	20.7[b]±1.8	85.2±25.3	73.1[ab]±8.7	65.3[b]±5.2	64.0[bc]±5.8
PI	17.8[b]±5.0	21.7[b]±5.1	27.9±1.4	50.7[b]±4.1	56.0[b]±6.5	44.0[c]±4.8
ANOVA *P*>F	0.0352	0.0286	0.2757	0.0021	0.0039	0.0007

GIT: gastrointestinal tract; AI: anterior intestine; MI: middle intestine; PI: posterior intestine; PC: pyloric caeca. Values are means±SEM of three replicates per species; each triplicate sample and its blank were run in duplicates. Means with different superscripts within the same column are significantly different (*P*≤0.05).

Table 4. Concentration (mg mL^{-1}) of trypsin within the different sections of the GIT of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus*.

GIT section	Trypsin concentration (mg mL^{-1})		
	C. othonopterus	*C. parvipinnis*	*C. xanthulus*
AI	0.94[a]±0.30	0.96[a]±0.32	1.61±0.80
PC	0.21[b]±0.09	0.42[ab]±0.07	0.82±0.19
MI	0.32[b]±0.11	0.27[b]±0.03	1.25±0.38
PI	0.22[b]±0.08	0.28[b]±0.08	0.38±0.02
ANOVA *P*>F	0.0346	0.0290	0.2766

GIT: gastrointestinal tract; AI: anterior intestine; MI: middle intestine; PI: posterior intestine; PC: pyloric caeca. Values are means±SEM of three replicates per species; each triplicate sample and its blank were run in duplicates. Means with different superscripts within the same column are significantly different (*P*≤0.05).

86.0 U mL^{-1}, respectively; *P*=0.0039) and *C. xanthulus* (113.6 and 89.0 U mL^{-1}, respectively; *P*=0.0007), as well as in *C. othonopterus*, but in this last sciaenid, the activity registered in MI (73.1 U mL^{-1}) was not significantly different (*P*=0.0021) from that recorded in the AI or PC (101.0 and 81.3 U mL^{-1}, respectively) (Table 3). The optimum temperature for lipase activity (Table 5) in *C. othonopterus* was 45°C (609.3 U mL^{-1}; *P*=0.0428); in *C. parvipinnis* a significantly higher activity was registered at 40°C (1,225.7 U mL^{-1}; *P*<0.0001), and in *C. xanthulus* no significant differences were observed between 40 (1,327.3 U mL^{-1}) and

45°C (1,299.8 U mL^{-1}). The optimum pH for activity of lipase in these sciaenids was 8.0 (1014.2-1185.6 U mL^{-1}), and in all three species, this pH was significantly higher (*P*<0.0001) than the rest of the pH values evaluated (Table 6).

DISCUSSION

The weights and lengths of the experimental organisms in this study corresponded to large commercial-size sciaenids. The HSI (1.2-1.6%), GSI (0.08-0.53%) and VSI (7.3-9.5%) reported in this study are comparable to values reported in the literature for these and other sciaenids; for *C. parvipinnis*, HSI and VSI values ranging from 0.9-2.6% and from 3.7-5.9%, respectively [35], and HSI values ranging from 2.2-2.9% for *C. othonopterus* [27] have been reported. For other sciaenids like *Totoaba macdonaldi*, also endemic to the Gulf of California, HSI values ranging from 1.6-2.2% and VSI of 3.6-4.5% [35] have been reported, as well as HSI ranging from 1.33-1.96% for *Sciaenops ocellatus* [36]. Although comparable, differences between the observed and reported values may be explained by the size differences, since the values reported in the previous studies corresponded mostly to juvenile fish. Their origin is also different; in the previous studies

Table 5. Activity (U mL^{-1}) of trypsin and lipase within AI of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* at different temperatures.

Temperature	Trypsin activity (U mL^{-1})			Lipase activity (U mL^{-1})		
	C. othonopterus	*C. parvipinnis*	*C. xanthulus*	*C. othonopterus*	*C. parvipinnis*	*C. xanthulus*
20°C	19.1[d]±0.8	15.8[g]±0.9	13.9[d]±1.9	62.1[cd]±24.9	219.1[efg]±28.8	57.2[d]±9.2
25°C	26.6[d]±3.6	18.8[g]±3.1	15.0[d]±1.4	148.9[abcd]±65.7	301.3[def]±17.5	135.5[d]±3.5
30°C	80.8[cd]±18.2	24.9[fg]±2.1	14.7[d]±0.6	271.7[abcd]±58.7	415.7[cde]±2.0	376.5[c]±10.8
35°C	100.5[cd]±18.5	27.8[fg]±4.8	20.7[cd]±1.6	339.0[abcd]±79.3	557.3[c]±48.1	628.6[b]±14.9
40°C	146.1[cd]±31.0	37.1[fg]±4.2	21.6[cd]±1.2	489.6[abc]±78.4	1225.7[a]±5.5	1327.3[a]±8.0
45°C	184.4[bc]±34.4	82.7[ef]±12.7	24.2[cd]±0.7	609.3[a]±90.8	880.4[b]±82.3	1299.8[a]±4.5
50°C	229.9[bc]±41.8	132.3[de]±16.9	30.3[cd]±0.8	560.3[ab]±74.3	439.6[cd]±71.7	536.9[b]±58.7
55°C	307.1[ab]±57.7	200.2[c]±28.6	42.1[bc]±5.6	307.3[abcd]±75.6	281.7[defg]±12.7	106.8[d]±14.7
60°C	426.3[a]±81.4	473.3[b]±8.7	62.6[b]±7.9	108.9[bcd]±29.7	172.4[fg]±50.3	38.8[d]±4.5
65°C	318.5[ab]±36.2	699.4[a]±27.6	160.7[a]±3.4	53.0[cd]±17.7	66.4[g]±23.5	33.7[d]±4.2
70°C	48.0[d]±2.9	146.2[cd]±11.5	64.9[b]±7.6	33.1[d]±17.5	38.5[g]±12.6	19.1[d]±5.0
75°C	36.2[d]±5.4	39.1[fg]±5.4	36.1[cd]±9.2	-	-	-
80°C	29.2[d]±5.3	38.7[fg]±5.9	35.3[cd]±5.5	-	-	-
85°C	28.9[d]±6.3	42.1[fg]±5.0	42.2[bc]±6.6	-	-	-
90°C	26.7[d]±5.8	40.6[fg]±5.0	27.2[cd]±6.6	-	-	-
ANOVA P>F	< 0.0001	< 0.0001	< 0.0001	< 0.0428	< 0.0001	< 0.0001

AI: anterior intestine. Values are means±SEM of three replicates per species; each triplicate sample and its blank were run in duplicates. Means with different superscripts within the same column are significantly different (*P*≤0.05).

Table 6. Activity (U mL^{-1}) of trypsin and lipase within AI of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* at different pH values.

pH	Trypsin activity (U mL^{-1})			Lipase activity (U mL^{-1})		
	C. othonopterus	*C. parvipinnis*	*C. xanthulus*	*C. othonopterus*	*C. parvipinnis*	*C. xanthulus*
5	-	-	-	12.7[cd]±2.9	37.9[e]±4.5	30.2[d]±7.9
6	291.1[b]±34.9	227.3[cd]±10.3	306.1[cd]±9.9	110.8[cd]±31.6	537.3[d]±17.9	179.4[c]±12.7
7	493.4[ab]±20.2	653.3[ab]±75.8	499.8[b]±44.2	587.3[b]±69.8	987.9[b]±63.5	715.6[b]±27.6
8	797.9[a]±31.7	845.2[a]±48.2	782.8[a]±2.4	1014.2[a]±114.6	1185.6[a]±7.9	1158.4[a]±23.2
9	527.9[a]±72.3	462.5[bc]±61.7	325.9[c]±16.0	215.3[c]±27.4	786.2[c]±8.3	730.4[b]±12.3
10	245.4[b]±6.3	211.5[d]±10.7	215.8[d]±11.3	6.9[d]±1.9	5.9[e]±1.6	11.1[d]±2.6
ANOVA P>F	0.0003	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

AI: anterior intestine. Values are means±SEM of three replicates per species; each triplicate sample and its blank were run in duplicates. Means with different superscripts within the same column are significantly different (*P*≤0.05).

the reports are for cultured fish, whereas wild organisms were used in this study. Nevertheless, it is evident from these biological indices that the experimental fish in this study were in good physiological state.

Lipases are present in the GIT of carnivorous fish because, in spite of consuming large amounts of protein in their natural diet, lipids are also a significant component. In *C. othonopterus*, the molecular weight of lipase was 65.8 kDa, fairly similar to the lipase of *Pagrus major* of 64 kDa [14]. In *C. parvipinnis*, two bands of 61.5 and 36.0 kDa showed lipase activity, similarly to the observation in *Oncorhynchus tshawytscha*, where two bands of 79.6 and 54.9 kDa were reported and thus, the presence of two isozymes of lipase was

proposed [15]. Lipase of Atlantic cod *Gadus morhua* has a molecular weight of 60 kDa [12], similar to the molecular weight of the first lipase observed in *C. parvipinnis*. Finally, lipase of *C. xanthulus* had a molecular weight of 69.6 kDa, close in value to the pancreatic lipase of *T. macdonaldi* of 70.4 kDa [19], and to the 70 kDa purified lipase of Indian carp *Catla catla* [37]. Additional studies have reported molecular weights of 80.3 kDa in *S. ocellatus* [20], 74 kDa in common carp *Cyprinus carpio* [38] and 44.6 kDa for *Macruronus novaezelandiae* [15]. The molecular weight of lipase in marine finfish varies according to species; differences in tridimensional structures may be attributed to differences in amino acid sequences constituting the primary protein structure as a result of genetic di-

versity, as well as the result of possible environmental pressures to which they have been subjected, resulting in evolutionary adaptations at a molecular level [39-41]; feeding habits most likely also play an important role in the structural differences observed in lipases and other digestive enzymes in finfish.

Lipase activity within the different sections of the GIT of *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* evaluated at 35°C confirmed that the AI is the section with the greatest enzymatic activity, though it is not significantly different from the activity in the PC. In general, activity gradually decreased towards the posterior end of the intestine in all three species. Higher lipolytic activities within the AI and PC in comparison with the distal sections of the intestine have also been reported in *T. macdonaldi*, *Sparus aurata* and *Thunnus orientalis* [19,42,43]. These observations coincide with earlier reports demonstrating that the digestion and absorption of lipids in marine finfish takes place within the AI [44,45]. The higher activity of lipase within the anterior sections of the GIT is explained by the fact that pancreatic enzymes are secreted within the AI of finfish. Moreover, enzymes are proteins also exposed to hydrolysis by other proteases in the GIT; thus, when they reach the MI and PI they have been subjected to hydrolysis to some extent, explaining the lower enzymatic activity within these sections. It is interesting to note that the lipases of all three sciaenids had catalytic activity towards fatty acids esterified to triacylglycerol molecules present in olive oil, which leads us to assume that the catalytic mechanism in the lipases of the three species is quite similar to that observed in higher vertebrates, and that under culture conditions, balanced diets could include this type of vegetable oil as long as their nutritional requirements for essential fatty acids (mainly n-3 highly unsaturated fatty acids) are satisfied by other lipid sources, fish oil for instance.

The optimum temperature for lipase activity of *C. parvipinnis* was 45°C; at 40 and 45°C *C. xanthulus* showed no differences in activity, and for *C. othonopterus*, 45°C was the temperature where the highest activity was recorded, quite similar to the observation for *T. macdonaldi* with optimum lipase activity at 45°C [18,19]. Optimum lipase activities between 35 to 45°C in *T. orientalis* and *Morone saxatilis* [18] have been reported, and 35°C for *O. tshawytscha* and

M. novaezelandiae [15]. Differences in optimum temperatures for pancreatic enzymes could be explained by the differences in environmental temperatures observed in the habitats of the species, which clearly lead to adaptations [46]. Sciaenids in this study inhabit a geographical area with average temperatures ranging between 19.8±2.3 and up to 27.8±1.9°C [47]. The optimum temperature for enzyme activity in finfish and higher vertebrates is usually higher than their optimum physiological temperature; this is explained by the increment observed in the molecular kinetic energy at higher temperatures, up until the point where denaturation of the protein occurs [48]. Optimum pH for lipase activity on the other hand was 8.0 for all three sciaenids, coinciding with the value reported for *T. macdonaldi*, *M. saxatilis* and *T. orientalis* [18,19]. For *O. tshawytscha* and *M. novaezelandiae*, the optimum pH was reported to be 8.0-8.5 [15]. Thus, the optimum pH for enzymatic activity of lipase does not vary much among these sciaenids or other marine finfish.

Trypsin molecular weights for *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* were 24.4, 23.6 and 23.7 kDa, respectively, comparable to mammalian and other marine finfish trypsins, such as that of *Sardinops sagax caerulea* with 25 kDa [49], or that of *Zosterisessor ophiocephalus* with 23.2 kDa [50]. The molecular weight of purified trypsin of *Clarias macrocephalus* × *Clarias gariepinus* is 24 kDa [51]. In *Thunnus alalunga*, trypsins A and B are 21 and 24 kDa, respectively [46]. In *Colossoma macropomum*, trypsin has a molecular weight of 23.9 kDa [52] and in *Diapterus rhombeus* 26.5 kDa [53]. As has been suggested, variability in molecular weights is expected even in closely related species because of genetic differences, differences in tridimensional structures and amino acid sequences in the proteins, as well as environmental adaptations [39-41], among other factors. Similar to lipase, the proteolytic activity of trypsin was higher in the AI of *C. othonopterus* and *C. parvipinnis*, although it was not significantly different from that in PC in the latter species. In *C. xanthulus* no significant differences were detected, but higher activity was also observed in the AI and PC. Higher activity of trypsin in the AI has also been described for *Salvelinus alpinus* [54] and for *Salmo salar* and *Oncorhynchus mykiss* [55]. Since trypsin is secreted by the pancreas in the anterior section of the GIT like lipase, it would also be expected

to be more active and to be more concentrated within this section of the tract [56].

The optimum temperature for trypsin activity in *C. othonopterus* was 60°C, but not significantly different from that at 55 or 65°C, coinciding with the values for two trypsin isoforms of *Katsuwonus pelamis*, 55 and 60°C [57] and the trypsins of *C. macrocephalus* × *C. gariepinus* [51], *O. tshawytscha* [15], *Sardina pilchardus* [58] and *Mugil cephalus* [59], with an optimum temperature at 60°C. In *C. parvipinnis* and *C. xanthulus*, the optimum temperature was 65°C, the same as for *Centropomus undecimalis* [60], *Atractosteus tropicus* [61], *Brycon orbignyanus* [62] and *T. thynnus* [63]. Lower optimum temperatures for trypsin activity have been described in the literature, such as those for *S. sagax caerulea* [49] and *Gadus macrocephalus* [64] at 50°C, or *Balistes capriscus* [41], *Pterygoplichthys disjunctivus* [65] and *Cirrhinus mrigala* [66] with optimum temperatures as low as 30-40°C. Optimum temperature for activity for all enzymes could still be related to environmental temperature, in spite of being higher than their optimum physiological temperature [46,48]. All sciaenids in this study showed a drastic decline in trypsin activity after reaching 70°C, similarly to observations for trypsins of *C. macrocephalus* × *C. gariepinus* and *T. alalunga* that also declined at 70°C [46,51] and of *C. undecimalis* at 75°C [60], which is explained by the thermal denaturation of the protein. Heat causes the enzyme molecule to unfold, breaking down the secondary and tertiary structures of the protein and the enzyme loses its function. It has been suggested that alkaline proteases of aquatic organisms are usually stable and active under adverse conditions, such as temperatures between 50-60°C [67]; this is the reason why trypsins from *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* are interesting candidates for the feed industry or for industrial applications such as the manufacture of detergents.

The optimum pH for trypsin activity in *C. othonopterus* was 8.0-9.0, in *C. parvipinnis* it was 7.0-8.0 and in *C. xanthulus* it was 8.0, all within the range proposed as the optimum pH for trypsins [49], 7.0-9.0. An optimum pH of 8.0 for trypsin activity of *S. sagax caerulea* has been reported [49], and the same value has been reported for *C. macrocephalus* × *C. gariepinus* [51] and *O. tshawytscha* [15]. An op-

timum pH value of 8.5 was reported for *T. alalunga* [46] and *D. rhombeus* [53], and a range of 8.0-9.0 for the trypsins of *S. pilchardus* [58]. Thus, the optimum pH values of trypsin activity for several marine and freshwater fish closely resemble those observed in this study for our three studied sciaenids. The activity of trypsin decreased at either lower or higher pH values, presumably as a result of conformational modifications resulting from changes in the electrical charge of side chains of amino acids, which in turn modify electrostatic interactions that stabilize the tertiary structure of proteins [48] or cause the denaturation of the protein under antagonist acid or alkaline conditions of the aqueous solutions, resulting in lower enzyme activity [68]. In general, trypsins of aquatic organisms are active at pH values ranging from 7.0 to 10.0 and can hydrolyze a diversity of substrates [49,69]. Consequently, the trypsins from *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* evaluated in this study may also be used as additives in detergent formulations, since they are all active at those alkaline pH values [70].

CONCLUSIONS

This study revealed that the molecular weights of lipases from *C. othonopterus*, *C. parvipinnis* and *C. xanthulus* were 65.8, 61.5 and 36.0 (two lipases) and 69.6 kDa, respectively, whereas the molecular weights of trypsins were 24.4, 23.6, and 23.7 kDa, respectively. All values were well within the range of values reported for marine fish and related sciaenids. In general, for both enzymes, greater activity was observed within the AI and PC in all sciaenids, and decreased towards the posterior end of the GIT. The optimum temperature for lipase activity in *C. parvipinnis* and *C. othonopterus* was 45°C and 40-45°C in *C. xanthulus*; on the other hand, the optimum pH for lipase activity was 8.0 in the three species. The optimum temperature for trypsin activity in *C. othonopterus* was 60°C, but not different from that at 55 or 65°C; for *C. parvipinnis* and *C. xanthulus* the optimum temperature was 65°C. The optimum pH for trypsin activity in *C. othonopterus* was 8.0-9.0, 7.0-8.0 in *C. parvipinnis* and 8.0 in *C. xanthulus*, all within the 7.0-9.0 range generally proposed as the optimum pH for trypsins. These enzymes meet certain criteria that make them poten-

tial candidates for some industrial applications, such as the food industry and the production of detergents.

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