Arch Biol Sci. 2018;70(3):489-496 https://doi.org/10.2298/ABS180202009G

Partial characterization, quantification and activity of pancreatic lipase in the gastrointestinal tract of *Totoaba macdonaldi*

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Received: February 2, 2018; **Revised**: March 16, 2018; **Accepted**: March 16, 2018; **Published online**: March 21, 2018

Abstract: Lipids are one of the main macronutrients that constitute balanced feeds used in aquaculture. Adequate utilization of dietary lipid is influenced by the activity of pancreatic lipase, one of the enzymes that promotes digestion of dietary lipids in the gastrointestinal tract of fish. The culture of *Totoaba macdonaldi* is quite recent; its nutritional requirements have been partially established. Knowing the characteristics of pancreatic lipase for this species could help optimize the dietary lipids included in balanced feeds for its culture. Therefore, the aim of this work is to partially characterize and evaluate the enzymatic activity of pancreatic lipase for *T. macdonaldi*. Biological indices showed that experimental organisms had a good nutritional status. Pancreatic lipase molecular weight was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and its activity was evaluated in crude enzymatic extracts from different gastrointestinal tract regions. The molecular weight of lipase was estimated to be 70.4 kDa; the highest lipolytic activity was observed at 45°C and at a pH optimum of 8.0 in the anterior intestine and pyloric caeca, where the concentration and activity of the enzyme was significantly higher (P=0.004) compared to the distal parts of the intestine. Biochemical characteristics observed for the pancreatic lipase of *T. macdonaldi* are quite similar to other lipases of fish cultured worldwide; results provided in this study will help understand the role this lipolytic enzyme plays in the digestive process of this species.

Key words: *Totoaba macdonaldi*; pancreatic lipase; enzymatic activity; gastrointestinal tract; lipids

INTRODUCTION

Understanding the utilization of dietary lipids in marine fish is crucial for understanding their role in energy metabolism [1]. Nevertheless, the knowledge of the biochemical mechanisms that allow marine fish the use of this particular nutrient is rather basic. Pancreatic lipase (PL) is one of the main digestive lipolytic enzymes in higher vertebrates secreted by the exocrine pancreas that has strong preference for acylglycerols over other lipids, and it may contribute to the *in vivo* hydrolysis of retinyl esters. It is also known as a colipase-dependent lipase because the coenzyme colipase, also secreted by the pancreas, is required for lipid binding and allows the enzyme to anchor to the water-lipid interface to carry out the hydrolysis of dietary lipid effectively [2-5]. Alternatively, the enzyme carboxyl ester lipase, also known as bile salt-activated or bile salt-dependent lipase (BSDL), is another pancreatic lipase that possesses the widest range of substrate specificity, being able to hydrolyze mono-, di-, and triacylglycerols, cholesteryl, aryl and wax esters, as well as vitamin A and E esters, phospholipids, lysophospholipids and ceramides. This enzyme requires the presence of primary bile salts to hydrolyze triacylglycerols or phospholipids with medium or long-chain fatty acids, but it has basal activity on molecules with short-chain fatty acids in the absence of bile salts [6,7].

Pancreatic lipase has been reported in zebrafish *Danio rerio* [8], rainbow trout *Oncorhynchus mykiss* [9], top minnow *Triportheus* sp. [10] and in the hepatopancreas of sardine *Sardinella longiceps* [11]. It has been proposed that two enzymes are present in

O. mykiss, one that predominantly hydrolyzes triacylglycerols and the other wax and steryl esters [9,12]. In turbot *Scophthalmus maximus*, a non-specific lipase with activity in the absence of bile salts was reported [13-15], although it was later confirmed to be a bile salt-dependent lipase [16,17].

Totoaba macdonaldi is a marine sciaenid of the Gulf of California with great potential for aquaculture, although knowledge about its digestive enzyme assembly, their activity, and physiology is still rather scarce. Development of aquafeeds for its culture is progressing as quantitative nutritional requirements are established, but lipid levels from 8 to 22% do not seem to affect its growth performance, similarly to red drum, *Sciaenops ocellatus*, another sciaenid for which lipase is apparently not stimulated by increasing dietary lipid content. Dietary lipid is one of the crucial macronutrients in balanced feeds for cultured fish species [18]; it plays an important role as a source of energy, as a constituent of cell membranes and tissues, as a supply of essential fatty acids, and as a precursor of molecules that have many important and diverse functions in their metabolism [19]. However, lipid requirement varies according to species, sex, developmental stage and habitat, among other variables [20]. When dietary lipid is provided to cultured fish in excess, it is stored in their bodies, e.g. in the liver, the peritoneal cavity and muscle, and could affect the quality and shelf-life of the marketed fillet [21,22]. Moreover, excess dietary lipid may limit feed consumption resulting in reduced fish growth during culture, as reported for *S. ocellatus* [23,24] and totoaba [25]. Knowing the type of lipase present in a particular fish species and its functional characteristics contributes to the understanding of the digestive physiology of the organism and could perhaps help develop strategies to improve feed formulations. The objective of the present work was to partially characterize, quantify and investigate the activity of pancreatic lipase along the gastrointestinal tract of *Totoaba macdonaldi*.

MATERIALS AND METHODS

Ethics statement

All fish were treated according to the Official Mexican Norm (NOM-062-ZOO-1999) on the technical specifications for the production, care and use of laboratory animals (in Spanish), issued by the Official Journal of the Federation on August 22, 2001, which is approved by the Ethics Committee of the University of Sonora.

Experimental fish and biological indices

Forty experimental fish were donated by the Center for Reproduction of Marine Species of the State of Sonora (CREMES) located in Kino Bay, Sonora, Mexico. *T. macdonaldi* individuals were killed by immersion in iced water (≈4°C). Individual weight (IW, g) and total length (IL, cm) were measured to determine the condition factor K=(wet body weight×100)/total length^3, cm [26]. Then, fish were eviscerated *in situ* and the gastrointestinal tract (GIT) was removed, separately stored in sealed plastic bags, and transported in an ice-filled cooler to the Nutrition Laboratory of the Department of Scientific and Technological Research of the University of Sonora (DICTUS). The weights of the liver and viscera were obtained for each individual to determine the hepatosomatic index HSI [%]=(liver weight/total weight)×100, and the viscerosomatic index VSI [%]=(viscera weight/total weight)×100. GIT samples were stored at -82.0°C until further analyses.

Enzyme extraction and SDS-PAGE

The GIT of fish was dissected into four sections: anterior, middle and posterior intestine, and pyloric caeca. Homogenates were obtained with approximately 0.5 g of tissue from each section, 1.3 mL of 50 mM Tris-HCl buffer solution (pH 7.5), and 0.7 mL of protease inhibitors (0.5 mL benzamidine-HCl 2mM, and 0.2 mL EDTA 1 mM) using an homogenizer (Ultra Turrax, T18 basic, IKAWorks Inc., Wilmington, NC, USA). The crude extract obtained was centrifuged (Heraeus Fresco 21, Thermo Fisher Scientific, Dreieich, Germany) at 21,000 × *g* and 4°C for 30 min, until the samples were clear. After centrifugation, the precipitate was discarded, and the supernatant was concentrated and semi-purified in using 30 kDa centrifugal filters (Amicon®Ultra-4, Centrifugal filter device, Millipore Corp., Billerica, MA, USA) at 1300 × *g* and 4°C for 30 min. The protein content of the semi-purified extract was analyzed in 100 µL of sample from each extract via combustion by the Dumas method, N factor=6.25; method 968.06 [27], with a Dumas Nitrogen Analyzer (Model NDA 702, VELP® Scientifica, Usmate, Italy).

In order to determine the molecular mass of lipase and to quantify its content in the different sections of the GIT of fish, SDS-PAGE was carried out in a four-gel vertical electrophoresis system (Mini-Protean Tetra Cell, Bio-Rad; CA, USA) using 10% polyacrylamide gels. The enzymes were resolved at a constant voltage of 115 V applied for about 2.5 h at 10°C. Gels were then fixed in a solution of 40% ethanol and 10% acetic acid, rinsed twice with distilled water, and stained overnight in gentle agitation with QC Colloidal Coomassie stain, then destained by rinsing again in distilled water. An internal standard (Precision Plus Protein Standard, Bio-Rad®, Hercules, CA, USA) with proteins of 10-250 kDa, and human pancreatic lipase (BCR-693, European Community Bureau of Reference) were used as a molecular weight marker and as a quantitative internal standard, respectively. Lastly, the gels were scanned with a GS-900 Calibrated Densitometer (Bio-Rad®, Hercules, CA, USA) for identification and quantification of protein bands using the ImageLab 5.0 software (Build 18, Bio-Rad®, Hercules, CA, USA).

Zymography

To confirm the band identified as PL in the SDS-PAGE gels, a zymogram was obtained following a previously described procedure [28]. Briefly, SDS-PAGE was done as previously described. After resolving at a constant voltage of 115 V, the gel was immediately rinsed three times with distilled water and equilibrated in a 50-mM Tris-HCl (pH 8) buffer for 30 min at 25°C. A chromogenic substrate solution was prepared, containing 0.01% phenol red, 1% oleic acid, and 2% agar dissolved in a 10-mM $CaCl_2$ solution with the pH adjusted to 8.2 using 0.1 N NaOH. This solution was warmed to 95°C for 2 min to dissolve the agar and cooled down to 40°C at ambient temperature. The gel was then covered with the chromogenic substrate and allowed to solidify during 30 min, followed by incubation for approximately 3.5 h at 37°C. After incubation, lipase activity was observed as a yellow spot on the fuchsia colored gel.

Activity of pancreatic lipase

Activity of PL within the different sections of the GIT was determined in quadruple samples by the enzymatic assay of lipase (EC 3.1.1.3) using olive oil as a substrate, with minor modifications. The homogenates were prepared using three volumes of 200 mM Tris-HCl buffer solution (pH 7.7) and centrifuging twice at 21000 × *g* and 4°C for 30 min, until the samples were clear. The supernatant was recovered and used to prepare a solution of 1 mL 200 mM Tris-HCl buffer (pH 7.7), 2.5 mL tridistilled water, 1 mL of crude extract, and 3 mL olive oil. Then, the samples were mixed vigorously and incubated in a shaker at 35°C and 120 rpm for exactly 30 min. The reaction was stopped by adding 3 mL of 95% ethanol, followed by addition of four drops 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 30-min incubation period. Each reaction sample and blanks were analyzed in duplicates. One unit of activity of lipase (U) per mL of crude extract was defined as the amount of enzyme that will hydrolyze 1.0 microequivalent of fatty acid from a triglyceride in one hour at pH 7.7 and 35°C, using the equation 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).

Effect of temperature on lipase activity

Enzymatic activity was higher in the anterior intestine and pyloric caeca as compared to the other two sections of the GIT. Thus, these two sections were used to evaluate the effect of temperature on enzymatic activity. The analysis was run in triplicate samples for each temperature tested: 20, 25, 30, 35, 40, 45, 50, 55 and 60°C. Each triplicate sample and its blank were run per duplicate, following the same method previously described.

Effect of pH on lipase activity

The anterior intestine and pyloric caeca were also used to evaluate the effect of pH on enzymatic activity as previously described, using a pH range of 6 to 10. Buffers were adjusted according to pH: a 200-mM citrate-phosphate buffer was used for pH 6.0, a 200-mM Tris-HCl buffer for pH 7.0 to 9.0, and a 200-mM glycine-NaOH buffer for pH 10. The temperature for incubation was adjusted to 45°C because, at this value, lipase activity was observed to be optimal in the previous test (2.4.1.).

Statistics

Biological indices, the molecular weight and concentration of pancreatic lipase determined by electrophoresis were analyzed by descriptive statistics, whereas enzymatic activity in 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 where 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

Biological indices

Individual mean average weight (±standard error of the mean, SEM) was 1477.25±22.98 g. The weight of the viscera, consisting of esophagus, stomach, pyloric caeca and intestine, as well as associated organs like heart, liver, swim bladder, gallbladder, etc., averaged 72.52±1.47 g. Fish total length averaged 52.86±0.31 cm. The condition factor (K) averaged 1.00±0.01, while hepatosomatic and viscerosomatic indices averaged 0.91±0.03% and 4.92±0.08%, respectively.

Molecular weight, concentration and activity of lipase in the GI

The mean molecular weight (±SEM) of the semi-purified pancreatic lipase of *T. macdonaldi* determined by SDS-PAGE was 70.4±0.26 kDa. This band was confirmed by zymography (Fig. 1). Coomassie blue-stained polyacrylamide gels also provided an estimated content of the enzyme along the GIT. The estimated content in the anterior, middle and posterior intestine, as well as the pyloric caeca were 11.0, 2.5, 2.4, and 2.7 mg of PL g^{-1} of tissue, respectively. Lipolytic activity was significantly higher (P=0.0004) in the anterior intestine, 35.6±5.4 U mL^{-1} of crude enzymatic extract, followed by pyloric caeca, 24.1±2.6 U mL^{-1}, middle intestine, 15.8±2.1 U mL^{-1}, and posterior intestine 8.0±1.3 U mL^{-1}, where activity was significantly lower compared to other sections of the GIT (Fig. 2).

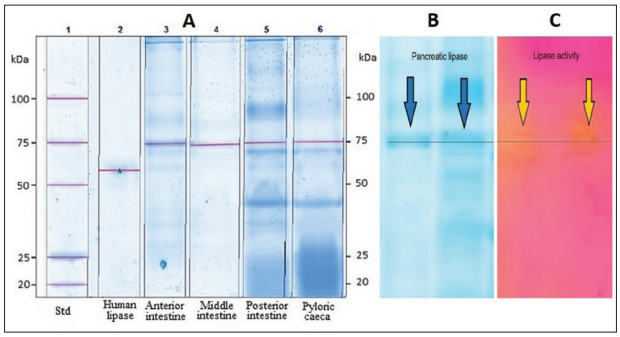

Fig. 1. A – PAGE gel of the semi-purified crude extract of pancreatic lipase from different sections of the gastrointestinal tract of *Totoaba macdonaldi*, with a molecular weight of 70.4 kDa. Std – Precision Plus Protein Standard (Bio-Rad®, Hercules, CA, USA). **B** – PAGE gel of pancreatic lipase of *T. macdonaldi* stained with QC Colloidal Coomassie. **C** – Zymogram demonstrating lipase activity towards oleic acid.

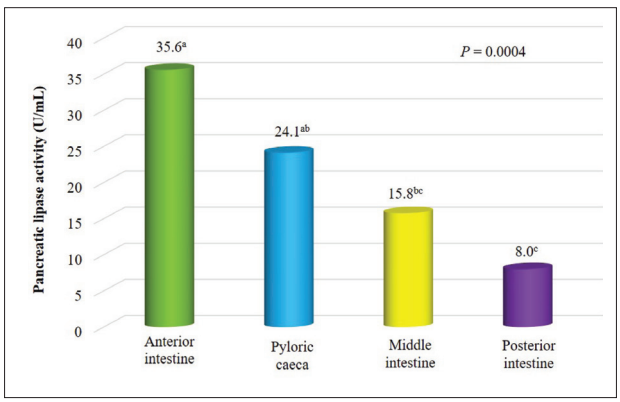

Fig. 2. Activity of pancreatic lipase in the different sections of the GIT of *Totoaba macdonaldi* at 35°C. Values of activity with different superindices are significantly different (P=0.0004).

Effect of temperature and pH on lipase activity

The anterior intestine and pyloric caeca were the sections of the GIT of *T. macdonaldi* where more lipase activity was observed. Thus, the effect of temperature and pH on enzyme activity was evaluated using both segments. Activity was consistently higher in the anterior intestine as compared to the pyloric caeca (Table 1), and significantly higher activity (P<0.0001) was observed at 45°C, with 70.3 U mL^{-1} of crude enzymatic extract in the anterior intestine compared to the rest of the evaluated temperatures (Fig. 3). In addition, significantly higher enzymatic activity (P<0.0001) was observed at the pH of 8.0 (Table 2), 73.8 U mL^{-1} in the anterior intestine, compared to rest of the evaluated values (Fig. 4).

Table 1. Pancreatic lipase activity in the anterior intestine and pyloric caeca of *Totoaba macdonaldi* at different temperatures.

	Anterior intestine Enzymatic activity U mL^{-1} of crude extract			**Pyloric caeca** Enzymatic activity U mL^{-1} of crude extract	
Temperature	**Mean***	**SEM**	**Temperature**	**Mean***	**SEM**
20°C	13.2^{de}	3.1	20°C	7.5^{ef}	1.4
25°C	14.8^{de}	2.6	25°C	10.0^{ef}	1.4
30°C	31.0^{bcde}	4.9	30°C	15.6^{def}	1.9
35°C	35.5^{bcd}	4.2	35°C	24.1^{bcd}	2.6
40°C	39.0^{bc}	4.1	40°C	28.8^{b}	1.6
45°C	70.3^{a}	7.8	45°C	43.1^{a}	2.8
50°C	45.8^{b}	8.1	50°C	26.3^{bc}	3.0
55°C	22.5^{cde}	1.4	55°C	16.9^{cde}	2.1
60°C	9.5^{e}	4.2	60°C	5.0^{f}	1.4
ANOVA Pr>F	<0.0001		*ANOVA Pr>F*	<0.0001	

*Means in each column with the same letter are not significantly different (P≤0.05).

Table 2. Pancreatic lipase activity in the anterior intestine and pyloric caeca of *Totoaba macdonaldi* at different pH.

	Anterior intestine Enzymatic activity U mL^{-1} of crude extract			**Pyloric caeca** Enzymatic activity U mL^{-1} of crude extract	
pH	**Mean***	**SEM**	**pH**	**Mean***	**SEM**
6	17.5^{e}	0.7	**6**	8.3^{d}	1.5
7	32.1^{c}	1.1	**7**	24.2^{b}	2.2
8	73.8^{a}	0.7	**8**	36.7^{a}	2.5
9	40.4^{b}	1.8	**9**	18.3^{bc}	2.2
10	24.6^{d}	1.1	**10**	11.3^{cd}	1.9
ANOVA Pr>F	<0.0001		*ANOVA Pr>F*	<0.0001	

*Means in each column with the same letter are not significantly different (P≤0.05).

DISCUSSION

Dietary protein has proven to be readily used as a source of energy in fish; however, dietary lipid and carbohydrate can have a protein-sparing effect, allowing protein to be preferentially used for somatic growth [25,29-31], a goal usually set for any cultured species in aquaculture. Juvenile *Totoaba macdonaldi* has shown similar growth performance when fed dietary lipid (provided as sardine fish oil) levels ranging from 8 to 22% at a fixed crude protein level of 46% [32], suggesting there is a limit in the capability to use this macronutrient, as has been observed in other species. For instance, in *Dicentrarchus labrax*, a plateau in enzymatic activity of lipase and phospholipase A2 was observed when supplementing more than 15% dietary triglycerides and more than 4.5% dietary phospholipid, respectively [33]. For *S. ocellatus*, maximal lipase activity was suggested to occur at a dietary inclusion of neutral lipid close to 11.8% [34]. This is an indication of a limited physiological ability in marine fish species to utilize this nutrient, during either digestion, absorption, transport or storage of dietary lipid. Since *T. macdonaldi* is a relatively new species in aquaculture, little is known with respect to the set of digestive enzymes present in the gastrointestinal tract of this species and their characteristics. Knowing these properties for its lipase will not only contribute to understanding the digestive physiology of this marine fish, but could also help improve feed formulations for its culture.

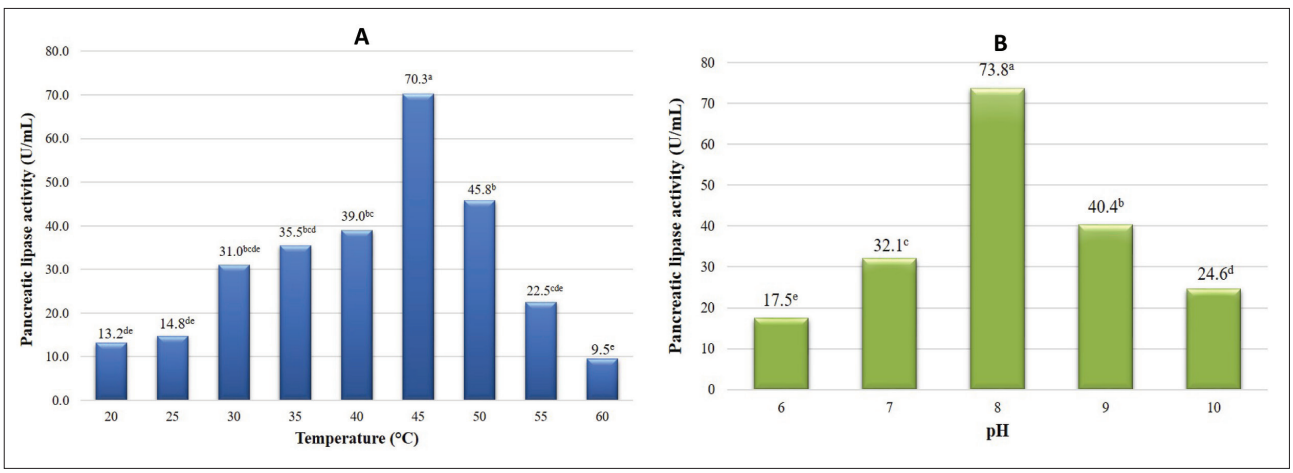

Fig. 3. Activity of pancreatic lipase in the anterior intestine of *Totoaba macdonaldi*. **A** – At different temperatures. **B** – At different pH. Values of activity with different superindices are significantly different (*P*<0.0001).

The molecular weights reported for lipases of marine fish vary according to species [35], as can be expected, and although evidence suggests the presence of bile salt-dependent lipase is more common, there is evidence of a PL-colipase system as well [19]. Molecular weights reported for BSDL of different marine fish species include those of 64 kDa in *Pagrus major* [36] and 60 kDa in *Gadus morhua* [37]. In the pyloric caeca of *Oncorhynchus tshawytscha*, two bands of 79.6 and 54.9 kDa were reported as possibly the uncleaved and the final form of its BSDL, respectively [38]. Other molecular weights reported for lipases include a 74 kDa hepatic lipase of *Cyprinus carpio* [35], and a 57.4 kDa pancreatic lipase in the intestine of *Cynoscion othonopterus* [18]. In this study, the molecular weight determined for pancreatic lipase of *T. macdonaldi* was 70.4 kDa, which is within the range of values previously mentioned for other fish species. For this sciaenid, there is a strong possibility that the pancreatic lipase described in the current study is not a BSDL because the activity of the enzyme was detected without supplementation of bile salts, and its activity did not increase in the presence of bile salts or natural bile extracts [39], although this inference requires further confirmation.

From the quantification of PL in the different regions of the gastrointestinal tract of *T. macdonaldi,* it was clear that the enzyme was more abundant within the anterior section of the intestine and the pyloric caeca. Moreover, the activity of PL was also significantly higher (P=0.0004) in the aforementioned sections. This observation coincides with the higher concentrations and activity of lipase also reported in the pancreas, anterior intestine and pyloric caeca of several fish species, like *S. aurata* [40,41], *Thunnus orientalis* [42], *Glyptosternum maculatum* [43] and the hybrid *Oreochromis niloticus* × *O. aureus* [44], among others. This is not surprising because pancreatic enzymes are secreted within the upper intestine of fish [45], thus, their concentration and activity would be more conspicuous in this area and would decrease towards the distal sections of the GIT. However, the activity of lipases and other enzymes is subjected to modifications by the influence of many factors, such as age, the source and quality of the dietary lipid, the prandial status, and the species [46].

It was established that the BSDL of *S. ocellatus* had the highest activity at a pH close to 8.0 and 50°C [47], the same temperature value reported for optimal lipolytic activity in *S. aurata* [41]. The maximum lipolytic activity from pancreatic crude extracts of *T. orientalis*, *T. macdonaldi* and *Morone saxatilis* was reported at pH 8.0 and temperatures ranging from 35 to 45°C [39]. Optimal lipolytic activity was observed at 40°C for *Sardinops sagax caerulea* [48], while the highest lipolytic activity was observed at 35°C and at a pH of 8-8.5 for *O. tshawytscha* and *Macruronus novaezelandiae* [38]. In this study, the highest lipolytic activity was observed at 45°C and a pH optimum of 8.0 in both anterior intestine and pyloric caeca of *T. macdonaldi*, confirming the observations provided previously for this species [39], and agreeing with the general observations of related sciaenids and marine fish. As expected, pancreatic enzymes, including PL and BSDL, would be more active in the alkaline pH range, between 7.0 and 9.0, values that may very well be expected in the intestine of marine teleosts. However, unlike pH, it is often observed that temperatures for optimal activities of some enzymes are higher than the physiological temperature of the organisms where they are found [49]; when temperature rises, the kinetic energy of the molecules increases, also increasing the reaction rate, but at higher temperature denaturation of the enzyme is more likely to occur [50], which explains to some extent why optimal enzymatic activities differ from optimal physiological temperatures.

The biological indices obtained from the experimental organisms are in agreement with values previously reported [32,51] for smaller *T. macdonaldi* of 74.7 and 128.3 g of initial individual weight, which ranged from 0.7-1.3%, 2.7-3.8% and 1.0-1.3 for HSI, VSI, and K, respectively, altogether comparable to the values of 0.91%, 4.92% and 1.0 in this study. A general well-being was observed in the experimental fish that was corroborated by the condition factor K, an index reflecting the effect of biotic and abiotic interactions on the physiological condition of cultured fish [52]. The HSI could be also used an indicator of the nutritional status of fish; fluctuation of this value can be an indication of seasonal or dietary changes, infections, etc., and an elevated value could be associated to a high consumption of an energy-dense or nutrient-dense diet [53]; however, this was not exhibited in the liver of *T. macdonaldi* from this study.

CONCLUSIONS

A pancreatic lipase of 70.4 kDa was identified in the GIT of *Totoaba macdonaldi* and confirmed through the colored trace of enzymatic activity observed in a zymogram. The concentration and activity of the enzyme were significantly higher in the anterior intestine and pyloric caeca, decreasing towards the middle and posterior sections of the digestive tract. Higher lipolytic activity was confirmed to be at 45°C and at a pH optimum of 8.0 in both anterior intestine and pyloric caeca. There is a strong possibility that the pancreatic lipase described for *T. macdonaldi* in this study is not a BSDL because the activity of the enzyme was detected without supplementation of bile salts, and its activity did not increase in the presence of bile salts or natural bile extracts as previously reported, although additional studies are required to confirm this inference, and to further characterize this enzyme. Fully understanding the enzymatic action and potential of pancreatic lipase will contribute to understanding the catabolism of lipid *in vivo* for this species and promote better formulations of balanced feeds, and may perhaps allow its utilization in industrial applications.

Acknowledgments: Funding for Mrs. Santana-Bejarano was provided by the Consejo Nacional de Ciencia y Tecnología (CONACYT-México). The authors would like to thank the Center for Reproduction of Marine Species of Sonora State (C.R.E.M.E.S.), Kino Bay, Sonora, Mexico, for donating the experimental fish. The mention of trademarks or proprietary products does not constitute an endorsement of the product and does not imply its approval to the exclusion of other products that may also be suitable.

Author contributions: Santana-Bejarano, M.Sc. contributed to all of the analytical determinations and data analysis. Dr. Perez-Velazquez procured the experimental organisms and contributed to their processing, estimation of biological indices, the editing of this manuscript and with financial support. Dr. Villalba-Villalba participated in the analytical determination of enzymatic activity. Dr. González-Félix participated in the SDS-PAGE analysis, the analysis of data, with the financial support, and with the writing of this manuscript.

Conflict of interest disclosure: The authors have no conflict of interest related to this work.

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