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Partial Characterization of Digestive Proteases in the Green Cichlid, *Cichlasoma beani*

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Abstract: This study undertakes the characterization of digestive proteases in the juvenile green cichlid, *Cichlasoma beani*. The results obtained showed a higher activity of alkaline proteases (0.14 ± 0.01 U mg protein^{-1}) compared to acid proteases (0.07 ± 0.01 U mg protein^{-1}) in this species. The optimum temperature of the alkaline proteases was 65 °C and these enzymes were more thermostable to temperature changes than the acid proteases, characterized by an optimal temperature of 55 °C. The pH optimum was 2 for acid proteases, and 11 for alkaline proteases, which were also more stable to changes in pH between 8 and 10. The use of specific inhibitors showed an acid protease inhibition of 88% with pepstatin A as inhibitor. In the zymogram SDS-PAGE analysis of alkaline proteases, five active fractions were revealed, indicating the presence of serine proteases. These results confirm that both alkaline and acid proteases are involved in the digestion of *C. beani*, and suggest that this species is omnivorous with carnivorous tendencies. The present study contributes to our knowledge about the digestive physiology of *C. beani*, and can be applied towards improved understanding of the kinds of protein sources that could be used in the development of inerts diets.

Keywords: *Cichlasoma beani*; digestive enzymes; inhibitors; proteases; SDS-PAGE

1. Introduction

The development of fish aquaculture in Mexico is mainly based on the culture of introduced species such as the Nile tilapia *Oreochromis niloticus*, despite the existence of a variety of native species with high potential for aquaculture [1]. It has recently become of interest to develop new cultures of species with regional commercial potential, in response to the depletion of wild populations, mainly caused by overfishing, pollution and habitat alteration [2].

The green cichlid or mojarra Sinaloa *Cichlasoma beani* is a euryhaline species with potential for farming in Northwest Mexico. In this context, a study by [3] focused on the in situ reproductive population structure and its relation to the ecology of this species. Prior studies examined the effects of stocking density on growth and survival of this species under culture conditions [4,5]. There are currently no studies on biochemistry and digestive physiology of this species, although studies have been conducted on digestive biochemistry in species of the genus *Cichlasoma* [6–8], which contribute

to the knowledge of the digestive physiology of these cichlids for studies related to other species and future references. Given the above consideration, understanding of species-specific digestive physiology in fish, i.e., the composition and factors that affect the activity of digestive enzymes, provides information useful for the formulation of diets, i.e., selection of the nutrients and the type of protein ingredient required for growth maximization. This depends on the enzymatic activity in the digestive system and knowledge of the species capacity to hydrolyze protein, the most costly component in fish diets [9–11]. Description of the operational parameters of digestive proteases is essential to understand their types and form of action, as well as their activity level. Determination of pH and temperature optima and stability to these factors, allows long-term maximization of enzymatic activity [12–14].

Studies of enzymatic characterization have been supplemented by the use of specific inhibitors in the application of biochemical and electrophoretic techniques, which affect the enzyme active centers and thus decrease enzymatic activity, allowing determination of the sensitivity and the mechanisms of action of digestive enzymes [11,13–15]. Due to the importance of the effect of inhibitors found in many ingredients (mainly vegetable), the characterization of digestive proteases have been studied in marine and freshwater fish species such as spotted sand bass *Paralabrax maculatofasciatus* [12], common snook *Centropomus undecimalis* [16], Atlantic bluefin tuna *Thunnus thynnus* [17], Asian bonytongue *Scleropages formosus* [18], Senegal sole *Solea senegalensis* [19], Nile tilapia *Oreochromis niloticus* [20], halibut *Scophthalmus maximus* [21], bay snook *Petenia splendida* [22], Albacore *Thunnus alalunga* [23], tropical gar *Atractosteus tropicus* [24] and three-spot cichlid *Cichlasoma trimaculatum* [25]. These studies have demonstrated that acidic proteases commonly have an optimal pH level of 2 and optimum temperatures between 35 and 65 °C. In contrast, most of the alkaline proteases have an optimal pH between 9 and 10, and optimum temperatures in the range of 45–65 °C. In some carnivorous species inhibition of the enzymatic activity of alkaline proteases are highly affected by inhibitors and can be reduced by 100%. Alkaline protease inhibition varies among species and can be influenced by the type of food item in the habitat [12]. Therefore, the main objective of this study was to characterize the digestive proteases in juvenile *C. beani*, and thus to generate basic information on this species' digestive physiology, namely the determination of the optimal conditions of pH and temperature of digestive proteases, their susceptibility to changes in these parameters, and the effects of specific inhibitors on enzymatic activity. This information will allow the selection of appropriate ingredients, and formulation of diets that meet the nutritional requirements of the species under culture conditions.

2. Results

The optimum pH for the activity of acid proteases was 2 (Figure 1A). With respect to changes in pH stability, the remaining enzyme activity was 56% for pH 3, while pH 4–7 had remaining activities around 15%–20%, starting at 90 min pre-incubation (Figure 1B). For alkaline proteases activity the pH optimum was 11 (Figure 1C). The changes in pH stabilities in alkaline proteases occurred at pH 8–11, reaching a stability around 60%–100% in 90 min of pre-incubation, while pH 12 showed a decrease in its residual activity until reaching to about 5% at 90 min of pre-incubation (Figure 1D).

The optimum temperature for acidic proteases was 55 °C (Figure 2A). The stability of the acidic proteases presented thermal variations around 60% at 55 °C, 50% at 45 °C, with the temperature of 65 °C showing a decrease of <10% at 90 min of pre-incubation (Figure 2B).The optimum temperature of alkaline protease activity increased rapidly above 35 °C, presenting two peaks of maximum activity at 45 and 65 °C (Figure 2C). Finally, residual alkaline protease activity decreased by 70% at 45 °C, while temperatures of 55 and 65 °C decreased approximately 35%–40% at 90 min of pre-incubation (Figure 2D).

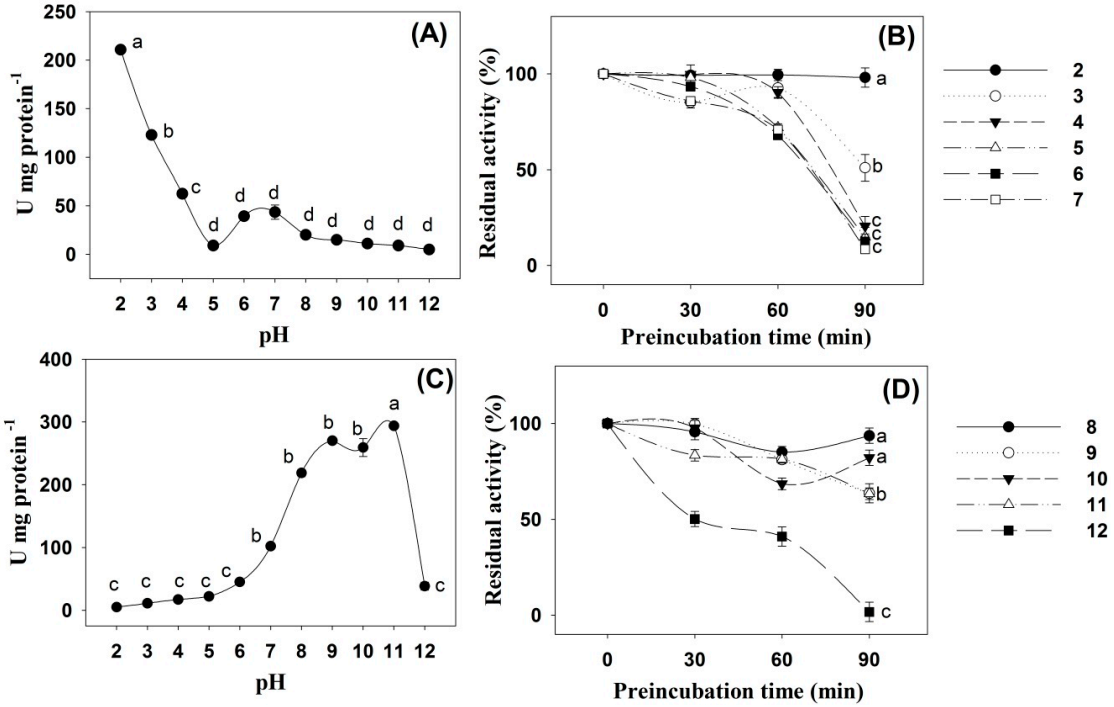

Figure 1. Effect of pH on the enzymatic activity of digestive proteases of juvenile *Cichlasoma beani* for the determination of: (**A**) optimal pH of acidic proteases; (**B**) pH stability of acidic proteases; (**C**) optimal pH of alkaline proteases; and (**D**) pH stability of alkaline proteases (mean ± SD, $n = 3$). Mean values denoted with unlike letters are statistically different, $p < 0.05$.

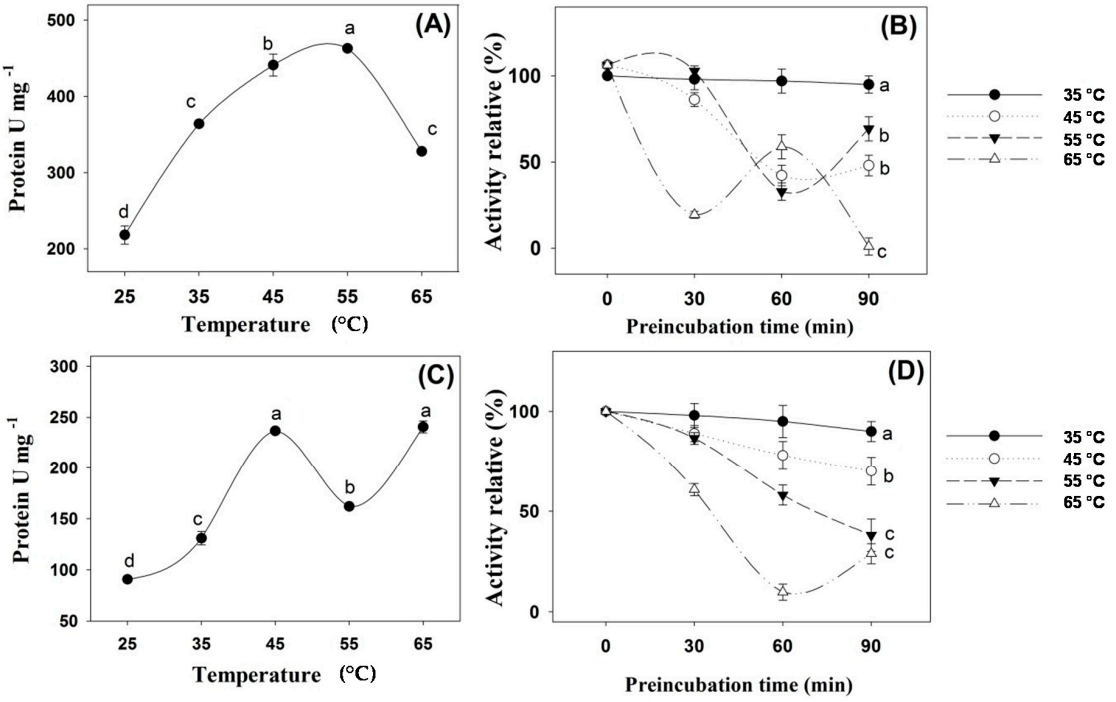

Figure 2. Effect of temperature on digestive proteases of juvenile of *Cichlasoma beani*: (**A**) optimum temperature of acidic proteases; (**B**) thermal stability of acidic proteases; (**C**) optimum temperature of alkaline proteases; and (**D**) thermal stability of alkaline proteases (mean ± SD, $n = 3$). Mean values denoted with unlike letters are statistically different, $p < 0.05$.

Analysis of the effects of inhibitors indicated that acid protease was inhibited by 85% by pepstatin A, a specific inhibitor of pepsin type A enzymes. Inhibitors of the activity of alkaline proteases showed an inhibition of 53% for TPCK, a specific inhibitor of chymotrypsin; inhibition of 74% for TLCK, a specific inhibitor of trypsin, 88% and 57% for Ovalbumin and SBT1, respectively, which inhibit serine proteases, a reduction of 87% and 55% for the metalo-protease inhibitors Phenanthroline and EDTA, respectively, and inhibition of 60% for PMSF, a general inhibitor of serine proteases (Figure 3).

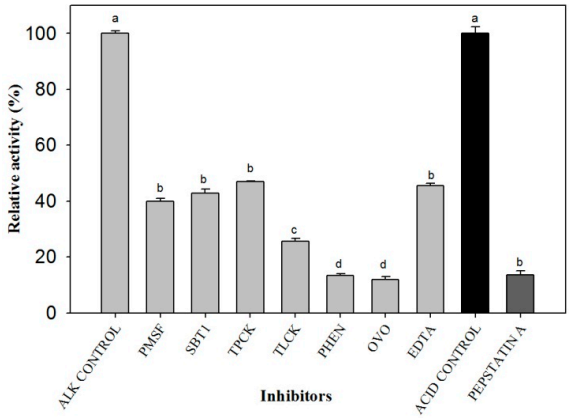

Figure 3. Residual activity (%) of digestive proteases using several inhibitors on multienzyme extracts of juvenile *Cichlasoma beani*. Alkaline proteases with no inhibitor (Alk control), tosylphenylanyl-chloromethyl ketone (TPCK), phenanthroline (PHEN), ethyl-diamine tetra-acetic acid (EDTA), tosyl-lysyl-chloromethyl ketone (TLCK), ovalbumin (OVO), soybean trypsin inhibitor (SBT1), phenyl methyl sulphonyl fluoride (PMSF), acidic proteases with no inhibitor (Acid control), pepstatin A (mean ± SD, $n = 3$). Columns with different letters show a significant difference ($p < 0.05$).

The analysis of SDS-PAGE zymograms for alkaline proteases revealed five active fractions in control without inhibitor (92.3, 60.1, 56.7, 38.2 and 35.4 kDa). Ovalbumin and TPCK inhibited three active fractions (92.3, 56.7 and 35.4 kDa), while PMSF and SBT1 inhibited only one active fraction (60.1 kDa). The TLCK inhibited three active fractions (92.3, 60.1 and 56.7 kDa). Phenanthroline and EDTA inhibited four active fractions (92.3, 60.1, 56.7 and 35.4 kDa, respectively) (Figure 4).

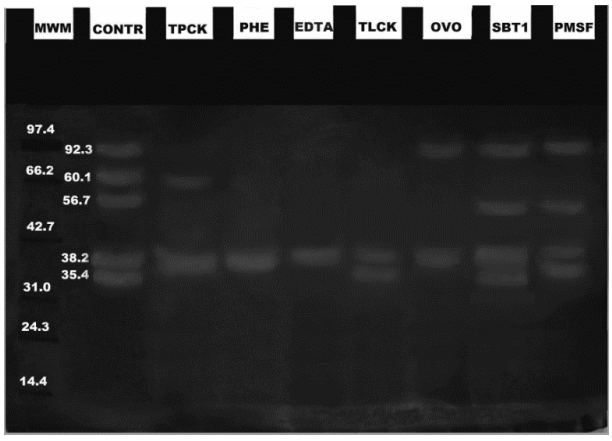

Figure 4. SDS-PAGE zymogram of alkaline digestive proteases of *Cichlasoma beani*: Molecular Weight Marker (MWM), rabbit phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbon anhydrase (31.0 kDa) and lysozyme (14.4 kDa); control (without inhibitor); inhibitors were the same as in Figure 3.

3. Discussion

Results obtained in the present study with the cichlid *C. beani*, show that this species exhibits a relatively high activity of digestive proteases, and that the alkaline protease activity was higher compared to that of acid proteases, which indicates that this species has omnivorous habits unlike other fish such as the Asian bonytongue *Scleropages formosus* [18] and turbot *Scophthalmus maximus* [21]. The latter two species have carnivorous habits characterized by high acid protease activities (>1500 UEA (Unit Enzymatic Activity) mg of protein^{-1}) in the stomach, the most important organ for the digestion of proteins, and a lower activity of alkaline proteases in the intestine (<250 UEA mg protein^{-1}), where fine hydrolysis of proteins to peptides and amino acids, absorbed by enterocytes, is performed [26].

In the present study, acid proteases reached their maximum activity at a pH level of 2, which is consistent with findings of the majority of prior studies for species such as Senegal sole *Solea senegalensis* [27], Blue disc *Symphysodon aequifasciata* [28], *Diplodus puntazzo* [29], coelacanth *Latimeria chalumnae* [30] *Nemipterus* spp. [31] and *Acipenser naccarii* [32]. The bay snook *Petenia splendida* and rainbow trout *Oncorhynchus mykiss* have optimum pH values of 5 and 4, respectively, uncommon for acid digestion [22,32]. For proteases the pH at which activity is maximized, is also that at which the enzymes' active centers have adequate ion formation to perform highly efficient catalysis [33].

In this study we found only a single active fraction of acid digestion using the inhibitor Pepstatin A. This protease acts first in protein hydrolysis under acidic conditions in the stomach, followed by digestion and nutrient absorption under alkaline conditions in the intestine. In *C. beani*, alkaline proteases showed a pH optimum of 11, which differed from the optimal pH (9 and 10) recorded for other marine and freshwater species, such as *Brycon orbignyanus* [26], *Symphysodon aequifasciata* [28]; *Catla catla*, *Labeo rohita* and *Hypophthalmichthys molitrix* [34]; *Thunnus orientalis* [35], *Ctenopharyngodon idella* [36], and *O. niloticus* × *O. aureus* [37], and *D. puntazzo* [29]. Some species, however, have been reported to exhibit different pH optimum; even in the same species two pH values of maximum activity may be recorded, as in *P. maculatofasciatus* [33] and *C. undecimalis* [16]. This is possible because alkaline proteases are composed of various types of enzymes, which have different pH optima [38]. Finding of a well-defined optimum pH in *C. beani* suggests a single type of enzyme or a major contribution of various types of enzymes that share the same pH optimum [35]. This could be the case of carboxypeptidase A, elastase or collagenase activities, which show maximum value in the pH range of 9–11 [35]. In *C. beani*, the greatest inhibition of enzymatic activity (55%) was found for phenanthroline and EDTA, which are specific inhibitor and chelating agent of metalloproteases.

Acid proteases are stable at acid pH and their activity decreases with increasing pH. In contrast alkaline proteases are stable at alkaline pH and are resistant to slightly acidic pH, conditions similar to those reported in the cichlid *P. splendida* [22]. In other marine fish species prone to carnivorous feeding behavior, however, acid proteases are stable for some time to alkaline pH, as reported for *T. thynnus* [17] and *S. maximus* [21]. This has also been found in omnivore marine species such as *S. senegalensis* [19] and carnivorous freshwater species such as *A. tropicus* [24]. From a physiological standpoint, the fact that acid proteases are resistant to alkaline pH, allows them to continue acting in the degradation of food when it passes into the intestine, until alimentary bolus alkalize, leads to hydrolysis by alkaline proteases. This could hardly occur in *C. beani* because acid proteases lose activity at a neutral or alkaline pH. This loss of activity is compensated, however, by the hydrolysis of alkaline proteases at an acidic pH, which is also compensated by a long intestine and strong sphincters that allow longer gut residence of the food, in addition to the joint action of digestive hormones such as cholecystokinin that causes peristalsis, increasing surface contact with the enzymatic substrate [33]. Thus, alkaline digestive proteases may start acting from the moment the food enters the intestine even at an acidic pH.

It is noteworthy that alkaline proteases are more stable at different pH values, because they are comprised by a greater number of isoforms (trypsin, chymotrypsin, L-aminopeptidase and

carboxypeptidase A). This is shown in the zymogram by the detection of five active fractions with activity relative to the control without inhibitor, which increases the possibility of hydrolyzing food proteins despite the pH variation [38]. Instead, acid proteases are often more susceptible to pH changes, since they only have an isoform corresponding to pepsin, which when subjected to different pH values, undergoes changes in its structural configuration or denaturation that leads to partial or total loss of activity [39]. These variations in digestive enzyme activity differ among species, depending on genetic makeup and habitat conditions. In this respect, the optimum temperature of alkaline proteases is typically 10 °C higher than the optimum temperature of acid proteases, and it is more thermostable. Usually, the difference between the optimum temperatures of alkaline and acid proteases have been described in carnivorous fish species such as *T. thynnus* [17], *B. orbignyanus* [26] and *P. maculatofasciatus* [33]. The optimum temperature of acid proteases in *C. beani* was 55 °C, comparable to that described in the hybrid *O. niloticus* × *O. aureus* [37], but higher than that found for *S. senegalensis* [19], *T. orientalis* [35] and *C. trimaculatum* [8], with temperature optima from 35 to 55 °C. The activity of acid proteases is stable up to 45 °C, resulting in values >100% for some of the incubation times, but over 60% of the activity is lost above 55 °C. This could be due to denaturation of pepsin at temperatures >55 °C, in agreement with results described by [32]. In contrast, alkaline proteases have an optimal temperature of 65 °C, which is greater than that reported for the crevalle jack *Caranx hippos*, spotted goatfish *Pseudupeneus maculatus*, parrotfish *Sparisoma* sp., trahira *Hoplias malabaricus* [40] and *O. niloticus* [20], species that show an optimum temperature ranging from 50 to 55 °C. The high thermostability of the alkaline proteases in *C. beani* is similar to that reported for *C. undecimalis* [16], *A. tropicus* [24] and *C. trimaculatum* [25]. It is noteworthy that the temperature optima and the thermal stability of the digestive proteases are only operational parameters of enzymes, rather than physiologically relevant results, since activities may vary with enzyme configuration, habitat, environment and genetic aspects of the species [30,32,38]. This deficiency in activity is compensated, however, by a longer retention of food in the digestive system to perform the hydrolysis of proteins at lower temperatures than optimal [10,32,39,41]. Determination of the number of isoforms that constitute acid proteases in *C. beani* was performed using the specific inhibitor pepstatin A, which inhibited the activity of acidic proteases by 86%. This reduction is lower than that found in carnivorous species such as *S. maximus* [21], the Mandarin fish *Siniperca chuatsi* [42] and Skipjack tuna *Katsuwonus pelamis* [39], in which the purified pepsin enzyme was completely inhibited by pepstatin A, thus indicating that acid hydrolysis is carried out mainly by a pepsin-type enzyme.

The seven types of specific inhibitors reduced the activity of alkaline proteases by at least 53%. Of these, the group of serine proteases was inhibited by 60% by Phenyl methyl sulphonyl fluoride (PMSF), and by 88% and 57% with ovalbumin and soybean trypsin inhibitor (SBT1), respectively. This pattern in which serine proteases were more markedly affected than the rest of the enzymes was similar to findings in *S. aequifasciata* [28], *T. thynnus* [17], *C. idella* [36] and *C. trimaculatum* [8]. The other enzyme group subject to high metal inhibition was that of the proteases, with 87% inhibition by Phenanthroline and 55% by ethyl-diamine tetra-acetic acid (EDTA). This indicates that via alkaline digestion *C. beani* has the ability to hydrolyze proteins from the carboxyl and amino ends, thus releasing amino acids. This must be considered in diet formulation to determine the composition of ingredients that do not contain inhibitors of metalloproteases or serine proteases. The tosylphenylanyl-chloromethyl ketone (TPCK) was inhibited 53% of chymotrypsin activity and tosyl-lysyl-chloromethyl ketone (TLCK) inhibited 74% of trypsin activity. This result was similar to that found in *B. orbignyanus* [26] and *S. formosus* [18], in which greater inhibition of trypsin than of chymotrypsin by TLCK was documented. Thus, it is confirmed the determination of the types of enzymes that comprise the digestive proteases of a species using specific inhibitors allows identification of dietary ingredients that may reduce the activity of specific enzymes and thus limit feed digestibility [17].

Specific inhibitors of alkaline proteases was performed on SDS-PAGE, five active fractions were detected in the control well, differentiating two groups of isoforms: the first group of three active fractions with molecular weights between 92.3, 60.1 and 56.7 kDa, and the second group of two active

fractions with molecular weights of 38.2 and 35.4 kDa. The number of active fractions found in *C. beani* was similar to that reported in marine species such as *P. maculatofasciatus* [33], and freshwater species such as *P. splendida* [22], *C. undecimalis* [16], and *A. tropicus* [24]. In other omnivorous species such as the South American pilchard *Sardinops sagax caerulea* [43] and round sardinella *Sardinella aurita* [44], trypsin showed active fractions with different molecular weights ranging between 35 and 38.8 kDa, while purified chymotrypsin from *S. sagax caerulea* had a molecular weight of 35.5 kDa [45]. The inhibitors SBT1 and PMSF, specific for serine protease, presented the highest percentages of inhibition as they eliminated three active fractions in *C. beani*, which could be trypsin-like or chymotrypsin-like enzymes [42,44–46]. On the other hand the inhibitors specific for metalloproteases phenanthroline and EDTA, inhibited two active fractions in the first group of enzymes. Therefore, future studies will be conducted with purified enzymes to determine the specific type of enzymes present in *C. beani*.

4. Materials and Methods

4.1. Sample Preparation

Samples were obtained from juveniles donated by the Universidad Autónoma de Nayarit, with an average wet weight of 28.71 ± 5.35 g (n = 35), which were euthanized with an overdose of methanesulfonate tricaine (MS-222, Sigma-Aldrich, Saint Louis, MI, USA). Weight and total body length were recorded for each juvenile and then the stomachs and intestines were extracted with ice; the stomachs and intestines were homogenized separately in a 200 mg mL^{-1} ratio, with 100 mmol L^{-1} glycine-HCl buffer at pH 2 for the stomachs and buffer Tris-HCl 12.5 mmol L^{-1} + $CaCl_2$ L^{-1} 30 mmol L^{-1} at pH 7.5, for the intestines respectively. The homogenate was centrifuged at 16,000× g for 30 min at 4 °C; the multienzyme extract thus obtained was stored at −80 °C for further analysis. The concentration of soluble protein in the multienzyme extracts of stomach and intestine was determined using the [47] technique and using bovine serum albumin as standard.

4.2. Biochemical Analyses

For measurement and determination of the pH and temperature optima, and enzymatic stability as well as inhibition assays, the enzymatic extracts of ten stomachs were used, and for the alkaline proteases enzymatic extracts were used from ten juvenile intestines respectively.

The total activity of the acid proteases was determined using 1% hemoglobin in a glycine-HCl 100 mM buffer at pH 2 following the method of [48]. The activity of the alkaline proteases was evaluated following the method of [25], as modified by [49], using 1% casein in a Tris-HCl 100 mmol L^{-1} + $CaCl_2$ 10 mmol L^{-1} buffer at pH 9. One unit of activity was defined as 1 µg of tyrosine liberated per minute with a molar extinction coefficient (MEC) of 0.005 mL^{-1} μg^{-1} cm^{-1}.

4.3. Effects of pH and Temperature on Digestive Enzyme Activity

The optimum pH of the acid and alkaline proteases was evaluated using a universal buffer [50] with a pH range of 2–12 following the previously described procedure to determine protease activity. The optimum temperature of the acid and alkaline proteases was established by testing different temperatures (35, 45, 55, 65 °C). The effect of pH and temperature on the stability of the acid and alkaline proteases was determined by pre-incubating the multienzyme extracts for 0, 30, 60 and 90 min with a buffer at the assay temperature [50]. For determination of the stability to pH and temperature the pre-incubation time 0 min value was used as a control to determine the residual activity expressed as a percentage of residual activity comparing with the extracts that were incubated at different pH and temperatures values.

4.4. Zymograms and Effects of Inhibitors

The enzymes in the multienzyme extracts were characterized following the method described by [51]. Pepstatin A 1 mmol L^{-1} was used as inhibitor in the case of acid proteases, and the following inhibitors were used for the alkaline proteases: phenyl-methanesulphonyl fluoride 100 mmol L^{-1} (PMSF), *N-q*-tosyl-llysine-chloromethyl ketone hydrochloride 10 mmol L^{-1} (TLCK), soybean trypsin inhibitor 250 mmol L^{-1} (SBT1), *N-q*-tosyl-L-phenylalanine chloromethyl ketone 10 mmol L^{-1} (TPCK), 1,10-phenanthroline 10 mmol L^{-1} (Phen), trypsin inhibitor, type II-T: turkey egg ovoalbumine 250 mmol L^{-1} (Ovo) and ethylene diamine tetra acetic acid 10 mmol L^{-1} (EDTA). Extracts were incubated in the presence of each inhibitor for 1 h (1:1 v/v) at pH 7.5, the value of intestine extracts, and at pH 2 for stomach extracts during sample preparation. In both tests a control without inhibitor was used as 100% of total activity and the residual activity was expressed as a percentage of reduction of the activity with the different inhibitors. All assays were conducted in triplicate.

The effect of inhibitors on the alkaline proteases was performed by SDS-PAGE electrophoretic analysis using a discontinuous polyacrylamide gel, which included a 4% stocking gel and 10% stocking gel, according to the methods of [51], as modified by [26]. The seven inhibitors: TPCK, PHEN, EDTA, TLCK, Ovo, SBT1, PMSF, described by [15] were used. They were pre-incubated in a 1:1 ratio (enzyme: inhibitor) for 1 h. The molecular weight marker Bio Basic Inc., BM523 and the program Quantity One 1-D Analysis Software de Bio-Rad® were used to calculate the molecular weight of each active fraction.

4.5. Statistical Analysis

All the enzymatic activities of pH and optimum temperature and stabilities, as well as the inhibitors were carried out in triplicate and were evaluated by means of non-parametric statistical analysis of Kruskal-Wallis test and a posteriori Nemenyi test as the data were not normally distributed. Statistical tests were conducted with the Statistica software v. 7.0 (Statsoft, Tulsa, OK, USA) using a significance value of 0.05.

5. Conclusions

In conclusion, results of the present study show that digestion in *C. beani*, as inferred from enzymatic activity, is carried out either by acid proteases or alkaline proteases; a pepsin-like enzyme is responsible for most of the hydrolysis, but it shows little resistance to pH and temperature. In contrast, digestion of alkaline proteases is more stable to these parameters. These biochemical conditions allow *C. beani* to hydrolyze proteins in formulated foods. Results of the present study also suggest that *C. beani* is an omnivorous species, with some tendency to carnivore, results which can be used to guide the development of specific diets for culture.

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