

Original Article

## Obtainment and characterization of digestive aspartic proteases from the fish *Caranx hippos* (Linnaeus, 1766)

Obtenção e caracterização de proteases aspárticas digestórias do peixe *Caranx hippos* (Linnaeus, 1766)

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### Abstract

This work aimed to obtain aspartic proteases of industrial and biotechnological interest from the stomach of the crevalle jack fish (*Caranx hippos*). In order to do so, a crude extract (CE) of the stomach was obtained and subjected to a partial purification by salting-out, which resulted in the enzyme extract (EE) obtainment. EE proteases were characterized physicochemically and by means of zymogram. In addition, the effect of chemical agents on their activity was also assessed. By means of salting-out it was possible to obtain a purification of 1.6 times with a yield of 49.4%. Two acid proteases present in the EE were observed in zymogram. The optimum temperature and thermal stability for EE acidic proteases were 55 °C and 45 °C, respectively. The optimum pH and pH stability found for these enzymes were pH 1.5 and 7.0, respectively. Total inhibition of EE acid proteolytic activity was observed in the presence of pepstatin A. dithiothreitol (DTT) and Ca<sup>2+</sup> did not promote a significant effect on enzyme activity. In the presence of heavy metals, such as Al<sup>3+</sup>, Cd<sup>2+</sup> and Hg<sup>2+</sup>, EE acidic proteases showed more than 70% of their enzymatic activity. The results show that it is possible to obtain, from the stomach of *C. hippos*, aspartic proteases with high proteolytic activity and characteristics that demonstrate potential for industrial and biotechnological applications.

**Keywords:** waste recovery, fish enzymes, marine fish, aspartic proteases.

### Resumo

Este trabalho objetivou obter proteases aspárticas de interesse industrial e biotecnológico a partir do estômago do peixe xaréu (*Caranx hippos*). Para isso, foi obtido um extrato bruto do estômago, o qual foi submetido a uma purificação parcial por salting-out onde se obteve o extrato enzimático (EE). As proteases do EE foram caracterizadas físico-quimicamente e através de zimograma. Além disso, o efeito de agentes químicos sobre sua atividade também foi avaliado. Através de salting-out foi possível obter uma purificação de 1,6 vezes com rendimento de 49,4%. Foram observadas duas proteases ácidas presentes no EE através de zimograma. A temperatura ótima e a estabilidade térmica para as proteases ácidas do EE foram de 55 °C e 45 °C, respectivamente. O pH ótimo e a estabilidade ao pH encontrados para estas enzimas foram o pH 1,5 e 7,0, respectivamente. Observou-se a inibição total da atividade proteolítica ácida do EE na presença de pepstatina A. O ditiotreitolo (DTT) e o Ca<sup>2+</sup> não promoveram efeito significativo na atividade enzimática. Na presença de metais pesados, como Al<sup>3+</sup>, Cd<sup>2+</sup> e Hg<sup>2+</sup>, o EE manteve mais de 70% de atividade enzimática do EE. Os resultados mostram que é possível obter, a partir do estômago de *C. hippos*, proteases aspárticas com alta atividade proteolítica e características que demonstram potencial para aplicações industriais e biotecnológicas.

**Palavras-chave:** aproveitamento de resíduos, enzimas de peixe, peixes marinhos, proteases aspárticas.

### 1. Introduction

The crevalle jack fish, *Caranx hippos* (Linnaeus, 1766), is a migratory species that belongs to the family Carangidae. They are predatory animals, feeding on smaller fish and some invertebrates, and when young they prefer

zooplankton and crustaceans (Luque and Alves, 2001). This species has a worldwide distribution and can easily be found in the American continent from the Caribbean to the coast of Uruguay. Hence, it is found throughout the Brazilian coastline (Costa et al., 2013). According to

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the latest report on extractive fishing and aquaculture carried out in Brazil (Brasil, 2011), the average national production of crevalle jack fish (*C. hippos*) between 2009 and 2011 was 2,509.1 ton, with no great variation in production during this period.

As fresh fish is very perishable foods and due to the current market requirements, most of the fish produced for human consumption receives some kind of processing before being commercialized. Consequently, a large amount of processing residues is produced, such as scales, skins, bones and viscera that are often discarded *in natura* in the environment, causing great pollution (Freitas-Júnior and Bezerra, 2015).

There is a growing need for the discovery of alternative sources for obtaining industrial enzymes and, on account of that, several studies have been carried out to use the viscera of tropical fish captured or produced in Brazil, including *C. hippos*, to obtain these molecules. Such studies have shown that these byproducts are important and low-cost sources for obtaining alkaline digestive peptidases with industrial and biotechnological potential (Espósito et al., 2010; Freitas-Júnior, et al., 2012; Costa et al., 2013; Medeiros et al., 2015; França et al., 2016). However, the obtaining of acidic proteases from the processing residues of these and other species commercialized in the country has been poorly studied.

Acidic proteases, mainly pepsin, in addition to being used in the food industry for the production of protein hydrolysates and dairy products, can also be used to optimize collagen extraction and the production of gelatine from fish (Zhao et al., 2011; Bkhairia et al., 2016). Pepsins and pepsinogens with industrial potential have been isolated and characterized from the gastric mucosa of several fish species (Zhao et al., 2011). However, the yield and the high costs of these purification processes still hinder the use of fish enzymes with a high degree of purity (Freitas-Júnior et al., 2012). However, many industrial applications, such as those mentioned above, do not need to use such pure enzymes (Freitas-Júnior and Bezerra, 2015). Recently, some researchers have shown the potential of using crude or partially purified enzymatic extracts, obtained from the fish stomach, to obtain gelatin and fish collagen (Bkhairia et al., 2016; Acevedo Gomez et al., 2018).

In view of this, the objective of this work was to evaluate the stomach potential of *C. hippos* fish as a source for obtaining acidic proteases with industrial and biotechnological potential.

## 2. Material and Methods

### 2.1. Obtaining crevalle jack stomachs and crude extract (CE)

The stomachs of *Caranx hippos* were donated by fishmongers in the metropolitan region of the city of João Pessoa, the State of Paraíba, Brazil, and taken under refrigeration at 4 °C to the Laboratório de Biomoléculas de Organismos Aquáticos (BIOAQUA) at Universidade Federal da Paraíba. In the laboratory, the stomachs were weighed, cut and frozen in liquid nitrogen. After freezing the pieces

were crushed and homogenized with 0.15 M NaCl solution in the proportion of 1:10 (g/mL). Then, the homogenate was centrifuged for 20 minutes, at 4 °C, at 10,000 g, and the collected supernatant was called crude extract (CE). CE was stored at -20 °C until further use.

### 2.2. Enzyme activity assay and soluble protein dosage

The acidic proteolytic activity was determined according to Pavlisko et al. (1997), using as substrate a 2% (w/v) hemoglobin solution in 0.03 M HCl. The result was determined in a Multiskan GO microplate reader (Thermo®). One unit of enzymatic activity was determined to be the amount of enzyme capable of promoting a change in absorbance, at 280 nm, of 0.1 per minute of reaction. The concentration of soluble proteins was determined using the method of Bradford (1976), using bovine serum albumin as a standard.

### 2.3. Activation of zymogens

The activation of zymogens, such as pepsinogen, present in CE, was carried out by acidifying it to pH 2.0 by adding 6 M HCl. Afterwards, the CE was incubated for 60 min. at 25 °C. Subsequently, the CE was centrifuged at 10,000 g, at 4 °C, for 10 min., and the supernatant was collected at the end.

### 2.4. Partial purification of acidic proteases present in acidified CE

The acidified CE was subjected to saline precipitation by the addition of ammonium sulfate until 60% saturation. After 2 h of incubation at 4 °C, at rest, the mixture was centrifuged at 8,000 g, at 4 °C, for 20 min. After centrifugation, the precipitate was collected, resolubilized in 0.15 M NaCl solution (pH 2.0) and dialyzed with the same solution for 24 h at 4 °C. The 0-60% dialyzed fraction was stored and called Enzyme Extract (EE).

### 2.5. Zymogram

The number of acidic proteases in EE was determined by zymogram using the methodology described by Garcia-Carreño et al. (1993), with modifications, in which after the gel electrophoresis and polyacrylamide run, it was washed in distilled H<sub>2</sub>O and incubated in 0.03 M HCl for 5 min. at 4 °C. Then the gel was incubated with a 2% hemoglobin solution in 0.03 M HCl for 1 h and 30 min at 37 °C. After incubation, the gel was washed in distilled H<sub>2</sub>O and stained with Comassie blue R-250 solution.

### 2.6. Physicochemical characterization of EE acidic proteases.

The effects of pH and temperature on the proteolytic activity of EE were evaluated using the methodology described by Acevedo Gomez et al. (2018), with minor modifications. To determine the optimal temperature and thermal stability, the temperature range of 25 to 70 °C was used. The optimal pH and pH stability determination were performed using the 0.2 M glycine-HCl (pH 1.5 to 2.5) and citrate-phosphate (pH 3.0 to 7.0) buffers. The stability tests were carried out for 30 minutes.

### 2.7. Effects of chemical agents on the activity of EE acidic proteases

The effect of chemical agents on enzyme activity was carried out according to Acevedo Gomez et al. (2018) using a 10 mM solution of aluminum ( $\text{Al}^{3+}$ ), copper ( $\text{Cu}^{2+}$ ), magnesium ( $\text{Mg}^{2+}$ ), lead ( $\text{Pb}^{2+}$ ), manganese ( $\text{Mn}^{2+}$ ), calcium ( $\text{Ca}^{2+}$ ), sodium ( $\text{Na}^+$ ), mercury ( $\text{Hg}^{2+}$ ), cadmium ( $\text{Cd}^{2+}$ ) chlorides; the specific inhibitor of aspartic proteases, pepstatin A (100  $\mu\text{g}$  / mL) and the reducer dithiotreitol (DTT) 1 mM.

### 2.8. Effect of NaCl

The effect of NaCl on the proteolytic activity of EE was evaluated by incubating, for 30 min., at 25 °C, aliquots of the EE, in the proportion of 1:1 (v/v), with NaCl solution in different concentrations (10, 20 and 30%) (w/v), for 30 min. Residual activity was determined at 37 °C and for 10 minutes, using 2% hemoglobin as substrate.

### 2.9. Statistical analysis

All the aforementioned analyzes were performed in triplicate ( $n = 3$ ). All data were analyzed using ANOVA analysis of variance and Tukey's test. The software used was OriginPro® 8.0 and all tests were performed with a significance level of  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Partial purification of the Crude Extract

The CE obtained from the stomach of *C. hippos* showed a total specific activity of 439  $\text{mU}\cdot\text{mg}^{-1}$  (table 1). Through a simple step, it was possible to achieve a purification of 1.6 times with a yield of 49.4%. The use of a simple step for partial purification of the crude extract and concentration of enzymes is an economic advantage for the commercial use of this material in industrial processes that do not demand a high degree of purity of the enzymes, such as in the extraction of collagen and production of fermented fish. Acevedo Gomez et al. (2018), through the same process, obtained a yield of 45.1% and a purification rate of 1.8 times for aspartic proteases from the stomach crude extract of the fish *Prochilodus lineatus* (Valenciennes, 1836).

### 3.2. Zimogram

It was possible to observe the presence of two acidic proteases, with bands of similar density and closely resembling characteristics of molecular mass and charge in EE (Figure 1). Cao et al. (2011), Wu et al. (2009), and

Zhou et al. (2008) found more than two acidic proteases in the stomachs of the fish *Lateolabrax japonicus* (Valenciennes, 1828), *Anguilla anguilla* (Linnaeus, 1758) and *Siniperca chuatsi* (Basilewsky, 1855), respectively. However, Bkhairia et al. (2016) observed only one protease in the stomach crude extract zymogram of the of *Liza aurata* (Risso, 1810).

### 3.3. Effect of temperature

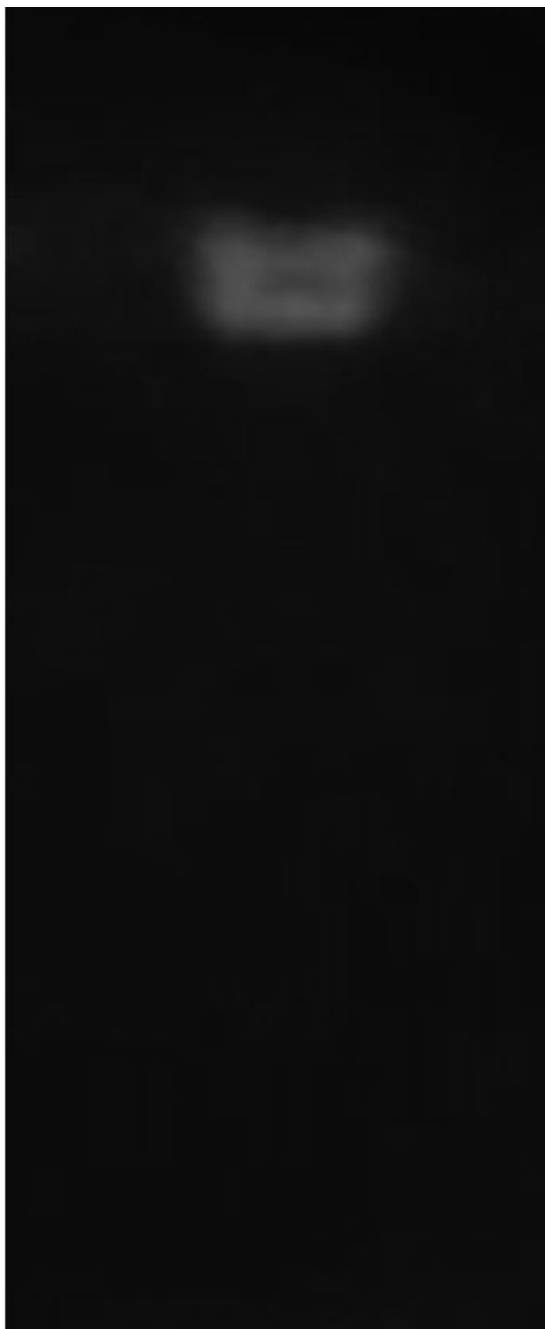
Fish are ectothermic animals, that is, they depend on the environment to maintain their body temperature. Therefore, the temperature is an important parameter to be investigated, given the diverse distribution of these animals across the globe. The optimum temperature determined for the maximum activity of EE acidic proteases was 55 °C (Figure 2A). However, at a 60 °C temperature, the fraction maintained approximately 50% of its proteolytic activity, which suggests the possibility to use them in industrial processes that involve mild to moderate heating. Zhao et al. (2011) state that the pepsin optimum temperature of different fish species range from 35 °C to 55 °C, and that there is a direct relationship between the optimum temperature and the water temperature where these species inhabit. Bkhairia et al. (2016) characterized an acidic protease in the stomach crude extract of *L. aurata* and found the optimum temperature of 40 °C. The same result was found by Bougateg et al. (2008) for *Mustelus mustelus* (Linnaeus, 1758) pepsins. Acevedo Gomez et al. (2018) obtained digestive aspartic proteases from *P. lineatus* and determined their optimum temperature to be 45 °C. The same result was found for acidic proteases present in the stomach crude extract of *Pangasianodon gigas* (Chevey, 1931) (Vannabun et al., 2014) and for pepsins from *Coryphaenoides pectoralis* (Gilbert, 1892) (Klomklao et al., 2007). Pepsins from the stomach of *Sparus latus* (Houttuyn, 1782) showed optimum temperature of 45 °C and 50 °C (Zhou et al., 2007). Similar results were obtained for pepsins from *Micropterus salmoides* (Lacepède, 1802) (Miura et al., 2015) and (40 and 50 °C) and *Thunnus alalunga* (Bonnaterre, 1788) (50 °C) (Nalinan et al., 2010). The same result was found for acidic proteases present in the stomach crude extract of *Paralichthys orbignyanus* (Valenciennes, 1839) (Candiotto et al., 2018).

In the same way, it is important to determine the optimum temperature of these enzymes, it is also necessary to establish their thermal stability in order to use this data in the optimization of their storage, transport and handling conditions, maintaining their native conformation preserved. The EE proteases were stable in the temperature

**Table 1.** Purification of acidic digestive proteases from the stomach of *C. hippos* fish.

Process step	Total Activity (U)	Total Protein (mg)	Specific activity ( $\text{mU}\cdot\text{mg}^{-1}$ )	Purification (fold)	Yield (%)
Crude Extract	1,546 ± 50	3,523 ± 102	439	1	100
EE	764 ± 36	1,103 ± 75	693	1.6	49.4

\* Parameters established for the process yield using 1L of crude extract. One unit of enzyme activity was considered to be the amount of enzyme capable of modifying the absorbance in 0.1 per minute of reaction.



**Figure 1.** EE Zymogram .

range of 25 °C to 45 °C (Figure 2B). Similar results (25 °C to 40 °C) have been reported for acidic digestive proteases of *L. aurata* (Bkhairia et al., 2016) and *P. orbignyanus* (Candiotta et al., 2018) which were stable up to 40 °C. The same result was observed for pepsins from *Sardinella aurita* (Valenciennes, 1847) (Khaled et al., 2011) and from *C. pectoralis* (Klomklao et al., 2007). Acevedo Gomez et al. (2018) observed that *P. lineatus* digestive aspartic proteases were stable up to 37 °C. The *T. alalunga* and *M. mustelus* pepsins were stable up to 50 °C (Bougatef et al., 2008; Nalinanon et al., 2010).

### 3.4. Effect of pH on enzyme extract activity and stability

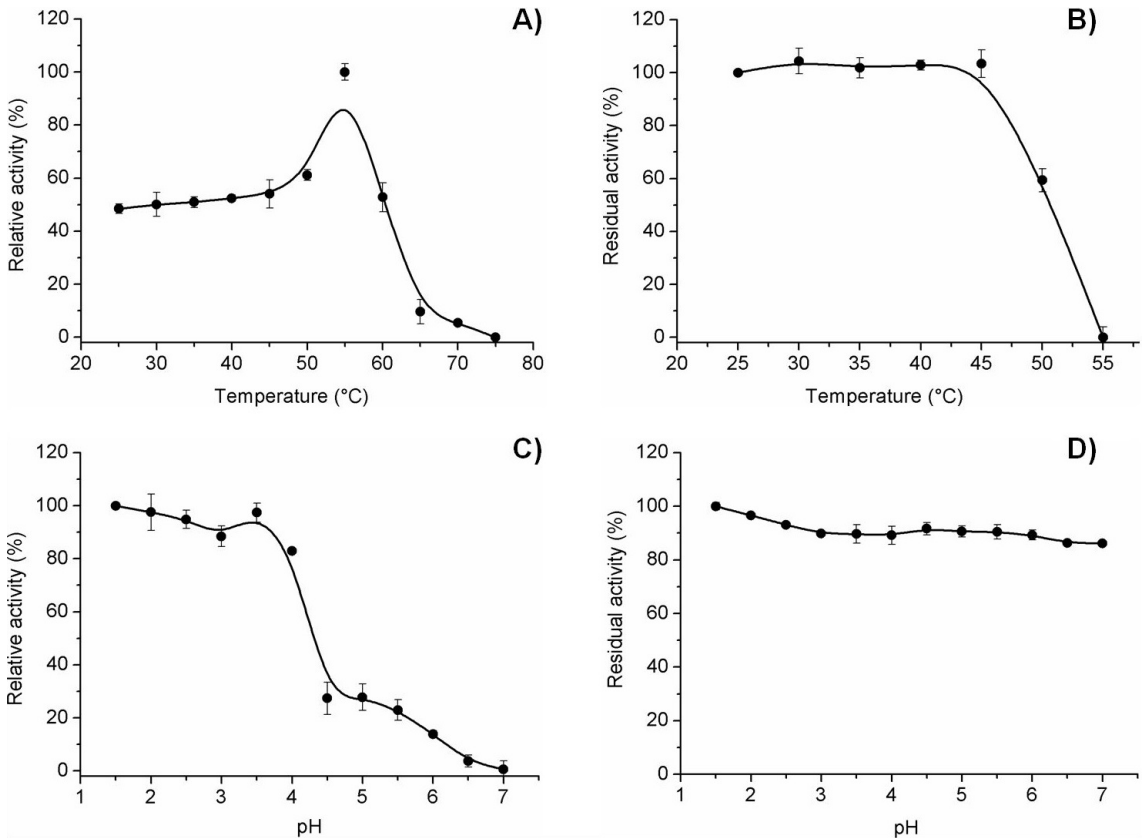
Knowledge of the optimum pH of an enzyme, that is, the pH in which the enzyme has greater catalytic activity, enables the determination of its potential industrial applications, in addition to providing insights on how to preserve its activity during storage and transportation in solution. The effect of pH on EE catalytic activity was determined and is shown in Figure 2C. The maximum activity was observed at pH 1.5. However, at pH 4.0 the proteolytic activity was greater than 80% demonstrating an optimum pH range between pH 1.5 and 4.0. Similar results were described by Miura et al. (2015) for two *M. Salmoides* pepsins (pH 1.5 and 2.0), for *P. lineatus* (Acevedo Gomez et al., 2018) and *P. orbignyanus* (Candiotta et al., 2018) digestive acidic proteases (pH 2.0), Bougatef et al. (2008) for *M. mustelus* pepsins (pH 2.0), Nalinanon et al. (2010) for *T. alalunga* pepsins (pH 2.0) and Tanji et al. (2007) for *Latimeria chalumnae* (Smith, 1939) pepsins (pH 2.0). Bkhairia et al. (2016) determined the optimum pH for *L. aurata* digestive acidic protease activity as pH 3.0, similarly to the report by Vannabun et al. (2014) for *P. gigas* digestive acidic proteases and by Khaled et al. (2011) for *S. aurita* pepsin. Klomklao et al. (2007) and Zhou et al. (2007) determined the maximum activity of *C. pectoralis* and *S. latus* pepsins, respectively, at pH of 3.5.

The pH stability profile is shown in Figure 2D. The enzymes, after a 30-minute incubation, maintained more than 80% of activity in the pH range 1.5 to 7.0. Since these are acidic proteases, they are not expected to be stable at pH far from their optimum pH. However, the proteolytic activity of EE was stable in the pH ranges between 1.5 and 7.0. Bkhairia et al. (2016) report that fish enzymes that inhabit warmer waters can be stable even at neutral pH. The same authors observed a similar result for the digestive acidic protease of *L. aurata*, which was stable in the range of pH 3.0 to 7.0. However, in the study by Klomklao et al. (2007), *C. pectoralis* pepsins were stable between pH 2.0 and 6.0. A similar result was reported by Nalinanon et al. (2010), in which *T. alalunga* pepsins were stable in a range of pH 2.0 to 6.0. Bougatef et al. (2008) characterized the pepsins of *M. mustelus* and observed that they remained stable only in the pH range between 1.0 and 4.0.

The effect of pH on *C. hippos* digestive acidic proteases indicates the possibility of storing EE in slightly acidic to neutral pH without proteolysis or loss of the native structure of these enzymes. In addition, the fact that these enzymes are very active at pH 4.0 indicates the possibility of their use in industrial and biotechnological processes that use weak acids, as in the extraction of collagen where acetic acid is used.

### 3.5. Effect of chemical agents on EE acidic proteases

The effect of some chemical agents on the EE acidic proteolytic activity is shown in Table 2. The effect of the specific aspartic protease inhibitor (which includes pepsin), pepstatin A, was evaluated on the activity of the enzymes present in the EE. Total inhibition of acidic proteolytic activity was observed in the presence of this inhibitor. According to Motwani et al. (2015), pepstatin A binds to the active enzyme site and prevents the



**Figure 2.** pH and temperature effect on EE acidic proteases. (A) Optimal temperature; (B) Thermal stability; (C) Optimal pH; (D) pH stability.

**Table 2.** Effect of enzyme inhibitors and metal ions on the proteolytic activity of EE.

Chemical Agent	Residual Activity (%)	Inhibition (%)
Control	100 ± 4.19 <sup>a</sup>	0
DTT	104 ± 3.25 <sup>b</sup>	0
Pepstatin A	0 <sup>c</sup>	100
Pb <sup>2+</sup>	85 ± 0.10 <sup>d</sup>	15
Mg <sup>2+</sup>	87 ± 3.35 <sup>e</sup>	13
Al <sup>3+</sup>	89 ± 1.20 <sup>f</sup>	11
Mn <sup>2+</sup>	90 ± 2.05 <sup>g</sup>	10
K <sup>+</sup>	91 ± 2.55 <sup>h</sup>	9
Hg <sup>2+</sup>	94 ± 0.72 <sup>i</sup>	6
Cd <sup>2+</sup>	97 ± 2.16 <sup>j</sup>	3
Cu <sup>2+</sup>	98 ± 5.51 <sup>k</sup>	2
Ca <sup>2+</sup>	101 ± 1.08 <sup>l</sup>	0

\*Different superscript letters mean statistical differences in relation to control, according to the Tukey test ( $p < 0.05$ ).

formation of the enzyme-substrate complex. Thus, the result of this test indicates that EE acidic proteases belong to the class of aspartic proteases, as well as pepsins, and because they are stomach enzymes, they are probably of the pepsin-like type. Similar results were found in studies using crude, or partially purified, stomach extracts from fish, in which a high inhibition of acidic proteolytic activity was observed (Bkhairia et al., 2016; Acevedo Gomez et al., 2018). Fish pepsins are strongly inhibited by pepstatin A (Klomklao et al., 2007; Wu et al., 2009; Nalinanon et al., 2010; Cao et al., 2011). The reducing agent dithiotreitol (DTT) did not promote significant changes in the proteolytic activity of EE, which indicates that these aspartic proteases present no disulfide bridges in their native structure.

It is widely known that calcium interacts with some proteins, enhancing its stability. The interaction of calcium with proteases has been reported in several studies, but the stabilization mechanism is not yet fully understood (Kotorman et al., 2003). However, for *C. hippos* digestive aspartic proteases, Ca<sup>2+</sup> did not promote a significant effect on the tested concentration. The proteolytic activity of *L. aurata* digestive acidic protease was also not affected by Ca<sup>2+</sup> and Bkhairia et al. (2016) stated that this finding presents a factor for a potential use of the enzyme in food processing, such as in the production of dairy products. All other metals tested promoted mild EE activity inhibition, which varied between 3% and 15%. The way

in which the activity of digestive proteases decreases in the presence of some metal ions is not fully understood. However, it is suggested that these metals may influence the interaction of the enzyme with the substrate at the active site (Sila et al., 2012) or they bind to other regions forming salts with these proteins leading to denaturation. The resistance to the presence of these elements is an interesting characteristic of *C. hippos* acidic proteases that could be used without the need to remove metals present in the process.

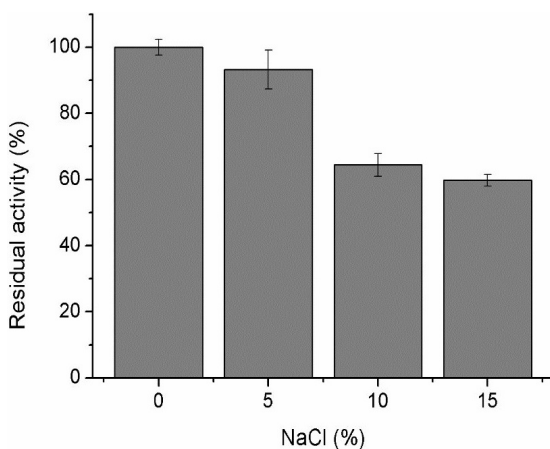
Acevedo Gomez et al. (2018) reported that the addition of  $Mg^{2+}$  stimulated the activity of *P. lineatus* digestive aspartic proteases, increasing the activity by approximately 10%, while in crevalle jack this ion inhibited 13% of the EE acidic proteolytic activity. Klomklao et al. (2007) showed that the activity of *C. pectoralis* pepsins was potentiated by the divalent cations  $Ca^{2+}$  and  $Mg^{2+}$ .

### 3.6. Effect of NaCl on EE acidic proteolytic activity

The effect of NaCl at different concentrations on the activity of EE acidic proteases is shown in Figure 3. It can be seen that the proteolytic activity decreased continuously with the increase in NaCl concentrations. However, the enzyme maintained a high activity even in the presence of 15% NaCl.

Similar results were found by Acevedo Gomez et al. (2018) for commercial porcine pepsin and digestive aspartic proteases from *P. lineatus*. It was similarly reported for acidic proteases from the stomach crude extract of *P. gigas* and for pepsins from *C. pectoralis* (Klomklao et al., 2007; Vannabun et al., 2014), whereas pepsins from *S. aurita*, after incubation with 10% of NaCl, showed proteolytic activity of approximately 20% (Khaled et al., 2011). Klomklao et al. (2007, 2009) emphasize that high NaCl concentration promotes an increase in protein-protein interactions, which may lead to precipitation. In addition, as they are hydrolase class enzymes, competition with salt for water molecules would hinder their catalysis.

In view of the results presented, it can be concluded that through a simple step of salting-out it was possible to obtain a protein concentrate containing two isoforms of aspartic proteases, with high proteolytic activity, from the *C. hippos*



**Figure 3.** Effect of NaCl on the EE acidic proteases.

stomach crude extract. The physicochemical characteristics determined demonstrate that these enzymes presenting similar characteristics found for pepsins isolated from fishes and have potential for industrial and biotechnological applications.

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