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Partial characterization of amylasic activity present in the intestine of *Pachymerus nucleorum* larvae (Fabricius, 1792)

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Abstract

Pachymerus nucleorum (Fabricius, 1792) is a beetle specie of the Bruchinae sub-family, family Chysomelidae and principal host is coconut of the palm babassu (Orbinya spp), typical of the varzea zones and river valleys. The objective in this work was identify and to characterize the amylolytic activity in *P. nucleorum* larvae guts. The guts were immediately homogenized in phosphatate buffer 50 mM pH 7,5 containing protease inhibitor (Aprotinine 0,2 ug/mL and Benzamidine 1 mM), sucrose 250 mM and EDTA 1 mM, after that the samples were centrifugated at 15000xg for 30 minutes, 4 ºC. The supernatant fraction (S1) presented higher amylolytic activity in relation to the precipitated fraction (P1). Analyses in SDS-PAGE revealed polypeptides of 62 kDa, in fraction P1 and in fraction S1 polypeptides of 12 and 40 kDa. The fraction S1 presented maximum amylolytic activity in 50°C, and stimulation of the activity in 20 % and 45 % in presence of calcium 0,5 mM or sodium chloride 14 mM, respectively. The preparation containing sucrose and EDTA presented higher amylolytic activity in relation to the preparation without sucrose and EDTA. An increase in the amylolytic activity was observed in both preparations when stored in refrigerator, however the preparation without sucrose and EDTA presented higher increase in this activity.

Keywords: Babassu; Bruchid; *Pachymerus nucleorum*; Activity; Amylase

1. Introduction

Pachymerus nucleorum (Fabricius, 1792) is a species of beetle included in the Bruchidae family, currently modified to the Bruchinae subfamily, of the Chrysomelidae family. This family is known for attacking seeds of *Phaseolus vulgaris* (1,2) and canopies of tropical plants such as *Acacia farnesiana* (3,4), *Erythrina abyssinica* (5) and *Attalea phalerata* (6). The main host of *P. nucleorum* larvae are coconuts from the babassu palm (Orbinya spp), a plant typical of the transition region between the cerrado, the Amazon rainforest and the northeastern semi-arid region, concentrated in the Brazilian state of Maranhão.

Beetle larvae of the Bruchinae family have α-amylases (EC 3.2.1.1), enzymes that degrade starch, hydrolyzing its α-1,4-glycosidic bonds, also classified as endoamylases (7–10). In the midgut of beetle larvae of the species *Zabrotes subfasciatus*, a pest of *Phaseolus vulgaris*, α-amylase isoforms with 95, 85 and 65 kDa are found. The 85 kDa isoform is sensitive to heat, being completely inactivated after 30 minutes of incubation at 65 ºC, while the 65 kDa isoform was more thermostable, remaining active after 2 hours. The 95 kDa isoform showed intermediate thermostability, being inactivated between 90 and 120 minutes (11,12). In 1990, Lemos and collaborators verified that fractions of α-amylases obtained from *Z. subfasciatus* were slightly inhibited at 60 ºC, but such activity was reestablished after the addition of 20 mM NaCl or 1.0 mM CaCl2. These researchers also observed that the addition of 2 mM EDTA stimulated amylase activity by 27% in some fractions, but inhibited others by 34%. In the intestine of *Z. subfasciatus* larvae, α-amylase

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inhibitors (αAI-1) or inhibitors extracted from *Triticum aestivum* showed different actions (11). This difference in inhibition was related to the ability of the larva to release different isoforms (12).

In the intestine of *Callosobruchus maculatus*, pests of *Vigna unguiculata* seeds, α-amylase isoforms of 56, 45 and 35 kDa were found that showed sensitivity to heat. The 35 kDa amylase showed greater sensitivity to heat while the 56 kDa amylase showed greater thermostability, but all were completely inactive at 65 ºC for a period of less than 10 minutes (11,12). In 1989, Campos and collaborators (13) showed the presence of similar isoforms in *C. maculatus* that were not inhibited by an α-amylase inhibitor (αAI-1), extracted from *Phaseolus vulgaris* seeds, but were inhibited by inhibitors extracted from *Triticum aestivum*.

All these data from the literature reinforce the importance of evaluating the presence and characterization of possible molecular markers that can potentially be used for biological control (7,14–16). As starch digestion is of fundamental importance for bruchids, the identification and characterization of amylasic activity in the intestine of larvae of the coleopteran *Pachymerus nucleorum* can clarify important aspects of this process and be useful in the control of these insects.

2. Material and methods

2.1. Reagents

Starch, monobasic and bibasic phosphate, EDTA, protease inhibitors, β-mercaptoethanol and DTT reagents were obtained from SIGMA Co. USA. The amylsase kit was obtained from Labtest Diagnóstica. The other reagents used were analytical grade and purchased commercially.

2.2. Preparation of the fraction with amylase activity

Pachymerus nucleorum (Fabricius, 1792) larvae were removed from babassu coconut (Orbinya spp), washed and dissected using a magnifying glass. 0.1g of intestine was immediately homogenized per ml of 50 mM Phosphate buffer, pH 7.5 containing 1 mM Benzamidine, 2 µg/ml Aprotinin and as indicated 250 mM Sucrose and 1 mM EDTA. The homogenate was centrifuged at 15,000xg for 30 minutes at 4 °C in a Sigma 3K30 centrifuge. The supernatant (S1) and precipitate (P1) fractions were recovered and kept at 4 °C.

2.3. Determination of Amylase Activity

Amylase activity was measured by the Caraway method (1959) (17) using the LABTEST amylase kit. The unit (U) was adopted as the amount of enzyme that cleaves 10 mg of starch in 30 minutes at 37 ºC.

2.4. Amylase activity calculations

$$\text{Hydrolyzed starch} = \frac{(Abs.contrl - Abs.da\ sample)}{Abs.contrl} \text{X [ ] of starch}$$

2.5. Protein Dosing Method

The protein concentration of the samples was determined according to the quantitative method of Bradford (1976) (18). Parallel to the protein dosage of the fractions, a standard bovine serum albumin (BSA) dosage curve was prepared.

2.6. Calcium Dosing Method

Calcium was measured by the method of Connerty (1966) (19) using the Bioclin calcium kit.

2.7. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The polypeptide profile of the protein fractions was analyzed on SDS-PAGE using the discontinuous buffer system of Laemmli and Favre (1973) (20) and the plate system employed by Studier (1973) (21) and mini-gels with gradient concentration from 5 to 22% and fixed of 10%. SDS-6H molecular weight standard from SIGMA Co. USA.

2.8. Zymogram

Mini-gels were prepared at a concentration of 10% (split gel), using 10 x 8 x 0.075 cm mini-gels. Protein samples were prepared by adding 90 mL of the respective fraction to 10 mL of sample buffer (6.5 mM Tris pH 6.8 containing 1% glycerol, 2% SDS, 1% Bromophenol), observing the absence of heating and b-mercaptoethanol. The electrophoretic separation of polypeptides was conducted under constant voltage of 150 V using a Bio-Rad Power Pac 1000 source.

After electrophoresis, the gel was placed in a 2.5% (w/v) Triton X-100 solution for 20 minutes at room temperature and subsequently transferred to a pH 7.0 phosphate buffer solution containing soluble starch at 1% (w/v) and incubated at 37°C for 24 hours. Amylasic activity was stopped with the addition of a dye solution (1.3% metallic iodine and 3% potassium iodide). After the reaction, clear bands against a dark background indicate the presence of active amylase.

2.9. Statistic

Statistical tests were performed from 3 experiments (n=3), analyzed using the t-student test, with a significance level of 5% (P=0.05). Means and standard errors are plotted

3. Results

3.1. Electrophoretic profile of fraction S1 and P1

In SDS-PAGE, it is observed that the supernatant fraction S1 presents a more evident band, which corresponds to two Mr polypeptides around 62 kDa. This still presents several other polypeptides, but they appear less intensely in the gel. Among these, one of high molecular weight (234 kDa) and some between 12 and 40 kDa stand out. In the precipitated fraction P1, the presence of 6 more evident polypeptides is verified, two paired around 63 kDa. The remainder is distributed below the molecular weight standard of 45 kDa, two with molecular weight of 31 and 27 kDa and two paired low molecular weight polypeptides around 21 kDa **(Figure 1)**.

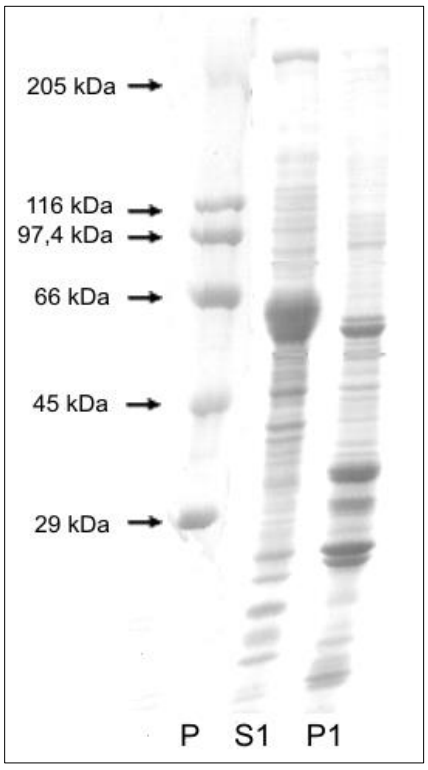

Figure 1 Polypeptide profile of the S1 and P1 fractions of *Pachymerus nucleorum* larval intestine. The following were applied: 8.01 µg of S1; 8.22 µg of P1. P - SDS-6H molecular weight standard

3.2. Analysis of the amylasic activity of fractions S1 and P1

The S1 fraction shows amylase activity of 187.74 mU/mg, corresponding to the hydrolysis of 11% of the starch in the reaction medium, while the precipitated fraction P1 does not show amylase activity **(Figure 2)**.

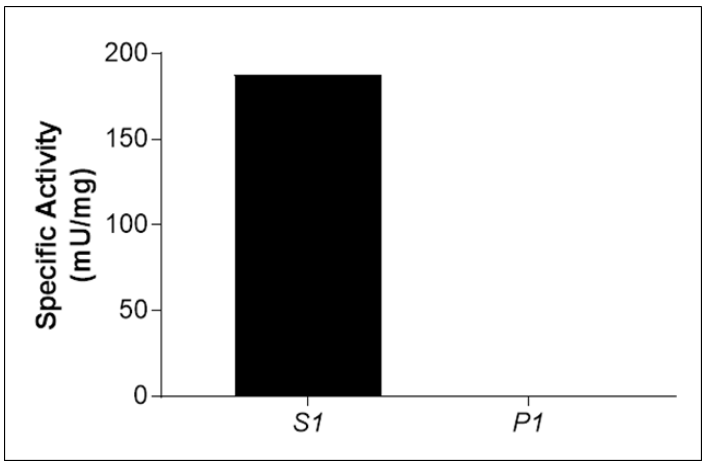

Figure 2 Amylasic activity of S1 and P1 fractions from *P. nucleorum* larval intestine. 0.2 mg of substrate was pre-incubated for 2 minutes at 37°C, then adding 0.17 mg of S1 or 0.12 mg of P1 as indicated. After incubation for 7 minutes and 30 seconds, 0.5 mL of 16.7 mM potassium iodate and 271 mM potassium iodide solution and 4 mL of water were added

The determination of the amylasic activity was carried out by reading the remaining starch in the reaction. To verify if the activity was underestimated, the endogenous starch content in the S1 and P1 fractions was analyzed. It was observed that the P1 fraction has 1.7 µg/µL of starch, while the S1 fraction only 0.3 µg/µL **(Figure 3)**. **Figure 4** shows that the P1 fraction caused the total disappearance of the amylasic activity of the S1 fraction. This is due to P1's endogenous starch (see discussion). Slight apparent inhibition was also detected when the P1 fraction was added to salivary amylase fractions **(Figure 5)**.

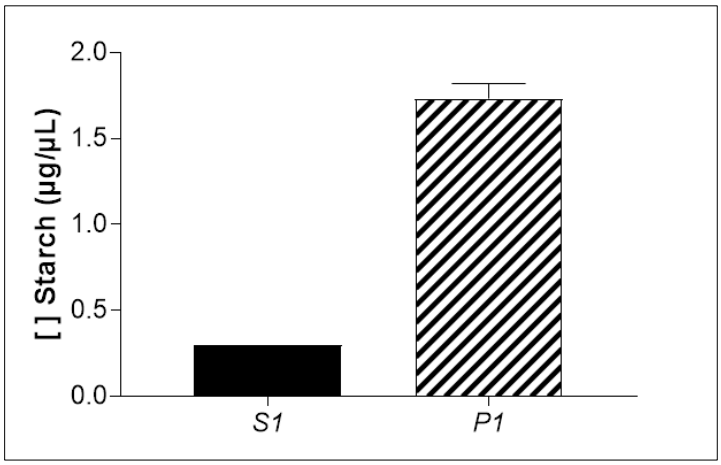

Figure 3 Starch concentration in fractions S1 and P1. 50 µL of the S1 or P1 fraction was added as indicated in 500 µL of water. Then, 0.5 mL of 16.7 mM potassium iodide and 271 mM potassium iodide solution and 4 mL of water were added. Starch standard curve from 0 to 0.2 mg was used

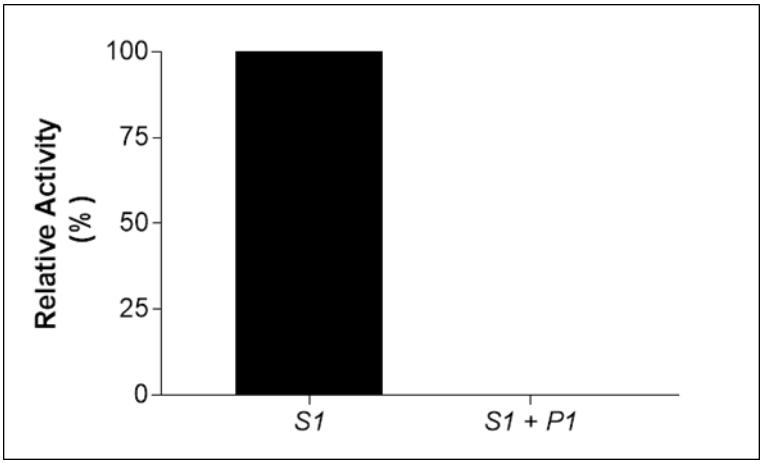

Figure 4 Effect of the P1 fraction on the amylase activity of the S1 fraction. 0.2 mg of substrate was pre-incubated for 2 minutes at 37°C, then adding 0.17 mg of S1 and 0.12 mg of P1 as indicated. After incubating the reaction for 7 minutes and 30 seconds, 0.5 mL of 16.7 mM potassium iodate and 271 mM potassium iodide solution and 4 mL of water were added

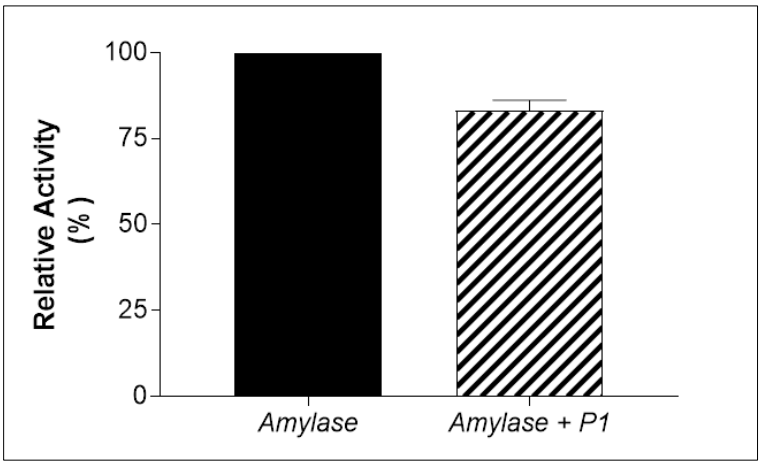

Figure 5 Effect of the P1 fraction on the amylasic activity of salivary amylase. 0.2 mg of substrate was pre-incubated for 2 minutes at 37 ºC, adding fractions of 0.88 µg of salivary amylase and 10 µL of P1 as indicated. After incubating the reaction for 7 minutes and 30 seconds, 0.5 mL of 16.7 mM potassium iodate and 271 mM potassium iodide solution and 4 mL of water were added

3.3. Effect of Sucrose and EDTA on the preparation of the S1 fraction

In the preparation of the S1 fraction, extraction buffers without or with 250 mM sucrose and 1 mM EDTA were used, which showed significant differences in relation to amylase activity. The preparation with sucrose and EDTA had a specific activity of 187.74 mU/mg, while the extraction without sucrose and EDTA had a specific activity of 60.82 mU/mg **(Figure 6)**.

As the preparation obtained with sucrose and EDTA presented amylase activity 3 times greater than the preparation without, the effect of sucrose and EDTA on the amylase activity of the latter was tested. Only 5% activation was observed when sucrose was added and 4% in relation to EDTA, compared with the control without sucrose and EDTA **(Figure 7)**.

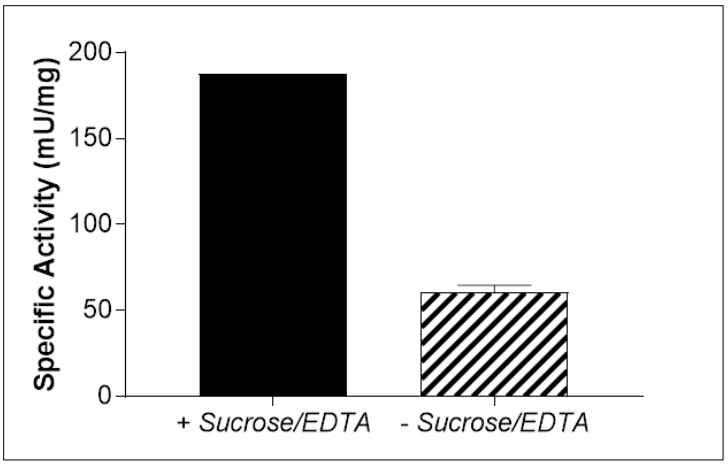

Figure 6 Amylasic activity of the S1 fractions obtained with and without sucrose and EDTA. 0.2 mg of substrate was pre-incubated for 2 minutes at 37°C, followed by addition of 0.12 or 0.17 mg of S1 protein, respectively, with or without sucrose and EDTA as indicated. After incubating the reaction for 7 minutes and 30 seconds, 0.5 mL of 16.7 mM potassium iodate and 271 mM potassium iodide solution and 4 mL of water were added

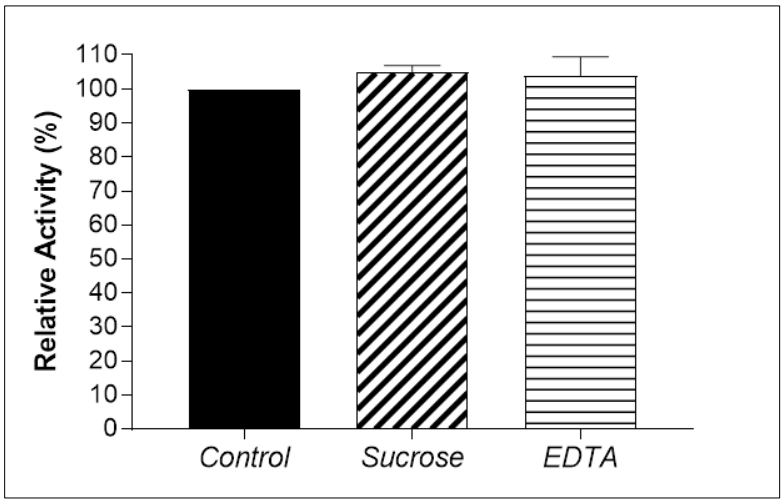

Figure 7 Effect of sucrose and EDTA on S1 amylase activity. 5 minutes before the activity assay, 250 mM of Sucrose or 1 mM of EDTA were added to S1 fractions obtained without sucrose and EDTA. 0.2 mg of substrate was pre-incubated for 2 minutes at 37°C, then 0.17 mg of protein was added. After incubation for 7 minutes and 30 seconds, 0.5 mL of 16.7 mM potassium iodate and 271 mM potassium iodide solution and 4 mL of water were added

It was also observed that the amylasic activity of the S1 fraction obtained with and without sucrose and EDTA showed, when stored at 4 ºC, an increase of around 20 and 90%, respectively **(Figure 8)**. As expected, the two S1 fractions also showed differences in free calcium concentration. In the fraction obtained in the presence of sucrose and EDTA, the concentration of free calcium was 63 µM, while the fraction obtained without sucrose and EDTA presented a concentration 4 times higher, corresponding to 242 µM **(Figure 9)**. As the S1 fraction showed amylasic activity, the polypeptides of this fraction were separated in SDS-PAGE and incubated with soluble starch (zymogram). On the zymogram, small clear dots above 58 kDa can be observed, which seems to correspond to starch cleavage. An industrial amylase (Termamyci) was used as a positive control **(Figure 10)**.

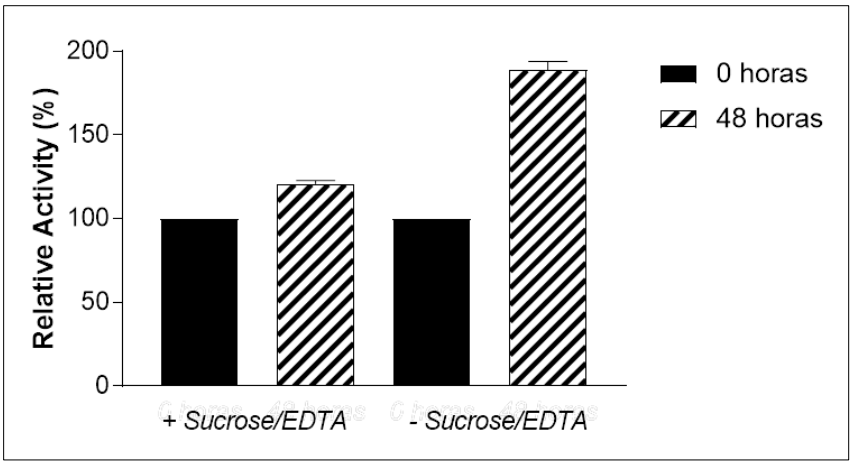

Figure 8 Stimulation of the amylasic activity of the S1 fractions with or without sucrose/EDTA after 48 hours at 4 °C. 0.2 mg of substrate was pre-incubated for 2 minutes at 37°C, then adding 0.12 or 0.17 mg of protein from the preparation with or without sucrose and EDTA as indicated. After incubating the reaction for 7 minutes and 30 seconds, 0.5 mL of 16.7 mM potassium iodate and 271 mM potassium iodide solution and 4 mL of water were added

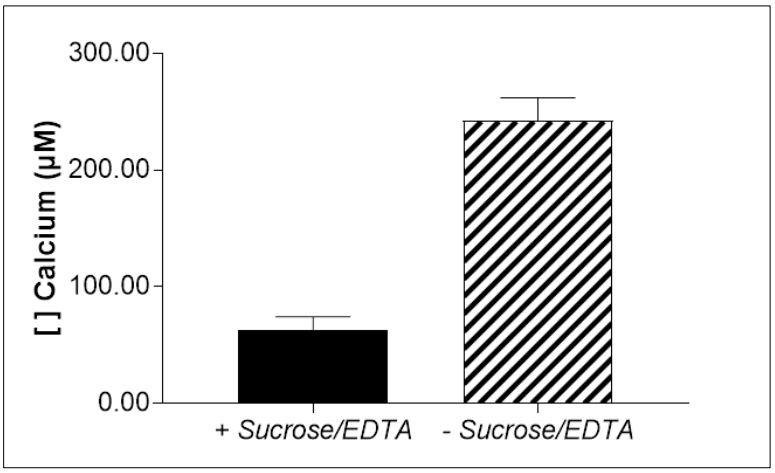

Figure 9 Calcium concentration in S1 fractions extracted with or without sucrose/EDTA. 20 µL of the S1 fraction were added to 1 mL of buffer and 1 mL of 0.5 mM)-cresolphalein and 0.7 mM 8-hydroxy quinoline solution. Calcium 10 mg/dL was used as standard.

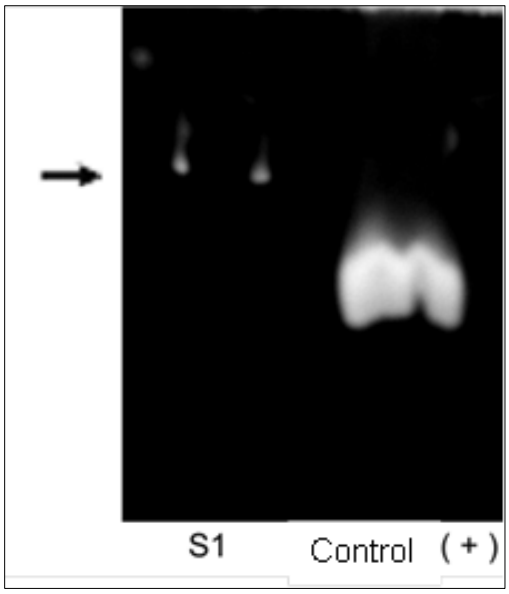

Figure 10 Zymogram of the S1 fraction of *P. nucleorum* larval intestine. 38 µg of S1 and 23 µg of control (+) were separated on SDS-PAGE. After electrophoresis, the gel was placed in a 2.5% (w/v) Triton X-100 solution for 20 minutes at room temperature, and then transferred to a pH 7.0 phosphate buffer solution containing soluble starch at 1% (w/v) and incubated at 37°C for 24 hours. After addition of dye solution (metallic iodine 1.3% and potassium iodide 3%) the clear bands against the dark background indicate the presence of active amylase. Industrial amylase was used as a positive control

3.4. Characterization of the amylasic activity of the S1 fraction

3.4.1. Temperature Effect

Amylase activity of S1 was analyzed at temperatures ranging from 24 to 80 ºC. As seen in Figure 11, the S1 fraction has an activity of 137 mU/mg at 24 ºC, gradually increasing to 235 mU/mg (50 ºC) and decreasing at higher temperatures (11). Above 70 °C, the enzyme is completely inactivated.

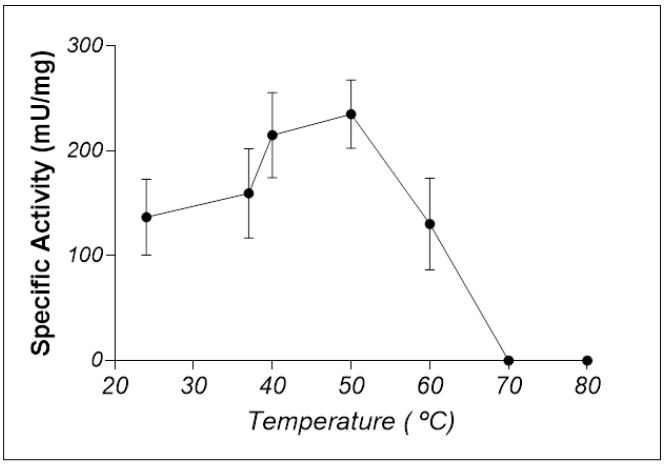

Figure 11 Effect of temperature on the amylase activity of the S1 fraction. 0.2 mg of substrate was pre-incubated for 2 minutes at the indicated temperature, then 0.17 mg of protein was added. After incubating the reaction for 7 minutes and 30 seconds, at the indicated temperature, 0.5 mL of 16.7 mM potassium iodide and 271 mM potassium iodide solution and 4 mL of water were added

3.4.2. Effect of Calcium

To verify the effect of the calcium cation on the amylasic activity of the S1 fraction, calcium chloride was added in increasing concentrations (0.05 to 2 mM) to the reaction medium. Activity did not change significantly with different calcium concentrations. Only a slight increase (20 %) in amylase activity was observed at 0.5 mM calcium, and a slight inhibition (16 %) at a concentration of 2 mM **(Figure 12)**.

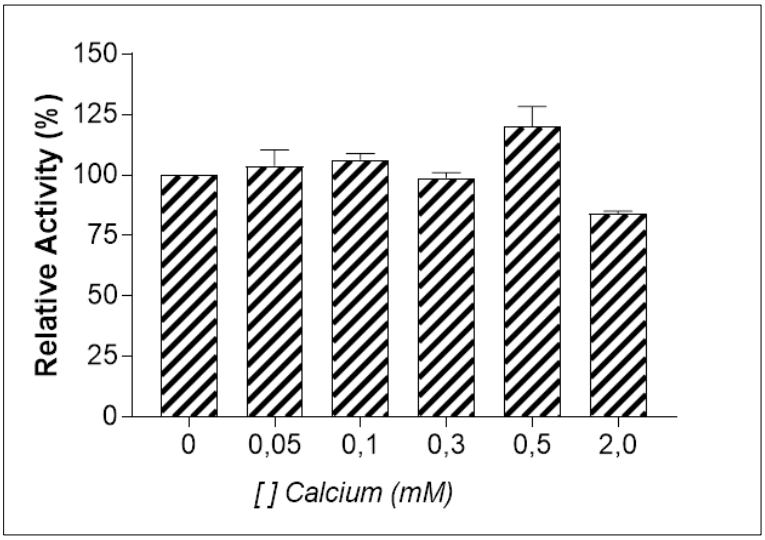

Figure 12 Effect of calcium ion on the amylase activity of the S1 fraction. Fraction S1 was pre-incubated for 5 minutes with different concentrations (0.05 to 2.0 mM) of calcium. 0.17 mg of protein from this fraction was added to 0.2 mg of substrate previously incubated for 2 minutes at 37 ºC. After incubating the reaction for 7 minutes and 30 seconds, 0.5 mL of potassium iodate solution 16 was added .7 mM and 271 mM potassium iodide and 4 mL of water

3.5. NaCl effect

NaCl also seems to affect the amylase activity of the S1 fraction. It is observed that the activity of the S1 fraction, at first increases with the increase of the sodium chloride concentration, culminating with 45 % of stimulation with 14 mM of NaCl. This remains stable around 120 % during subsequent concentrations **(Figure 13)**.

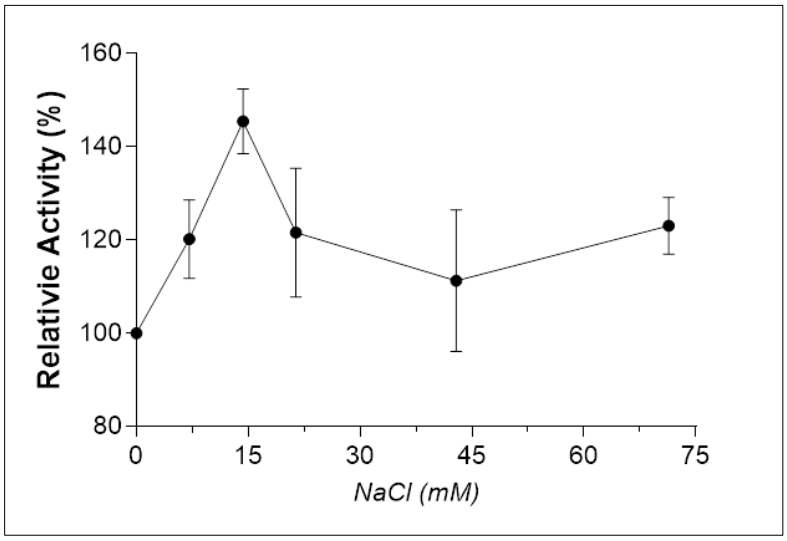

Figure 13 Effect of sodium chloride on the amylase activity of the S1 fraction. 0.2 mg of substrate, containing sodium chloride as indicated, was pre-incubated for 2 minutes at 37°C, followed by addition of 0.17 mg protein fractions. After incubating the reaction for 7 minutes and 30 seconds, 0.5 mL of 16.7 mM potassium iodate and 271 mM potassium iodide solution and 4 mL of water were added

4. Discussion

The soluble fraction of *Pachymerus nucleorum* showed greater amylase activity compared to the precipitated fraction (Fig. 2), similar to the species *Zabrotes subfasciatus* whose soluble fraction presents amylase activity about 10 times greater than the precipitated fraction (12). Despite the species *Callosobruchus maculatus* showing no difference between the amylasic activity of the soluble and precipitated fractions, the specific activity of these fractions, around 210 mU/mg, is close to that found in the soluble fraction of *Pachymerum nucleorum* (12).

According to the zymogram, it seems that a polypeptide around 60 kDa is responsible for the amylasic activity of the soluble fraction of *P. nucleorum* (Fig. 9). Amylase with relative mobility similar to that of this polypeptide was observed in *Z. subfasciatus* (12).

The results showed the presence of endogenous starch both in S1 and P1 (fig. 3). Regarding S1, the amount of starch present, 0.3 µg/µL, when considered in the specific activity calculations, increased the amylase activity by about 50%, that is, the activity of S1 is underestimated. Likewise, the P1 fraction showed amylase activity different from zero, when the presence of endogenous starch was considered in the amylase activity calculations.

Under the test conditions used, the S1 fraction hydrolyzed about 16% of the substrate (endogenous starch to S1 + added starch). This hydrolysis was completely inhibited when the S1 fraction was incubated together with the P1 fraction. In this test, 0.1 mg of starch, endogenous to the P1 fraction, were added to the reaction medium. As the reaction medium contains 0.2 mg of starch, the starch from P1 corresponds to 50% of the starch in the medium and is greater than the amount of starch (0.032 mg) that the S1 fraction hydrolyses under the test conditions. Therefore, the apparent inhibition of the amylasic activity of the S1 fraction by the P1 fraction can be explained by the presence of endogenous starch in the P1 fraction and not due to any amylase inhibitor present in P1. Starch endogenous to P1 also explains the slight inhibition that this fraction causes in salivary amylase activity.

The increase in amylase activity of the S1 fraction, observed after 48 hours at 4 ºC, may also be related to the amount of starch present in the S1 fraction (Fig. 3). At low temperatures, despite the decrease in amylase activity, the exposure time of the starch substrate to the amylase enzyme is very long (48 hours) and the hydrolysis of endogenous starch may explain the small increase in amylase activity of S1 in preparations with sucrose and EDTA (Fig. 8).

In the preparation without sucrose and EDTA, the increase in amylase activity, around 90 %, cannot be explained by previous degradation of endogenous starch to the S1 fraction. One possibility is the degradation of amylase inhibitors of the S1 fraction by endogenous proteases. Such inhibitors could come from the diet (nut) of the larva. At the pH of the S1 fraction (7.5), the action of proteinases may be favored, as observed for the intestine of *Tenebrio molitor* beetle larvae, which showed active trypsin and chymotrypsin under these conditions (22). Fraction S1 showed polypeptides with relative mobility similar to that of amylase inhibitors. The 45 kDa polypeptide, for example, has relative mobility similar to that of phaseolamine, an amylase inhibitor extracted from *Phaseolus vulgaris* (23,24). Polypeptides with relative mobility similar to subunits of the lectin extracted from *Talisia esculenta* were also observed. This lectin is an amylase inhibitor and its introduction into the diet caused a 50% increase in mortality in bruchids such as *Z. subfasciatus* and *C. maculatus* (8,25,26). A polypeptide with relative mobility *like* that of the inhibitor extracted from *Triticum aestivum* is also observed in the soluble fraction of *P. nucleorum*. This inhibitor inhibits the amylase activity of amylases from insects such as *Acanthoscelides obtectus*, *Callosobruchus maculatus* and *Zabrotes subfasciatus* (8,26).

The amylase activity of the soluble fraction of *P. nucleorum* showed optimal activity around 50 °C. At 60 °C an inhibition of 40% was observed and at 70 °C the activity is completely inhibited. Amylases from *Z. subfaciatus* were also inhibited by approximately 85 % at 60 °C, but such activity was reestablished after the addition of 20 mM NaCl or 1.0 mM CaCl2 (11). Amylases from *C. maculatus* are also inactivated around 60 °C (12).

The increase in S1 amylase activity in the presence of 0.5 mM calcium (Fig. 13) may be related to the functional structure of the α-amylase family (7,9,10,27,28). The link between domains A and B of amylases, a highly conserved region, has a binding site for calcium cations, which act by stabilizing the enzymatic structure, keeping this region more flexible and stable (7,9,27–30). At 2 mM of calcium (Fig. 13), the slight inhibition of activity may be related to other calcium binding sites in the enzyme that are less sensitive, as in the case of Taka-amylase, which has a second site below the binding region of the substrate, and which is responsible for inhibiting the enzyme at high concentrations of calcium (7,10). Yeast and bacterial amylases were also inhibited by millimolar calcium concentrations (31,32).

Sodium at a concentration of 14 mM caused a 45 % increase in amylase activity (Fig. 15), which may be related to the presence of ions in the active sites of amylases such as *Bacillus licheniformis*, which has a triad composed of calcium–

sodium–calcium promoting a greater space to the substrate binding site and causing greater stability of the negative charges of the side chains of Asp, Glu and Asp residues (29,30). Or, as observed by Nonaka (2003) (33), the presence of sodium in place of calcium, in the highly conserved sites of α-amylase, is also responsible for the stability of the structure

5. Conclusion

In this work, the presence of amylasic activity in the intestine of *Pachymerus nucleorum* larvae was shown. Amylase activity has an optimal temperature of 50 ºC and is stimulated by sodium chloride and calcium at low concentrations, but inhibited by calcium at higher concentrations.

Compliance with ethical standards

Disclosure of conflict of interest

The authors have no other conflicts of interest.

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