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Research Article

IDENTIFICATION AND CHARACTERIZATION OF P. NUCLEORUM LARVAE MYOSIN

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ABSTRACT

The objective of this work was to isolate myosin-like ATPase from the soluble fraction of P. nucleorum larvae. The soluble fraction was frozen at -20 oC, thawed after 48 h and centrifuged at 45,000g x 40 minutes. The precipitate was successively washed with buffer (pH 7.5 and 9.0) and ATPase was then solubilized with buffer (pH 9.0) containing 0.5M NaCl. The main polypeptides presented Mr of 205 and 43 kDa and identification by PMF shows similarity of p205 with myosin heavy chain of Tribolium castaneum. That fraction expressed Ca2+-ATPase activity and actin-stimulated Mg2+-ATPase activity. The Ca2+-ATPase activity was inhibited by magnesium but not by 140 µM thapsigargin, 1.7 mM ouabain or 1 mM azide.

INTRODUCTION

The babassu palm (Orbygnia spp) is an important natural source of oil. It has great energy generation potential and is suitable for the production of bio-diesel [1]. It is found in abundance in Brazil in babassu zones of southeastern Amazon, an area that covers 9.57 million ha, with an estimated potential of 10.6 billion tons of fruit annually [2]. Besides the chestnut, other palm components are used by the local population and many families depend on these products in Brazil's poorest regions, where the babassu palm plays a very important role in the economy of hundreds of thousands of people [3]. Pachymerus nucleorum is a beetle that eats fruit from several palm species, among them the babassu [4]. In the babassu zone of southeastern Amazon about 70% of the babassu fruits have at least one chestnut destroyed by the larvae of this insect.

ATPases are enzymes that hydrolyze ATP and utilize the energy released in this process to carry out some kind of cellular work [5, 6, 7, 8]. Some ATPases are insecticide targets [9, 10, 11, 12, 13]. The difenil-2-penten-1one, a natural insecticide extracted from Stellera chamaejasme, for example, inhibits the Ca2+/ Mg2+-ATPase of the insect's plasmatic membrane [12]. The actin, a protein that constitute the microfilaments and stimulate myosin Mg2+-ATPase activity, also are a botanic insecticide target [14, 15]. Therefore, the identification and characterization of insects ATPases can be important to control their populations.

ATPase has been obtained the particulate fraction of P. nucleorum larvae and it expressed high Ca2+-ATPase activity and this activity was significantly inhibited by magnesium, copper and zinc [16]. Myosin is an ATPase implicated in muscular contraction and several other cellular motility processes [17, 18]. Myosin II participates in the final process of the cellularization and the first cytokinesis after cellularization in Drosophila embryos [19] and myosin V is required for larval development and spermatid individualization [20].

Myosin can precipitate as result of the freezing of rat brain [21], testis and skeletal muscle [22] soluble fraction and the precipitate fractions exhibit high ATPase activity, which indicates that the freezing/thawing procedure does not destroy

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such activity. In this work, we used the freezing method to isolate ATPase from the soluble fraction of P. nucleorum larvae.

MATERIALS AND METHODS

2.1 Purification of ATPase from Pachymerus nucleorum larvae
The larvae were removed from babassu palm fruits, washed in Milli-Q water and immediately dissected for the extraction of the digestive system. The remaining tissues were homogenized (0.1g/mL) in extraction buffer (50mM imidazole-HCl pH 7.5, 250mM sucrose, 10mM ethylenediaminetetraacetic acid – EDTA, 10mM ethylene glycol tetraacetic acid – EGTA, 1mM dithiothreitol – DTT, 2µg/mL aprotinin and 1mM benzamidin) with a two-speed electric homogenizer and, immediately after that, with a potter-type glass homogenizer. The homogenate was centrifuged at 45,000g for 40 minutes and the supernatant fraction was recovered and frozen at -20 °C. After at least 48 hours, the S1 fraction was thawed in 27 °C bath and centrifuged at 45,000g x 40 minutes. The P2 fraction was homogenized in 10mL buffer I (20mM Imidazole-HCl pH 8.0, 1mM EDTA and 0.1mM DTT), centrifuged at 45,000g x 40 minutes and the P3 fraction was recovered, homogenized in 5mL buffer II (10mM CAPS pH 9.0, 1mM EDTA and 0.1mM DTT) and centrifuged at 45,000g x 40 minutes. The P4 fraction was homogenized with buffer II containing 500mM NaCl and was centrifuged again at 45,000g x 40 minutes. The P5 fraction was homogenized with buffer II for analysis. The centrifugations were carried out at 4 °C and the homogenizations were done in ice bath.

ATP ase Assays

ATPase activity was determined by the quantification of the inorganic phosphate (Pi) released by ATP hydrolysis using the colorimetric method of [23]. The assays were done at 37 °C in a final volume of 200µL and the absorbance reading was done at 355 nm. Reaction mediums used: Ca2+- or Mg2+-ATPase (25mM Imidazole-HCl pH 7.5, 1mM DTT, 1mM EDTA, 60mM KCl containing 4mM CaCl2 or MgCl2, respectively) and K+/EDTA-ATPase (25mM Imidazole-HCl pH 7.5, 2mM EDTA, 1mM DTT and 60 or 600mM KCl). Rabbit skeletal muscle actin, prepared as described by Spudich & Watt (1971), was added at the Mg2+-ATPase reaction medium for assay of F-actin stimulated Mg2+-ATPase activity.

Dosage of Proteins

The protein concentration in the fractions was determined in duplicate according to the Bradford method [24] using BSA (bovine serum albumin) as the standard. Aliquots of the respective fraction were previously diluted to 100 µL with Milli-Q water and, later, 3 mL of Bradford Reagent was added.

Sds-Page

The polypeptide profile of fractions was analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 5 to 22% linear gradient gels, using the method described by [25] and the plate system employed by Studier [26].

Peptide Mass Finger printing (PMF) Analysis

Bands from SDS-PAGE (p205 and p43) were excised and destained using 50% acetonitrile and 25mM NH4HCO3 for 3-4 times (10 min each) and washed for 10 min with 100% acetonitrile. The gels were dried for 30 min using a Speed Vac system (Savant, Farmingdale, NY). The reduction of proteins was performed with 10mM DTT in 100mM NH4HCO3 at 56 oC for 1 h followed by alkylation with 55mM iodoacetamide in 100mM NH4HCO3 in the dark at room temperature. Bands were then washed twice using 100mM NH4HCO3 and 100% acetonitrile, alternately, and subsequently dried in a Speed Vac system. Tryptic peptides from each band were obtained using sequencing grade modified trypsin (Promega, Madison, WI). Each sample was prepared onto an AnchorChip plate (Bruker Daltonics, Bremen, Germany) according to the modified method of [27]. One microliter of the supernatant from the in gel digestions was loaded on the target and allowed to dry completely. Then 1 µL of freshly prepared matrix solution (0.5 mg/mL α-cyano-4-hydroxycinnamic acid in 0.1% TFA and 90% acetonitrile) was applied to the same spot and left to dry. The samples were desalted on plate by dropping 2 µL of 0.1% TFA on the matrix deposit. After 10 s, the remaining solution was removed with a pipette.

Peptide masses were then analyzed by Matrix assisted laser desorption/ionization - time-of-flight mass spectrometry (MALDI-TOF MS) using an Autoflex II MALDI-TOF/TOF (Bruker Daltonics) mass spectrometer at the Universidade de Brasília – UnB. Generated mass lists were used to database searching of proteins by Peptide Mass Fingerprinting (PMF) using Mascot Server v2.2. The searches were carried out in the NCBInr database. The following parameters were set for the search: carbamidomethyl (Cys) was set as fixed; variable modifications included methionine oxidation and N-terminal acetylation of the protein. Only one missed cleavage was allowed and fragment mass tolerance was set between 0.1 and 0.25 Daltons. Hits were considered significant if the protein score exceeded the threshold score calculated by Mascot software assuming p-value <0.05.

RESULTS

Most of the Ca2+- and Mg2+-ATPase activities present in the homogenate appeared in the particulate fraction (P1 fraction), while only a small part of these activities appeared in the soluble fraction (S1 fraction) and the Mg2+-ATPase activity of that fraction was greater than the Ca2+-ATPase activity (date not shown). The Ca2+- and Mg2+-ATPase activities of the S1 fraction were, respectively, 6.6% and 34.7% of the activities of the P1 fraction (date not shown). Generally, the freezing of the S1 fraction generated a precipitate (P2 fraction), which contained less than 5% of the proteins of the S1 fraction, but presented specific Ca2+-ATPase activity up to 100 times higher than the S1 fraction (Fig. 1A). On the other hand, the Mg2+-ATPase activity of the S1 fraction was totally recovered in the S2 fraction (Fig. 1B). After washing the P2 fraction, the majority of the polypeptides were recovered in the soluble fraction (Fig. 2A), but most of the total Ca2+-ATPase activity of the P2 fraction was recovered in the precipitate fraction. Only a small part of the total Ca2+-ATPase activity was solubilized in this step (data not shown). The main polypeptides of the P3 fraction presented Mr of approximately 205 (p205), 57 (p57) and 43 (p43) kDa (Fig. 2A). This polypeptide profile was maintained in the P4 fraction. The Ca2+-ATPase activity of P. nucleorum larvae and most of the polypeptides p205 and p43 were solubilized with the treatment

of the P4 fraction with CAPS buffer pH 9.0 containing 0.5M (Fig. 2B). These two polypeptides and p57 were the single polypeptides bands that appeared intensely staining in the S5 fraction (Fig. 2A). A 145 kDa polypeptide band and a polypeptide of low molecular weight (like myosin light chain) also can be observed in the gel (fig. 2A).

situ and in silico digestions, and represented 16% coverage of the total sequence (Table 1). Identified protein presents a theoretical mass close to that calculated for the SDS-PAGE band (p205). The same procedure was made for identification of p43, and the results showed 19 matched peptides with

Table 1 Protein bands analyzed by MALDI-TOF MS and identified by searching the NCBI nonredundant protein database using Mascot

Spot	Mr (SDS-PAGE) kDa	Identified protein	Organism / Protein ID	Score Mascot	Matched Peptides	Coverage (%)	Mr (MALDI-TOF-MS) kDa
P205	211.882	Myosin heavy chain CG17927-PF isoform 2	Tribolium castaneum / gi|189239935	133	31	16	224.781
P43	42.603	actin-87E isoform 1	Tribolium castaneum / gi|91078486	200	19	44	42.256

The SDS-PAGE spots (p205 e p43) were cutted, digested and desalted using trypsin and ZipTips® C_{18} respectively. The digested fragments were analyzed by Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and Mascot v2.2 server was applied to proceed peptides identification and the searches was done using NCBI database 20080924.

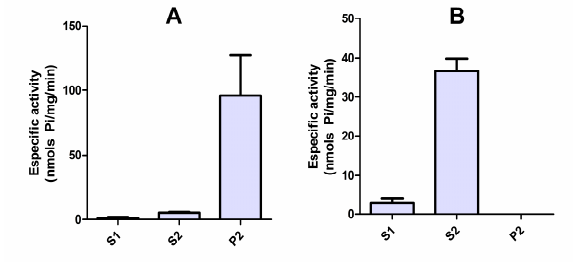

Figure 1 Separation of Ca^{2+} and Mg^{2+}-ATPase activity from the *P. nucleorum* larvae soluble fraction by freezing/thawing

About 25 µg of S1, 23 µg of S2 and 11 µg of P2 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 1 mM EDTA and 60 mM KCl) containing 4 mM $CaCl_2$ (A) or 4 mM $MgCl_2$ (B). The reaction was initiated by the addition of 1 mM ATP and stopped with 2 ml of phosphate dosing solution. The bars indicate the standard error calculated for n = 3.

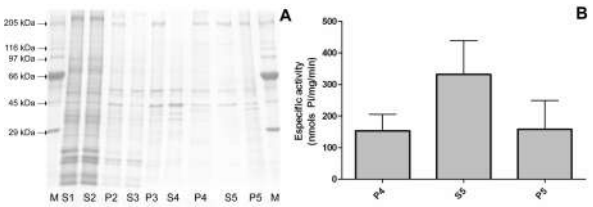

Figure 2 Ca^{2+}-ATPase purification from the *P. nucleorum* larvae soluble fraction

A. SDS-PAGE of the purification - Approximately 3 µL of S1 and S2, 5 µL of P2, S3 and P3, 15 µL of S4 and 10 µL of P4, S5 and P5 were applied on 5-24% polyacrylamide gel. (M) Molecular weight standard: myosin, β-galactosidase, phosphorilase b, bovine albumin, ovalbumin and carbon anyhdrase. The gel was stained with Coomassie blue R-250. ***B.Solubilization of Ca^{2+}-ATPase activity of the P4 fraction*** - About 7.5 µg of P4, 5.2 µg of S5 and 6.3 µg of P5 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 1 mM EDTA and 60 mM KCl) containing 4 mM $CaCl_2$. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 ml of phosphate dosing solution. The bars indicate the standard error calculated for n = 3.

The p205 band from SDS-PAGE was excised, digested in-gel with trypsin, and analyzed by MALDI-TOF MS. The results obtained showed a high score identification of p205 as being myosin heavy chain CG17927-PF isoform 2 (GenBank accession no. 189239935) from another beetle, Tribolium castaneum. A total of 31 tryptic peptides matched between in

Tribolium castaneum actin-87E isoform 1 (GenBank accession no. 91078486), representing 44% coverage of the total sequence (Table 1). Again the theoretical mass of identified protein is very close to the observed one (p43) (Table 1).

Myosins are ATPases that express actin-stimulated Mg2+-ATPase activity under physiologic conditions. In absence of F-actin, the S5 fraction exhibited low Mg2+-ATPase activity that was stimulated in about three times by F-actin (Fig. 3). These data confirm that the S5 fraction containing myosin family ATPase and this enzyme remain active. The S5 fraction exhibited high Ca2+-ATPase activity, but did not express ATPase activity in absence of bivalent cations and presence of high salt, K+/EDTA-ATPase activity (Fig. 4). This is a not physiologic activity exhibited by some myosin family members.

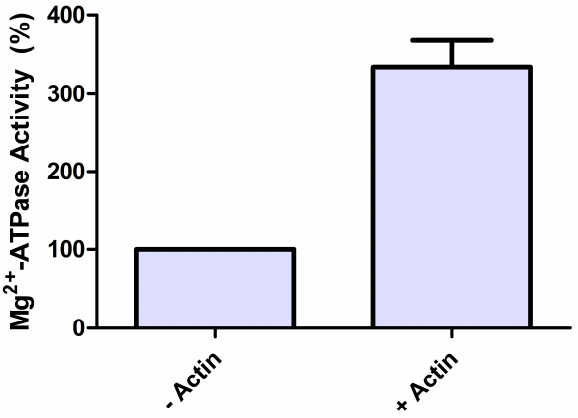

Figure 3 Actin-stimulated Mg^{2+}-ATPase activity from the S5 fraction About 5 µg of S5 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 1 mM EDTA and 60 mM KCl) containing 4 mM $MgCl_2$ and about 500 µg of actin. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 mL of phosphate dosing solution. The bars indicate the standard error calculated for n = 3.

Membrane ATPase inhibitors were added at the Ca2+-ATPase reaction medium for verified the presence of any membrane ATPase in the S5 fraction. We didn't detect any inhibition of Ca2+-ATPase activity of the S5 fraction in reaction medium containing 140µM thapsigargin (Fig. 5), an inhibitor of

reticulum Ca2+-ATPase. Azide, inhibitor of F1-ATPase, and ouabain, inhibitor of Na+/K+-ATPase also did not inhibit the Ca2+-ATPase activity of the S5 fraction isolated from P. nucleorum larvae (Fig. 5).

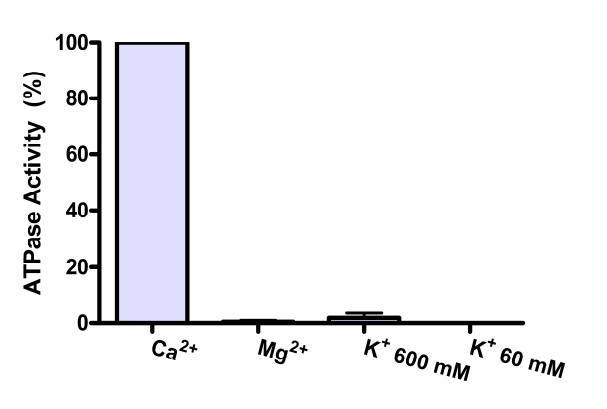

Figure 4 Ca^{2+} and K^{+}-EDTA-ATPase activities from the S5 fraction
About 2 µg of S5 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 1 mM EDTA and 60 mM KCl) containing 4 mM $CaCl_2$ or 4 mM $MgCl_2$ or reaction medium II (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 2 mM EDTA) with KCl 60 or 600 mM. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 mL of phosphate dosing solution. The basal specific Ca^{2+}-ATPase activity was 1564.5 nmols Pi/ mg Prot./ min. The bars indicate the standard error calculated for n = 3.

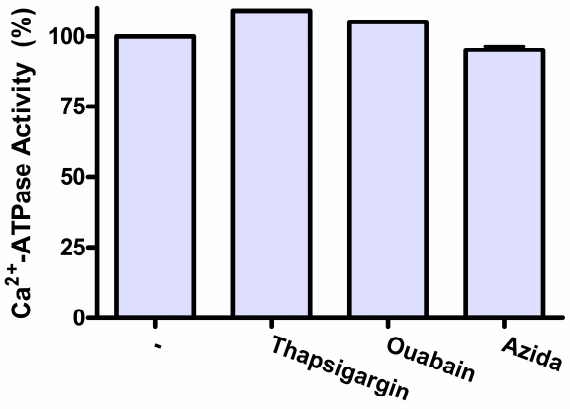

Figure 5 Effect of ATPases inhibitors in Ca^{2+}-ATPase activity from the S5 fraction
About 2 µg de S5 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 60 mM KCl) containing 4 mM $CaCl_2$ and 140 µM thapsigargin, 1.7 mM ouabain or 1 mM azide where indicated. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 mL of phosphate dosing solution. The basal specific Ca^{2+}-ATPase activity was 1564.5 nmols Pi/ mg Prot./ min. The bars indicate the standard error calculated for n = 3.

Calcium-dependent ATPase of particulate fraction of P. nucleorum larvae was inhibited by bivalent cations as copper, magnesium and zinc. These cations were used in the assay to verify their effect in the Ca2+-ATPase activity of the myosin isolated of Pachymerus nucleorum larvae. The Ca2+-ATPase activity practically not was inhibited by 1mM zinc, but was inhibited above of 50% by 1mM magnesium. Copper, in this same concentration, inhibited only 23% of the Ca2+-ATPase activity (Fig. 6). In the assay conditions, the P. nucleorum larvae myosin not hydrolyzed AMP and PPi and it just slightly hydrolyzed the ADP and the GTP (Fig. 6).

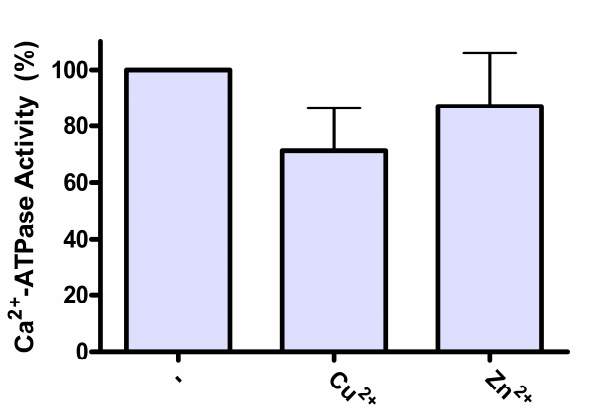

Figure 6 Effect of copper, magnesium and zinc in Ca^{2+}-ATPase activity from the S5 fraction
About 2 µg of S5 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 60 mM KCl) containing 4 mM $CaCl_2$ and 1 mM $CuCl_2$ or $ZnCl_2$ where indicated. The reaction was initiated by the addition of 1 mM ATP and stopped with 2 mL of phosphate dosing solution. The basal specific Ca^{2+}-ATPase activity was 1564.5 nmols Pi/ mg Prot./ min. The bars indicate the standard error calculated for n = 3.

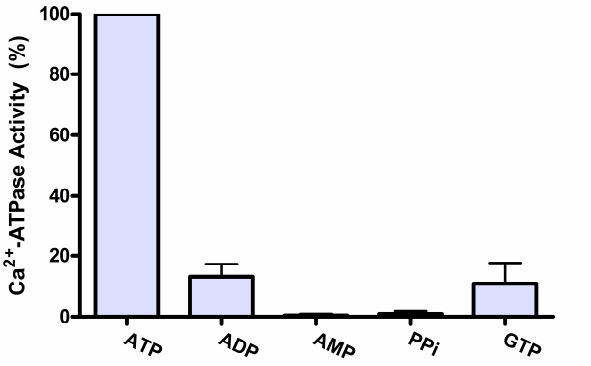

Figure 7 Substrate specificity of the enzyme from the S5 fraction
About 2 µg of S5 was incubated at 37°C for 20 min in reaction medium I (25 mM imidazole-HCl pH 8.0, 1 mM DTT, 1 mM EDTA, 60 mM KCl) containing 4 mM $CaCl_2$ and the respective substrate at 1 mM. The reaction was initiated by the addition of S5 and stopped with 2 mL of phosphate dosing solution. The basal specific Ca^{2+}-ATPase activity was 1564.5 nmols Pi/ mg Prot./ min. The bars indicate the standard error calculated for n = 3.

DISCUSSION

The freezing and thawing of the S1 fraction separated the Mg2+- and Ca2+-ATPase activities and enriched the Ca2+-ATPase activity in the P2 fraction. While the specific Ca2+- and Mg2+-ATPase activities of the S1 fraction were, respectively, 1.2 and 18.2 nmols Pi⁄mg prot. ⁄min, the P2 activities were, respectively, 96 and 0 nmols Pi⁄mg prot. ⁄min. (Fig. 1). The treatment of the P3 fraction with CAPS buffer pH 9.0 solubilized most of the polypeptides of 57 and 43 kDa, which is probably not related to ATPase activity, because that activity was totally recovered in the P4 fraction (data not show). p205, the main polypeptide of the P4 fraction, has Mr similar to the heavy chain of myosins II and V and these myosins can be precipitated by freezing the soluble fraction of different tissues as well [21, 22]. Besides the 205 kDa

polypeptide, the S5 fraction has a polypeptide with Mr similar to that of the actin, 43 kDa, and another of about 145 kDa (Fig. 2). Actin constitute the microfilaments and is known to purify along with the myosin [28, 29].

In order to identify the polypeptides p205 and p43, the bands correspondents were excised and used for in-gel tryptic digestion and MS/MS analysis. The polypeptides were identified with a high Mascot score and sequence coverage higher than 30% (Table I). p205 of P. nucleorum larvae was identified as been myosin heavy chain, whereas p43 was identified as been actin.

It is a unique behavior of myosins to have its Mg2+-ATPase activity stimulated by F-actin [28, 29]. The presence of actin-stimulated Mg2+-ATPase activity in the S5 fraction shown that the myosin purification method, that involve the soluble fraction freezing and thawing of P. nucleorum larvae, did not destroy its activity. The K+/EDTA-ATPase activity is a non-physiological activity used to identify members of the myosin family, because other ATPases do not present this kind of activity. Though P. nucleorum larvae S5 fraction not presented K+/EDTA-ATPase activity the MS/MS analysis showed that p205 correspond to myosin heavy chain. It is known that various tissues, despite possessing members of the myosin family, present low or even absence of K+/EDTA-ATPase activity [30]. Although the ATPase activity of myosins is considered sensitive to freezing, the low K+/EDTA-ATPase activity observed here seems to be a characteristic of P. nucleorum larvae myosin, since skeletal muscle myosin II obtained by freezing method showed high K+/EDTA-ATPase activity (Dias & Coelho, 2007b). Testis myosin II also does not seem to present K+/EDTA-ATPase activity (Dias & Coelho, 2007b) and it is important to mention that some myosin V preparations do not express K+/EDTA-ATPase activity either [31, 32].

ATPase isolated of P. nucleorum larvae particulate fraction expressed high ATPase activity in the presence of calcium and that activity was significantly inhibited by thapsigargin (Duarte & Coelho, manuscript in preparation), a specific inhibitor of Ca2+-ATPase from the endoplasmatic reticulum [33, 34]. The fact of the S5 fraction don't show inhibition of Ca2+-ATPase activity by 140 μM thapsigargin (Fig. 5) indicates that a Ca2+-ATPase of endoplasmatic reticulum not is present in that fraction. At the same time, the S5 fraction of P. nucleorum larvae did not present any polypeptide with Mr around 100 kDa (Fig. 2), which corresponds to Mr of Ca2+-ATPase of the endoplasmatic reticulum [35]. Ca2+-ATPase activity was also not sensitive to calmodulin (data not shown), a calcium biding protein that connects to the C-terminal region of plasma membrane Ca2+-ATPases and stimulate their activity [36]. Azide, a F1-ATPase inhibitor [37, 38], and ouabain, a Na+/K+-ATPase inhibitor [39] also did not inhibit the Ca2+-ATPase activity of the P. nucleorum larvae S5 fraction (Fig. 5), suggesting that those enzymes also are not present in the ATPase fraction obtained from P. nucleorum larvae cytosol. Ions magnesium inhibit Ca2+-ATPase activity isolated of P. nucleorum larvae particulate fraction (Cruz & Coelho, manuscript in preparation) and inhibited the myosin isolated in this work, but unlike of the particulate fraction ATPase [22], the myosin isolated of P. nucleorum soluble fraction not was inhibited by zinc and it was only slightly inhibited by copper (Fig. 6). In order to verify the specificity of substrate of the enzyme isolated from the P. nucleorum larvae soluble fraction, the S5 fraction was incubated in a reaction medium containing AMP, ADP or GTP instead of ATP. Apyrase hydrolyze the terminal phosphate of both ATP and ADP [40] and also did not seem to be present in the ATPase fraction of P. nucleorum larvae. Not was detected hydrolysis of pyrophosphate in the S5 fraction suggested the absence of pyrophosphatase.

In this work, we isolated a polypeptide (p205) from the soluble fraction of P. nucleorum larvae, which present Mr similar the myosin II and V weight chain. Similar myosin II and V, p205 was precipitated by freezing and thawing of soluble fractions and expressed actin-stimulated Mg-ATPase activity. p205 co-purified with a polypeptide that presented Mr similar the actin (43kDa). p205 and p43 were identified, respectively, as myosin and actin though of PMF analysis.

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