Agelotoxin: a phospholipase A$_2$ from the venom of the neotropical social wasp cassununga (*Agelaia pallipes pallipes*) (Hymenoptera-Vespidae)

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Abstract

The neotropical wasp *Agelaia pallipes pallipes* is aggressive and endemic in southeast of Brazil, where very often it causes stinging accidents in rural areas. By using gel filtration on Sephadex G-100, followed by high performance reversed phase chromatography in a C-18 column under acetonitrile/water gradient, the agelotoxin was purified: a toxin presenting phospholipase A$_2$ (PLA$_2$) activity, which occurs under equilibrium of three different aggregation states: monomer (mol. wt 14 kDa), trimer (mol. wt 42 kDa) and pentamer (mol. wt 74 kDa).

The enzyme presents high sugar contents attached to the protein chain (22% [w/w]) and a transition of the values of pH optimum for the substrate hydrolysis from 7.5 to 9.0, under aggregation from monomer to pentamer. All the aggregation states present Michaelian steady-state kinetic behavior and the monomer polymerization caused a decreasing of phospholipasic activity due a non-competitive inhibition promoted by the formation of a quaternary structure. The PLA$_2$ catalytic activity of agelotoxin changes according to its state of aggregation (from 833 to 12533 μmol mg$^{-1}$ min$^{-1}$) and both the monomeric and oligomeric forms present lowest activities than the PLA$_2$ from *Apis mellifera* venom and hornetin from *Vespa basalis*. Agelotoxin is also a very potent direct hemolysin; the monomer of agelotoxin presented hemolytic actions until 200 times higher than the PbTx from *P. paulista*, 740 times higher than the PLA$_2$ from *A. mellifera*, 570 times higher than that of neutral PLA$_2$ from *N. nigricolis* and about 1250 times than that of carditoxin from *Naja naja atra* venom. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Hymenoptera venoms are complex mixtures of biochemically and pharmacologically active components such as biogenic amines, peptides and proteins (Nakajima, 1986). The composition of vespid venoms have been subject to little investigation, since the production of venoms by the social wasps is very reduced and there is a limited availability of vespid venoms as raw materials.

It has been shown that the Vespinae venoms contain many different components such as phospholipases A and B, hyaluronidases, acid phosphatases, proteases and nucleotidases (Nakajima, 1986). The compositions of the various Vespinae venoms are rather similar each other (Habermann, 1972), however, very few is known about the neotropical Polistinae venoms.

It has been demonstrated that vespid venoms frequently cause allergic reactions in humans (Hoffman, 1985; Reisman and Osur, 1987; Castro et al., 1994) and the PLA2 are recognized as one of the major allergens from these venoms (Hoffman, 1978; 1985).

PLA2 (E.C. 3.1.1.4) catalyzes the specific hydrolysis of ester bonds at the C2 position of 1,2-diacyl-3-sn-glycerophospholipids into their corresponding lyso compounds with release of free fatty acids. Thus, PLA2 is able to disrupt the phospholipid packings from several types of biological membranes, leading to pore formation and/or cell lysis (Dotimas and Hider, 1987).

Recently we described the purification and biochemical characterization of the polybitoxins from the venom of the South American social wasp Polybia paulista, presenting PLA2 activity and very potent hemolytic actions in washed red cells (Oliveira and Palma, 1998). The aim of present paper is to describe the purification and some biochemical properties of a novel toxin from the venom of a neotropical social wasp presenting PLA2 and hemolytic activities: the agelotoxin from Agelaia pallipes pallipes, which naturally occurs in different states of aggregation (monomer, trimer and pentamer). This toxin is probably among the most powerful hemolysins of animal venoms already known.

2. Material and methods

2.1. Biological material and venom extraction

Workers of A. pallipes pallipes were captured in the University Campus, at Rio Claro, SP, southeast of Brazil. The freshly collected wasps were immediately frozen and dissected. The venom reservoirs were removed from the sting apparatuses by pulling with forceps and cutting with microscissors, under a stereomicroscope, minimizing contamination from extraneous tissues. The reservoirs were then carefully washed in a small volume of isotonic solution, thawed, punctured, followed by several washings with distilled water to extract the venom and followed by centrifugation at 12,000g, during 15 min at 4°C; the supernatant was freeze dried and kept at −80°C until be used.
2.2. Protein assay

Protein was determined by the method of Lowry (Hartree, 1972), using BSA as standard.

2.3. Determination of phospholipase specificity

In order to determine the type of phospholipase activity the crude venom of *A. pallipes pallipes* and the purified toxins were incubated at 37°C in presence of natural and synthetic phospholipids as substrates: egg phosphatydylcholine, egg lysophosphatydylcholine, 1-stearoyl-2-oleoyl-3-sn-glycero phosphophoryl choline and 1-oleoyl-2-stearoyl-3-sn-glycerophosphorylcholine (Sigma Chem. Co.). Fine suspensions of phospholipids were prepared by sonication in 1 mM Tris–HCl (pH 7.9), containing 100 mM sodium chloride, 20 mM potassium chloride, 10 mM calcium chloride and 0.5% (v/v) Triton X-100 in an ultrasonic bath. For the purpose of identification of products formed by phospholipase digestion, the same buffer as described above was used, but the concentration of Tris–HCl was increased to 50 mM. The digests were examined by thin-layer chromatography (TLC) on silica gel plates (Whatman LK6DF) as described by King et al. (1984): 35 µl (1.5 mg ml⁻¹ phospholipid) was applied to the pre-adsorbent zone and the plate was developed in chloroform–methanol–0.1 N HCl (60:35:5). The spots were visualized by exposure to iodine vapor for detection of monoacyl phospholipids. The digest were also examined directly for the presence of saturated fatty acids by chromatography on freshly prepared silica gel plates which had been dipped in 5% (w/v) AgNO₃ and then dried at 110°C for 1 h. After developing in presence of hexane–diethyl ether–acetic acid (70:30:1), the spots were visualized under UV light after spraying with 0.2% (w/v) dichlorofluorescein in ethanol. The procedure described above was also applied to PLA₂ from honeybee venom (Sigma Chem. Co.) in order to be used as a control experiment.

2.4. Phospholipase A₂ activity

The assays were routinely carried out by using a spectrophotometric method based on pH change due to the liberation of fatty acids, as described by Araujo and Radvanyi (1987). The reaction medium contained 15 µmol phosphatidylycholine, 18 µmol Triton X-100, 5 µmol calcium chloride, 80 µmol phenol red and 7.5 µmol Tris in a final volume of 2.5 ml, at pH 7.9. The absorbance was initially read at 558 nm against a proper reference; the reaction was initiated by addition of either crude venom or purified toxins. The decrease in the absorbance of phenol red, caused by the acidification of medium was measured after 5 min minutes of incubation at 37°C. The ΔA₅₅₈ was proportional to the liberation of fatty acids in the assay conditions. One unit of PLA₂ activity was defined as the amount of enzyme necessary to hydrolyse 1 µmol phosphatidylycholine h⁻¹ in 1 ml of the reactional medium at pH 7.9 and 37°C.
2.5. Hemolysis

Direct hemolytic activity was assayed on washed red cells of mouse by slight modifications in the procedure described by Ho and Ko (1988). The toxins were dissolved in 0.14 M saline Tris (0.01 M) buffered at pH 7.4 and the red cells, suspended in the same solution at the hematocrit 50%, were mixed and incubated at 37°C for 60 min. The hemolysis was stopped by addition of cooled (4°C) Tris buffered saline to a final volume of 5 ml and the degree of hemolysis was determined by measuring the released hemoglobin at 545 nm. Similarly, control samples were incubated in absence of the toxins, by hemolysing the red cells in water under similar conditions. The hemolytic potency was expressed as percent of hemolysis, assuming the lysis in water to be equal 100% in the given incubation time. The determinations above were run in triplicate at the end of five independent preparations. The results are expressed as means ± S.E.

2.6. Ageletoxin purification

All purification steps were carried out at 0–4°C, unless specified. The freeze dried venom (11.65 mg) was solubilized in 5 mM ammonium acetate pH 6.8 and applied to a Sephadex G-100 (51.0 × 2.5 cm) previously equilibrated with the same buffer. Elution was performed with 5 mM ammonium acetate pH 6.8 at a flow rate of 12 ml h⁻¹ and fractions of 3 ml were collected in presence of 100 μl of 10% (v/v) glycerol (previously added to each collection tube). Protein elution was monitored along the profile of elution by measuring the absorbance at 280 nm and PLA₂ activity assayed as described above for each collected fraction. The fractions presenting phospholipasic activity were pooled and freeze dried. The pool containing the PLA₂ activity (3.57 mg protein) was then solubilized in 5% (v/v) acetonitrile containing 0.04% TFA and then chromatographed under high performance reversed phase in a semipreparative packed column TOSOH (TSK gel 80-TM)-ODS (300 × 7.8 mm, 10 μm). The elution was performed by using a gradient from 5% (v/v) to 60% MeCN (containing 0.04% TFA), at 38°C during 50 min, under a flow rate of 1.0 ml min⁻¹ and monitored by measurement of absorbance at 280 nm. The fractions of 1.0 ml were collected and those presenting phospholipasic activity were pooled, freeze dried and kept at −16°C until be used. This procedure was carried out in at least five independent preparations.

2.7. Kinetic analysis

Initial velocities (V) were plotted as function of substrate concentration in Lineweaver and Burk plots and the interaction constants for the substrate (h) were determined by the Hill procedure as described by Koshland (1970). The Kₘ and Vₘₐₓ values given in this paper were obtained from linear-square analysis for one of the components of the curve, calculated in at least three independent experiments.
2.8. Molecular weight determination

The molecular weight of the agelotoxins were estimated by chromatography of molecular exclusion in Sephadex G-100 (Andrews, 1964). The reference proteins used were alcohol dehydrogenase, Yeast (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa) and cytochrome C (12.4 kDa).

2.9. Analytical assays

The possible presence of inorganic ions acting as cofactors of PLA2 was analytically assayed by using ICP analysis of the native toxin extensively dialyzed against distilled water. The possible presence of calcium, barium, magnesium, zinc, cobalt and cooper was investigated after acidic acidic digestion of AgTX in closed Teflon pumps in an inductively coupled plasma argon emission spectrometer Perkin Elmer Optima 3000).

2.10. Homogeneity

After purified the homogeneity of agelotoxin was examined by using reduced (pretreated with 5% mercaptoethanol) samples on PAGE-SDS electrophoresis (5–20%) as described by Weber and Osborn (1969).

3. Results

3.1. Purification and molecular properties of PLA2

Qualitative estimation of TLC showed that after 4 h digestion both the phospholipase from the crude venom and purified toxins presented the same results: only unsaturated fatty acid (oleic acid) was released from 1-stearoyl-2-oleoyl-3-\textit{sn}-glycerophosphorylcholine; only saturated fatty acid (stearic acid) was released from 1-oleoyl-2-stearoyl-3-\textit{sn}-gliserophosphorylcholine; only unsaturated fatty acids were released from phosphatidylycholine and no fatty acid was released from the digestion with lysophosphatidylycholine (results not shown). The analysis of digests of PLA2 from honeybee venom revealed very similar results; since the hydrolytic and specificity of this enzyme is well established the results of these control experiments may be used as structural proof of the substrates used and products delivered during the digestions.

The venom from 10,000 workers (11.58 mg protein) was fractionated on a Sephadex G-100 gel filtration column (Fig. 1). About 3.57 mg of protein was eluted into three large overlapped peaks presenting PLA2 activity, with mol. wt changing from 14 to 74 kDa. The active fractions were pooled, lyophilized and PLA2 was further purified by high performance reversed phase chromatography (Fig. 2). Three different fractions presenting PLA2 activities were eluted at different concentrations of MeCN and designated as fractions I, II and III,
respectively. The three fractions together represent 0.1% of the total protein of crude venom (Table 1).

The mol wt. of the three fractions of PLA2 determined through molecular exclusion chromatography in Sephadex G-100 were 14, 42 and 74 kDa, respectively (Fig. 3). Meanwhile, the SDS-PAGE of these fractions resulted in a single and apparently homogeneous protein band, corresponding to the mol. wt of 14 kDa (Fig. 4). The content of carbohydrate of agelotoxin was determined as 22% (w/w) in the three fractions; and in spite to this high carbohydrate content no heterogeneity was observed.

After a week of storage at 4°C, AgTX lost up to 50% of PLA2 activity.

Table 1
Purification records of PLA2 from the venom of the social wasp Agelaia p. pallipes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (µg ml⁻¹)</th>
<th>Total protein (µg)</th>
<th>Activity (U ml⁻¹)</th>
<th>Total units (U)</th>
<th>Specific activity (U µg⁻¹)</th>
<th>Purification (factor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude venom</td>
<td>2.2</td>
<td>5299</td>
<td>11658.0</td>
<td>2756</td>
<td>6063</td>
<td>0.52</td>
<td>–</td>
</tr>
<tr>
<td>Sephadex G-100 and freeze drying</td>
<td>3.0</td>
<td>1190</td>
<td>3570.0</td>
<td>1773</td>
<td>5319</td>
<td>1.49</td>
<td>3</td>
</tr>
<tr>
<td>RP (C 18)-HPLC:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AgTX-I</td>
<td>1.5</td>
<td>5.0a</td>
<td>7.5a</td>
<td>315</td>
<td>473</td>
<td>63</td>
<td>121</td>
</tr>
<tr>
<td>AgTX-II</td>
<td>1.6</td>
<td>2.0a</td>
<td>3.2a</td>
<td>101</td>
<td>161</td>
<td>50</td>
<td>97</td>
</tr>
<tr>
<td>AgTX-III</td>
<td>2.7</td>
<td>0.4a</td>
<td>1.1a</td>
<td>306</td>
<td>826</td>
<td>751</td>
<td>1445</td>
</tr>
</tbody>
</table>

a These values were measured and corrected by calculations, taking into account the relative numbers of molecular species, due to the contribution of monomers into oligomeric forms.
However, when kept at 4°C in presence of glycerol, the phospholipasic activity was maintained for at least one month. To remove glycerol, the preparation was quickly dialysed in the presence of 40 mM Tris, pH 7.8–8.3. The specific activities of the agelotoxins in presence of 2.5 mM Ca²⁺ ions, were 63, 50 and 32 μmol mg⁻¹ min⁻¹ for the fractions I, II and III, respectively.

The purified toxin as fractions I, II and III were submitted to atomic absorption analysis revealing the presence of Ca²⁺ ions associated to the three fractions presenting PLA₂ activities. No other inorganic cofactor was observed in the native agelotoxin under the analytical conditions.
Fig. 3. MW determination of native AgTX under molecular exclusion chromatography in Sephadex G-100 column.

Fig. 4. SDS-PAGE under reducing conditions of purified agelotoxin (monomer, trimer and pentamer), obtained in a 5-20% linear gradient gel. The lane of standards contained the following MW markers: bovine serum albumin (66 kDa), ovalbumin (45 kDa), trypsinogen (24 kDa), β-lactoglobulin (18 kDa) and lysozyme (14 kDa).
3.2. Kinetic characterization of PLA₂ activity

The PLA₂ activities of the three fractions are dependent of low Ca²⁺ ions concentration (0.2 mM). When the assays were repeated in presence both of 0.2 mM of Ca²⁺ and 1 mM EDTA, no activity was observed. Assays in the presence of 1 mM of Zn²⁺, 2 mM Cu²⁺ and Co²⁺ inhibited all the phospholipasic A₂ activities. The optimal pH values for the hydrolysis of phosphatydylcholine by the fractions I, II and III were 7.5, 8.0 and 9.0, respectively (not shown results).

Fig. 5 shows double-reciprocal plots of initial velocities, at pH 7.9, as a function of phosphatydylcholine concentration for each fraction of PLA₂. It can be observed that these plots produced straight lines, whose were characterized by Hill coefficients around 1.0 (Table 2). Since the kinetics of phosphatydylcholine hydrolysis were Michaelian, the constant values expressing the affinity for the substrate binding to the enzyme were expressed as $K_m$, with a value of $2.2 \times 10^{-4}$ M for all fractions (Table 2). The $V_{max}$ values decreased from the fraction I to fractions II and III, like a non-competitive inhibition; the $V_{max}$ values obtained for the fractions I, II and III were 330, 200 and 163 μmol ml⁻¹ h⁻¹, respectively.

Table 2
Summary of some steady state kinetic parameters for the hydrolysis of phosphatidylcholine as substrate at pH 7.9 by the different states of aggregation of agelotoxins from the venom of A. pallipes pallipes

<table>
<thead>
<tr>
<th>AgTX form</th>
<th>$K_m$ (M)</th>
<th>$V_{max}$ (μmol ml⁻¹ h⁻¹)</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>$2.2 \times 10^{-4}$</td>
<td>330</td>
<td>1.0</td>
</tr>
<tr>
<td>Trimer</td>
<td>$2.2 \times 10^{-4}$</td>
<td>200</td>
<td>1.2</td>
</tr>
<tr>
<td>Pentamer</td>
<td>$2.2 \times 10^{-4}$</td>
<td>163</td>
<td>1.1</td>
</tr>
</tbody>
</table>
3.3. Hemolysis

The hemolytic activities of AgTX were dependent on Ca\(^{2+}\) ions (0.2 mM). In Table 3, it may be observed that AgTX was extremely potent in lysing red cells in Tris-buffered saline (direct hemolysis). It must be emphasized that AgTX was capable of lysing the membrane phospholipids per se, i.e., without combination with any other component. The direct hemolytic activities on mouse RBC of agelotoxins were 100% at 3.3 \(\mu\text{g} \text{ml}^{-1}\) fraction I, 33% at 0.5 \(\mu\text{g} \text{ml}^{-1}\) fraction II and 19% at 0.7 \(\mu\text{g} \text{ml}^{-1}\) fraction III (Table 3).

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Concentration ((\mu\text{g} \text{ml}^{-1}))</th>
<th>Direct hemolysis (%) mouse RBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA2 from A. pallipes pallipes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monomer</td>
<td>3.3</td>
<td>100</td>
</tr>
<tr>
<td>Trimer</td>
<td>0.5</td>
<td>33</td>
</tr>
<tr>
<td>Pentamer</td>
<td>0.7</td>
<td>19</td>
</tr>
<tr>
<td>Polybitoxins from the social wasp P. paulista(^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PbTX-I</td>
<td>100.0</td>
<td>62</td>
</tr>
<tr>
<td>PbTX-II</td>
<td>100.0</td>
<td>57</td>
</tr>
<tr>
<td>PbTX-III</td>
<td>100.0</td>
<td>90</td>
</tr>
<tr>
<td>PbTX-IV</td>
<td>1.0</td>
<td>60</td>
</tr>
<tr>
<td>Hornetin from the wasp V. flavitarsus(^b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lethal Toxin from V. basalis(^b)</td>
<td>100.0</td>
<td>96</td>
</tr>
<tr>
<td>PLA2 from the honey bee Apis mellifera</td>
<td>100.0</td>
<td>27</td>
</tr>
<tr>
<td>Neutral PLA2 from the snake N. nigricolis(^b)</td>
<td>100.0</td>
<td>35</td>
</tr>
<tr>
<td>Basic PLA2 from the snake N. nigricolis(^b)</td>
<td>100.0</td>
<td>92</td>
</tr>
<tr>
<td>Cobra cardotoxin from the snake N. naja atra(^b)</td>
<td>100.0</td>
<td>16</td>
</tr>
</tbody>
</table>

\(^a\) From Oliveira and Palma (1998).
\(^b\) From Ho and Ko (1988).

4. Discussion

Phospholipases A have been detected in several different organisms, specially the enzymes of A\(_2\)-type from animal venoms, that are responsible for several myotoxic and/or neurotoxic effects of these venoms (Ho and Ko, 1988). However, the most of PLA\(_2\) from the Hymenopteran venoms have been poorly characterized from a biochemical point of view and the most of attention has been focused on their immunological properties.

From the characterization of phospholipase type experiments performed in the present study, is evident that the site of hydrolysis of the acyl group is at 2-position, both for the phospholipase activity in the crude venom and for the purified toxins. Thus, we purified and characterized three different aggregation
states of a PLA$_2$ toxin from the venom of a neotropical social wasp _A. pallipes_.

About 67% of the original activity was delivered from the crude venom with a degree of purification that changed from 62 to 121-fold, depending on aggregation state (Table 1). The total amount of AgTX in the crude venom of _A. pallipes_ (0.10%), is more reduced than that observed for the PLA$_2$ from the venoms of _P. paulista_ (1.1%) (Oliveira and Palma, 1998), _Apis mellifera_ (10–12%) (Dotimas and Hider, 1987) and _Vespa basalis_ (6%) (Ho and Ko, 1988).

The mol wt. determination of native toxin clearly suggests that the AgTX do exist in three different aggregation states: monomeric, trimeric and pentameric, with mol wt. 14 kDa, 42 kDa and 74 kDa, respectively, where the monomer is the predominant form (Table 1). The dissociation of polymeric forms of AgTX to the monomeric state required denaturating conditions, suggesting that the trimer and pentamer are stable enough to be considered as artifact of manipulation. These results suggest that the monomers, trimers and pentamers of native agelotoxin may coexist under equilibrium in physiological conditions.

The mol. wt of AgTX is different from those previously described for the phospholipases from other Hymenopteran venoms, such as: from 115 to 132 kDa for PbTX (Oliveira and Palma, 1998), 36 kDa for PLA$_2$ of _Polistes exclamans_ (King et al., 1984) and 32 kDa for lethal protein of _V. basalis_ (Ho and Ko, 1988).

The relative amount of carbohydrates attached to the protein [22% (w/w)] is lower than those observed for PbTX-I, -II and III (from 29 to 43%; Oliveira and Palma, 1998); however, is similar to that observed for PbTX-IV (22%; Oliveira and Palma, 1998) and much higher than the 8% previously described for the PLA$_2$ from the venom of _A. mellifera_ (Banks and Shipolini, 1986).

The specific activities of the different aggregational states of agelotoxin for the hydrolysis of phosphatydylcholine were lower than those described for the hornetin from _V. basalis_ (3800 μmol mg$^{-1}$ min$^{-1}$) and PLA$_2$ from honey bee venom (1471 μmol mg$^{-1}$ min$^{-1}$); however AgTX is both more active than PbTX (II, III and IV) from _P. paulista_ venom (from 28 to 372 μmol mg$^{-1}$ min$^{-1}$) and the basic PLA$_2$ from _Naja nigrilolis_ venom (323 μmol mg$^{-1}$ min$^{-1}$) (Ho and Ko, 1988).

The phospholipase A$_2$ activities of AgTX are dependent of calcium ions. Copper, zinc, cobalt and silver ions were potent inhibitors of the catalytic activities of AgTX as previously described for the PLA$_2$ polybitoxins from the venom of _P. paulista_ (Oliveira and Palma, 1999) and PLA$_2$ orientotoxin from the venom of giant hornet _Vespa orientalis_ (Tuichibaev et al., 1988). The kinetics of hydrolysis of phosphatydylcholine at pH 7.9 by AgTX producing straight lines in the double reciprocal plots (Fig. 4) with Hill coefficients around 1.0 suggests a Michaelian behavior and the existence of only one catalytic site for the hydrolysis of phosphatydylcholine. The PLA$_2$ activity of monomeric AgTX suffered a non-competitive inhibition when polymerized into trimer and/or pentamer, suggesting that the interactions between the tertiary structures of each monomer occurs in a region which influences directly the catalytic site, decreasing its activity. In spite to
this, apparently no effect occurred at level of substrate binding site since the \( K_m \) value was maintained constant.

The AgTX exhibited extremely potent hemolytic action on red cells incubated in Tris-buffered saline (direct hemolysis) (Table 3). The agelotoxin is a hemolysin more potent than the most of lytic factor known from social wasps, honeybees and snake venoms.

It should be noted that AgTX exhibits hemolytic action from 10 to 200 times higher than the polybitoxins from \( P. \) Paulista, from 24 to 740 times that of PLA\(_2\) from \( A. \) mellifera, from 20 to 570 times higher than that of neutral PLA\(_2\) from \( N. \) nigricolis and from 41 to 1250 times than that of cardiotxin from \( Naja \) naja atra.

The high hemolytic activities of AgTX against washed erythrocytes suggest that this toxin is capable of lysing biological membranes without the presence of exogenous phospholipids or special polypeptides as is required by the PLA\(_2\) from honey bee venom (Lawrence and Moores, 1975; Banks and Shipolini, 1986); this activity is inhibited by Cu\(^+2\) and Zn\(^+2\). Thus, in regard to these aspects, the agelotoxin seems to be similar to other PLA\(_2\) toxins from Vespidae/Vespinae venoms.

The results above suggest that AgTX, purified from the venom of the neotropical social wasp \( A. \) pallipes pallipes, occurs in three different aggregation states (monomer, trimer and pentamer), presents PLA\(_2\) activity and some molecular properties, such as: mol. wt and content of sugar attached to the molecules, different from the most Hymenopteran PLA\(_2\) known actually. A comparison of the direct hemolytic activities of AgTX with the hemolytic factors from other sources revealed that the agelotoxin probably is the most potent hemolysin known in the animal venoms. The toxin polymerization seems to be followed by decreasing both of its catalytic activity for phosphatidylcholine hydrolysis and hemolysis of mouse RBC, suggesting that the aggregation may be part of the mechanism of regulation of AgTX activity. \( A. \) pallipes pallipes is a very aggressive social wasp found in southeast of Brazil, that causes several stinging accidents throughout the year, especially in the rural area. In spite of this, and considering its high hemolytic activity, the low incidence of fatal stingings caused by this wasp may be explained by the very reduced amount of AgTX in this venom (0.10\% of the total protein from the crude venom). However, cases of multiple stingings are not uncommon and are generally followed by severe hemolysis and myocardial dysfunction. In this situation, the described symptoms may be caused by the accumulation of high levels of AgTX.

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