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Nucleotidase and DNase activities in Brazilian snake venoms

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Abstract

Among the myriad of enzymes present in animal venoms, nucleotidases and nucleases are poorly investigated. Herein, we studied such enzymes in 28 crude venoms of animals found in Brazil. Higher levels of ATPase, 5′-nucleotidase, ADPase, phosphodiesterase and DNase activities were observed in snake venoms belonging to *Bothrops*, *Crotalus* and *Lachesis* genera than to *Micrurus* genus. The venom of *Bothrops brazili* snake showed the highest nucleotidase and DNase activities, whereas that of *Micrurus frontalis* snake the highest alkaline phosphatase activity. On the other hand, the venoms of the snake *Philodryas olfersii* and the spider *Loxosceles gaucho* were devoid of most nucleotidase and DNase activities. Species that exhibited similar nucleotidase activities by colorimetric assays showed different banding pattern by zymography, suggesting the occurrence of structural differences among them. Hydrolysis of nucleotides showed that 1 mol of ATP is cleaved in 1 mol of pyrophosphate and 1 mol of orthophosphate, whereas 1 mol of ADP is cleaved exclusively in 2 mol of orthophosphates. Pyrophosphate is barely hydrolyzed by snake venoms. Phosphodiesterase activity was better correlated with 5′-nucleotidase, ADPase and ATPase activities than with DNase activity, evidencing that phosphodiesterases are not the main agent of DNA hydrolysis in animal venoms. The omnipresence of nucleotidase and DNase activities in viperid venoms implies a role for them within the repertoire of enzymes involved in immobilization and death of preys.

Keywords: Phosphomonoesterase; Exonuclease; Nucleoside triphosphate diphosphohydrolase (NTPDase); Nucleotide pyrophosphatase/phosphodiesterase (NPP); Apyrase; CD39; CD73; Snake; Venom

1. Introduction

Venom delivery systems have evolved in reptiles, spiders and other animals so that the injection of their venomous secretion serves as an effective means to immobilize and kill preys. To accomplish that function, a variety of proteins and enzymes have evolved in venoms (Escoubas, 2006; Fry et al., 2006). Among them, enzymes that are capable of hydrolyzing nucleic acids and their derivatives, the nucleotides, are also present in animal venoms (Mackessy, 1998; Rael, 1998; Aird, 2002; Rash and Hodgson, 2002; Hogan et al., 2004). Phosphodiesterases have been reported in snake venoms since 1930s (Gulland and Jackson, 1938), and henceforth 5′-nucleotidases, ADPases, ATPases, endonucleases, exonucleases, and alkaline and acid phosphatases have also been reported therein. Phosphodiesterases have also been known as exonucleases due to their capacity to hydrolyze phosphodiester bonds in a progressive fashion, beginning at the 3′-end of nucleic acids (Mackessy, 1998). However, many snake venoms also contain enzymes that can cleave internal phosphodiester bonds in nucleic acids (Georgatsos and Laskowski, 1962; Sittenfeld et al., 1991; Mackessy, 1998; de Roodt et al., 2003). Phosphodiesterases have also been shown to degrade ADP and ATP (Razzell, 1963), but *Bitis arietans* snake venom contains an enzyme with ADPase activity without phosphodiesterase activity (Schenberg et al., 1978).

Phosphate-releasing enzymes present in venoms can interfere with signaling and balance of extracellular nucleotides and nucleosides, not only by cleaving phosphate esters of nucleic acids, but also by degrading nucleoside 5′-tri, -di, and -monophosphates, namely adenylated nucleotides (ATP, ADP and AMP), and thereby evoking several pathophysiological disturbances in preys (Aird, 2002). Single enzymes were initially assumed to be capable of hydrolyzing ATP and ADP (ATPases and ADPases, respectively), and in fact this classification still prevails in

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literature. However, nucleotides are currently known to be hydrolyzed by four families of enzymes in mammalians. (1) Members of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family present not only alkaline phosphodiesterase, but also nucleotide pyrophosphatase activity, which are properties of the same enzyme molecule. They are capable of hydrolyzing 3′,5′-cAMP to AMP, ATP to AMP and pyrophosphate, and ADP to AMP and orthophosphate. Moreover, they can hydrolyze phosphodiester bonds of nucleic acids and the pyrophosphate linkages of nucleotide sugars (Zimmermann, 2000, 2001; Stefan et al., 2005; Zimmermann, 2006). (2) Alkaline phosphatases represent a protein family of nonspecific ecto-phosphomonoesterases. They reveal broad substrate specificity, degrading nucleoside 5′-tri, -di, and -monophosphates, as well as releasing inorganic phosphate from pyrophosphate and a variety of organic compounds, including proteins. One single enzyme could thus catalyze the entire hydrolysis chain from a nucleoside-5′-triphosphate to the respective nucleoside (Fernley, 1971; Zimmermann, 2001, 2006). (3) Ecto-5′-nucleotidases catalyze the hydrolysis of nucleoside 5′-monophosphates to the respective nucleosides and orthophosphate. Nucleoside 5′-monophosphates are also subject to hydrolysis by alkaline phosphatases and by some members of the E-NPP family, especially autotoxin, a secreted form of NPP2. However, ecto-5′-nucleotidases likely represent the major enzyme responsible for the formation of extracellular nucleoside from nucleoside 5′-monophosphates (Zimmermann, 1992; Clair et al., 1997; Zimmermann, 2000, 2001, 2006). (4) Members of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family hydrolyze nucleoside 5′-triphosphates and -diphosphates, albeit with considerable difference in their preference for the individual type of nucleotide. E-NTPDases are firmly anchored to the plasma membrane (Zimmermann, 2001, 2006).

Despite the ubiquity of 5′-nucleotidases, phosphodiesterases and alkaline phosphatases in animal venoms (Tan and Ponnudurai, 1991a,b; Tan and Ponnudurai, 1992a,b; Aird, 2002), questions about how these enzymes pass into venoms or what are their function therein have been poorly discussed in literature. Moreover, no research has globally compared and correlated nucleotidase, DNase, phosphodiesterase and alkaline phosphatase activities in animal venoms. Thus, herein we screened these activities in venoms from several animals found in Brazil, including species never tested earlier for phosphate-releasing enzymes, using a variety of methodological approaches, and thereafter we verified if they act similarly as the above mentioned family of mammalian nucleotidases. This is an initial effort to characterize such enzymes in snake venoms and their roles.

2. Material and methods

2.1. Venoms

Lyophilized or dried snake venoms were obtained from the Laboratory of Herpetology, Institute Butantan, and maintained at −20 °C. From native Brazilian venomous snakes, the following venoms were used: (1) rattlesnakes (Viperidae, Crotalinae) — *Crotalus durissus cascavella*, *Crotalus durissus collilineatus* and *Crotalus durissus terrificus;* (2) lance-headed snakes (Viperidae, Crotalinae) — *Bothrops alcatraz*, *Bothrops alternatus*, *Bothrops atrox*, *Bothrops brazili*, *Bothrops cotiara*, *Bothrops diporus*, *Bothrops erythromelas*, *Bothrops fonsecai*, *Bothrops insularis*, *Bothrops itapetiningae*, *Bothrops jararaca*, *Bothrops jararacussu*, *Bothrops mattogrossensis*, *Bothrops marajoensis*, *Bothrops moojeni*, *Bothrops neuwiedi*, *Bothrops pauloensis*, *Bothrops pirajai*, *Bothrops pradoi*, *Bothrops pubescens* and *Bothrops taeniatus* (formerly *Bothrops castelnaudi*); (3) bushmaster (Viperidae, Crotalinae) — *Lachesis muta muta*; (4) coral snake (Elapidae, Elapinae) — *Micrurus frontalis*. Besides, we also analyzed venoms from the (5) green snake *Philodryas olfersii* (opisthoglypha) (Colubridae, Xenodontinae) and the (6) brown spider *Loxosceles gaucho* (Sicariidae). The following venoms from exotic species were analyzed for comparison: *Crotalus adamanteus* (a North American rattlesnake) and *Crotalus durissus cumanensis* (Venezuelan rattlesnake).

2.2. Colorimetric assay of orthophosphate

To assay ATPase, ADPase and 5′-nucleotidase activities in venoms, we modified a previously described methodology (Sulkowski et al., 1963), which determines orthophosphate liberation from nucleotides. Each venom was two-fold serially diluted in incubation buffer (0.1 M glycine–NaOH, pH 8.9), in the range of 2 mg/mL to 1 μg/mL, and maintained in an ice bath until use. Samples (50 μL) of each venom dilution and blank (incubation buffer) were added in triplicate to 96-well microplates (Nunc, USA), and incubated with 50 μL of 1 mM ATP (disodium salt, Sigma A5394, USA), ADP (sodium salt, Sigma A2754, USA) or AMP (sodium salt, Sigma A1752, USA) in incubation buffer containing 3.8 mM $MgCl_2$. Aliquots (100 μL, triplicate) of orthophosphate solutions (KH_2PO_4, ranging from 0.8 to 48.4 nmol) were used for construction of standard curves. Microplates were incubated at 37 °C for 15 min in a humid chamber, and then the liberated orthophosphate was assayed by adding 140 μL of color reagent (81.1 mM ascorbic acid, 3 mM ammonium molybdate, and 0.5 M H_2SO_4) (Ames, 1966). Microplates were placed in the center of a household microwave oven and irradiated for 30 s on the highest potency, accompanied by a 250-mL erlenmeyer filled with 200 mL of water at room temperature. Absorbance was immediately read at 820 nm in a SpectraMax 190 microplate reader (Molecular Devices, USA), and values were plotted versus venom concentration; the amount of venom that liberated 20 nmol orthophosphate was used for comparison. Thereafter, specific activities of nucleotidases on ATP, ADP and AMP were calculated and expressed in μmol of liberated orthophosphate/min/mg of venom.

Pyrophosphatase activity in venoms of representative species (*B. jararaca*, *B. brazili*, *L. muta muta*, *C. adamanteus*, *C. durissus terrificus* and *M. frontalis*) was determined similarly as described above, but the substrate used for the assay was 1 mM sodium pyrophosphate.

2.3. Nucleotidase assay by zymography

Zymography (Picher et al., 1993) was also employed to characterize ATPase, ADPase and 5′-nucleotidase activities of