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Purification and structural characterisation of phospholipase A_1 (Vespapase, Ves a 1) from Thai banded tiger wasp (*Vespa affinis*) venom

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ABSTRACT

The Thai banded tiger wasp (*Vespa affinis*) is one of the most dangerous vespid species in Southeast Asia, and stinging accidents involving this species still cause fatalities. In the present study, four forms of *V. affinis* phospholipase A_1 were identified through a proteomics approach. Two of these enzymes were purified by reverse-phase chromatography, and their biochemical properties were characterised. These enzymes, designated Ves a 1s, are not glycoproteins and exist as 33441.5 and 33474.4 Da proteins, which corresponded with the 34-kDa band observed via SDS-PAGE. The thermal stabilities of these enzymes were stronger than snake venom. Using an *in vivo* assay, no difference was found in the toxicities of the different isoforms. Furthermore, the toxicity of these enzymes does not appear to be correlated with their PLA_1 activity. The cDNAs of the full-length version of Ves a 1s revealed that the Ves a 1 gene consists of a 1005-bp ORF, which encodes 334 amino acid residues, and 67- and 227-bp 5′ and 3′ UTRs, respectively. The two isoforms are different by three nucleotide substitutions, resulting in the replacement of two amino acids. Through sequence alignment, these enzymes were classified as members of the pancreatic lipase family. The structural modelling of Ves a 1 used the rat pancreatic lipase-related protein 2 (1bu8A) as a template because it has PLA_1 activity, which demonstrated that this enzyme belongs to the α/β hydrolase fold family. The Ves a 1 structure, which is composed of seven α-helixes and eleven β-strands, contains the β-strand/εSer/α-helix structural motif, which contains the Gly-X-Ser-X-Gly consensus sequence. The typical surface structures that play important roles in substrate selectivity (the lid domain and the β9 loop) were shortened in the Ves a 1 structure, which suggests that this enzyme may only exhibit phospholipase activity. Moreover, the observed insertion of proline into the lid domain of the Ves a 1 structure is rare. We therefore propose that this proline residue might be involved in the stability and activity of Ves a 1s.

1. Introduction

Hymenoptera venoms are well-known to contain a variety of major protein allergens, including antigen 5, phospholipase A_1B, hyaluronidase and protease (Abe et al., 2000; Hoffman, 1978; King et al., 1984). These components are reported to be life-threatening and accelerate fatal anaphylactic reactions in allergic patients. Phospholipase A_1s (PLA_1s) hydrolyse the *sn*-1 position of the phospholipid packing of several types of biological membranes, thereby releasing lysophospholipids and free fatty acids as reaction products. Vespid PLA_1s cause local inflammatory reactions

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(Ho et al., 1993; King et al., 2003) and act as allergens. In animals, these PLA_1s can also cause severe haemolysis with consequent cardiac dysfunction and death (Ho and Hwang, 1991; Ho et al., 1993; Ho and Ko, 1988). In addition, these enzymes have been reported to activate platelet aggregation and induce thrombosis *in vivo* (Yang et al., 2008). However, until now, the characterisation of these proteins was still incomplete, and their biological functions remained unclear.

All of the extracellular PLA_1s, such as human pancreatic lipase (HPL), lipoprotein lipase (LPL), endothelial lipase (EL), pancreatic lipase-related protein 2 (PLRP2), phosphatidylserine (PS)-specific PLA_1 (PS-PLA_1), and the PLA_1 in vespid venoms, belong to the pancreatic lipase gene family. Carriere et al. (1998) divided the pancreatic lipase gene family into eight subfamilies. Vespid PLA_1s form part of subgroup number eight. PLA_1s from different sources exhibit significant conserved region similarity to HPL and PLRP2. These enzymes all contain the typical catalytic triad residues Ser-His-Asp (Aoki et al., 2007). The activity of these enzymes significantly increases in the presence of Ca^{2+}. Crystallographic studies of HPL show that each lipase is composed of a large N-terminal domain and a smaller C-terminal domain. The large N-terminal domain belongs to the α/β hydrolase fold and is essential for catalytic activity. The Gly-X-Ser-X-Gly region is a consensus sequence that, to date, has only been found in lipases and esterases. The tertiary structure dominants, the β5 and β9 loops and the lid domain, are believed to play an important role in catalysis. The β5 loop is involved in the formation of an oxyanion hole, whereas the β9 loop and the lid domain are involved in substrate recognition and substrate selectivity (Carriere et al., 1998; Winkler et al., 1990).

The Vespinae subfamily of wasps in Thailand includes 18 species. However, the biochemical, pharmacological and immunological characteristics of their venoms are still unclear. *Vespa affinis*, the banded tiger wasp, is mostly distributed in forests throughout Thailand. *V. affinis* is considered to be one of the most dangerous species among vespids. Land abuse contributes to the invasion of these species in human habitats, which results in the many out-of-record stinging accidents that occur every year. In addition, two Asian wasp envenomation cases of *V. affinis* in Nepal have been reported. The symptoms found in these cases were very serious and showed evidence of haemolysis, hepatic dysfunction, oligoanuria and azotaemia (Das and Mukherjee, 2008). A better understanding of PLA_1 wasp venom would prove valuable for both therapeutics and commercial applications. Therefore, in the present work, we have identified, purified, biochemically characterised, sequenced and modelled the PLA_1 isoforms present in the venom of the Thai banded tiger wasp, *V. affinis*.

2. Materials and methods

2.1. Venom collection

Worker wasps were collected from Siang Sao Village (Sri Songkram District, Nakornpanom Province, Thailand) and immediately shocked in ice. The venom reservoirs of the wasps were pulled out and gently squeezed. The droplet of venom that appeared at the tip of the sting was immediately collected in a 1.5-mL microcentrifuge tube. The crude venom was then maintained at −80 °C until use. The protein contents were quantitatively determined by the Bradford method (1976) using bovine serum albumin as the standard.

2.2. Polyacrylamide gel electrophoresis (PAGE)

One-dimensional SDS-PAGE was performed following the standard method using a 13% (w/v) separating gel and a 4% (w/v) stacking gel. Phosphorylase B (97 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as the standards. After the samples were applied to the gel, the proteins were resolved at 150 V for 1 h. The gels were stained with silver staining solution.

For the two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), the crude venom protein (80 μg) or purified protein (20 μg) were mixed with a rehydration solution containing 7 M urea, 2 M thiourea, 2% CHAPS, 7 mg/2.5 mL DTT, 2% IPG buffer and 1% bromophenol blue to obtain a total volume of 125 μL. This solution was applied to IPG dry strips (pH 3-11 NL, GE Healthcare). After rehydration for 12 h, isoelectric focussing (IEF) was performed through the application of 500 V for 30 min, 1000 V for 30 min and 5000 V for 1.4 h at a charge of 50 mA per strip. After the IEF, reduction and alkylation steps were completed, the IPG strips were placed on a 13% (w/v) SDS-PAGE gel, which was used for the second dimension. The electrophoresis was performed for 15 min at 10 mA/gel and then at 20 mA/gel until the dye front reached the bottom of the gel. After staining with colloidal Coomassie Brilliant Blue G-250 or silver nitrate, the gels were scanned using a flatbed scanner. The data were analysed using ImageMaster 2D Platinum, version 5.0 (GE Healthcare, Sweden). The identities of the proteins were determined by mass spectrometry.

2.3. Protein identification by liquid chromatography coupled with mass spectrometry (LC–MS/MS)

This procedure was previously described by Sukprasert et al. (2012). Briefly, the excised spots were washed and trypsinised overnight at 37 °C. After extraction and washing, the digested peptides were separated by nanoscale LC using a NanoAcquity system (Waters Corp., USA), which was equipped with a Symmetry C18 (5 μm, 180-μm × 200-μm) Trap column and a BEH130C18 (1.7 μm, 100-μm × 100-μm) analytical reversed-phase column (Waters Corp., USA). A flow rate of 350 mL/min was used in this separation. Water and acetonitrile, both of which contained 0.1% formic acid, were used as solvents A and B, respectively. All of the samples were analysed in triplicate. The analysis of the tryptic peptides was performed using a SYNAPT™ HDMS mass spectrometer (Waters Corp., UK).

All of the analyses were performed using the positive nanoelectrospray ion mode. The time-of-flight analyser of the mass spectrometer was externally calibrated with [Glu^1] fibrinopeptide B from *m*/*z* 50 to 1600, and the