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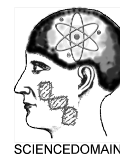

Structure-Activity Relationship of Phospholipase A_2s Isolated from *Vipera aspis* Venom

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Research Article

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ABSTRACT

Aims: To elucidate the structure-activity relationship of snake venom phospholipase A_2, the primary structures of two phospholipase A_2s from *V. aspis* venom were analyzed, and the three-dimensional conformation models were compared.
Study Design: Cross-sectional study.
Place and Duration of Study: Department of Microbiology, Faculty of Pharmacy, Meijo University, between August 2009 and June 2011.
Methodology: The primary structures of purified phospholipase A_2-II and -III were analyzed by Edman sequencing. Three-dimensional models of these enzymes and previously reported phospholipase A_2-I (Vaspin) were constructed by the homology modeling method.
Results: Both phospholipase A_2-II and –III were found to be monomeric proteins which consist of 121 and 122 amino acid residues, respectively. Their primary structures were consistent with the deduced sequence obtained from genomic DNA analysis. The molecular models of both enzymes indicated that the substitution of important amino acid residues for anticoagulant and lethal activity might have caused the relatively weak toxicity.
Conclusion: The structure-activity relationship of PLA_2s was clarified by using molecular models, and clear understanding was obtained.

Keywords: Phospholipase A_2; snake venom; structure; computer model.

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1. INTRODUCTION

Phospholipase A_2 (PLA_2) is the most common enzyme in snake venoms which hydrolyze glycerophospholipids at the *sn*-2 ester bond in a Ca^{2+}-dependent manner (van Deenen and de Haas, 1963). PLA_2s in snake venoms are generally involved with digestive and toxic functions (Valentin and Lambeau, 2000), and more than 150 enzymes have been characterized. These venom PLA_2s are low molecular weight proteins (13-15 kDa) which contain 6-7 disulfide bonds (Six and Dennis, 2000; Schaloske and Dennis, 2006; Burke and Dennis, 2009). They exhibit significant similarity in their three dimensional folding (Scott, 1997), and possess a conserved catalytic network formed by four amino acid residues, His48, Asp49, Tyr52, and Asp99 (Arni and Ward, 1996).

Various biological activities have been found in snake venom PLA_2s such as neurotoxicity, myotoxicity, and anticoagulant activity (Kini, 2003; Lomonte et al., 2009). There are several reports dealing with neurotoxic heterodimeric (or heterotrimeric) PLA_2s which contain an enzymatically active subunit and less active subunit (Mancheva et al., 1984 and 1987; Aird et al., 1985 and 1986; Komori et al., 1996). The primary structures of less active subunits are homologous to PLA_2s; however, they usually lack catalytically important amino acid residues for enzyme activity.

In our previous study, three PLA_2 homologues (PLA_2-I, -II and -III) from *V. aspis aspis* venom were reported (Komori et al., 1990). PLA_2-I was also found in *V. aspis zinnikeri* venom and the isolated enzyme was found to be a heterodimeric neurotoxic lethal enzyme which consists of noncovalently-bound acidic and basic subunits (subunit A and B). The complete primary structure PLA_2-I was reported (Komori et al., 1996), and later, the genes encoding both subunits were identified (Guillemin et al., 2003). Interestingly, genes encoding subunit A and B of PLA_2-I were also found in *V. aspis aspis* and *V. berus berus* snakes (Guillemin et al., 2003), and PLA_2 heterodimer identified in *V. aspis aspis* venom was reported as Vaspin (Jan et al., 2002).

Both PLA_2-II and -III were shown to be monomeric proteins which possess equivalent enzymatic activity with PLA_2-I, however, lethality was not found in either enzyme. Both of them had anticoagulant nature and myotoxicity, but their activities were relatively weak (Komori et al., 1990). To clarify the relationship between the structure and activity, we analyzed the complete amino acid sequence of PLA_2-II and -III the N-terminal sequences of which have been reported previously (Komori et al., 1990). In addition, the molecular models of PLA_2s were constructed and compared.

2. MATERIALS AND METHODS

2.1 Materials

Lyophilized crude venom of *Vipera aspis* was purchased from Latoxan (Rosans, France). Endoproteinase Lys-C (from *Clostridium histolyticum*), chymotrypsin and endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) were purchased from Roche Diagnostics. Metalloendopeptidase (from *Grifola flondosa*) was from Seikagaku Corporation (Tokyo, Japan). The reagents for protein sequencing were supplied by Applied Biosystems. Other chemicals used were of analytical grade and obtained from commercial sources.

2.2 Isolation and S-Pyridylethylation

PLA_2-II and –III were purified from crude venom by the previously reported method (Komori et al., 1990). Isolated enzymes (2 mg of each sample) were dissolved in 1.6 ml of 5 mM Tris-HCl buffer (pH 7.5) containing 8 M urea. 2-Mercaptoethnol (200 µl) was added to each sample and incubated at 37°C for 1 hour under nitrogen. 4-Vinylpyridine (200 µl) was then added to reduce samples and stirred at room temperature for 2 hours. The excess reagents were removed by dialysis.

2.3 Enzymatic Cleavage

Pyridylethylated samples were lyophilized, and digested with various proteases as described below:

Endoproteinase Lys-C:

Samples were incubated with enzyme (4 % by weight) for 5 hours at 37°C in 25 mM Tris-HCl buffer (pH 7.8) containing 1 mM EDTA and 1 M urea.

Chymotrypsin:

Samples were incubated with enzyme (8 % by weight) for 5 hours at 25°C in 0.1 M Tris-HCl buffer (pH 7.8) containing 10 mM $CaCl_2$ and 1 M urea.

Endoproteinase Glu-C:

Samples were incubated with enzyme (2 % by weight) for 5 hours at 25°C in 25 mM ammonium carbonate buffer (pH 7.8) containing 4 M urea.

Metalloendopeptidase:

Samples were incubated with enzyme (1 % by weight) for 10 hours at 70°C in 0.1 M TEA-HCl buffer (pH 9.0) containing 1 M urea.

All digests were separated by reversed-phase HPLC using a Develosil 300 ODS-7 column (4.6 × 250 mm)

2.4 Sequence Analysis

The amino terminal sequence analysis of intact PLA_2s and enzymatically digested fragments were performed with an Applied Biosystems model 491 protein sequencer and model 610A PTH analyzer in accordance with the manufacturer's instructions.

2.5 Homology Modeling

MOE^{TM} (a molecular simulation and modeling software; purchased from Chemical Computing Group Inc.) was used for construction of protein models.

3. RESULTS AND DISCUSSION

3.1 Primary Structure of PLA_2s

The direct amino acid sequence analysis of intact PLA_2-II and -III was performed and the sequence up to residues 46 and 42 were determined, respectively. The enzymatically digested fragments were also analyzed, and by combining all the data, the complete amino acid sequences of PLA_2s were established (Figures 1 and 2). PLA_2-II is composed of 121 amino acids residues and its molecular mass was calculated to be 13566.7 Da. PLA_2-III is composed of 122 amino acids residues and the molecular mass was calculated to be 13653.9 Da. These primary structures are consistent with the deduced sequence obtained from genomic DNA analysis and PLA_2-II and -III are homologous to Ammodytin I2 and Ammodytin I1a identified in *V. ammodytes ammodytes* venom, respectively (Guillemin et al., 2003; Jan et al., 2007).

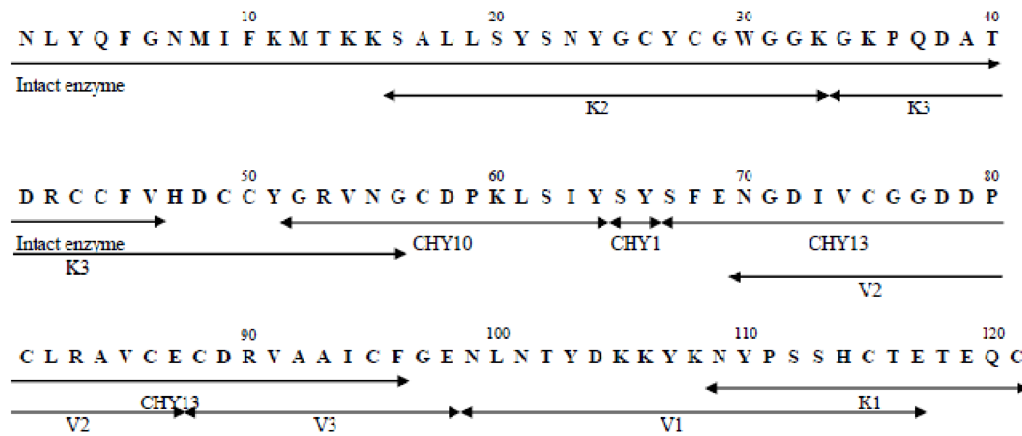

Figure 1. Amino acid sequence of PLA_2-II (Ammodytin I2)
Arrows indicate residues determined by sequence analysis
The following abbreviations are used for the various peptides:
K, endoproteinase Lys-C; CHY, chymotrypsin; V, endoproteinase Glu-C.

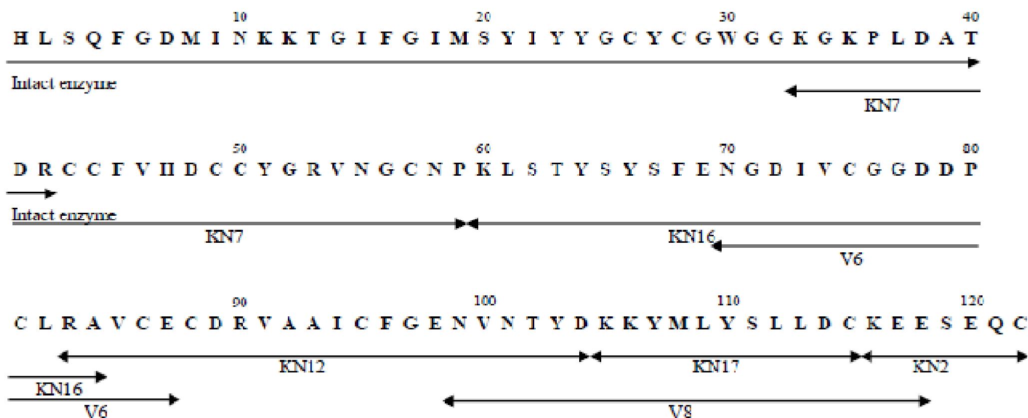

Figure 2. Amino acid sequence of PLA_2–III (Ammodytin I1)
Arrows indicate residues determined by sequence analysis
The following abbreviations are used for the various peptides:KN, metalloendopeptidase; V, endoproteinase Glu-C.

3.2 Comparison of Primary Structures of Pla$_2$s from Snake Venoms

The primary structures of PLA$_2$-II and –III were compared with PLA$_2$-I (Vaspin), and other venom PLA$_2$s (Figure 3). Sequences were aligned according to the numbering system of Renetseder et al., 1985. Both PLA$_2$-II and –III possess a conserved catalytic network formed by four amino acid residues (His48, Asp49, Tyr52 and Asp99), and calcium-binding site (Tyr28, Gly30, Gly32 and Asp49).

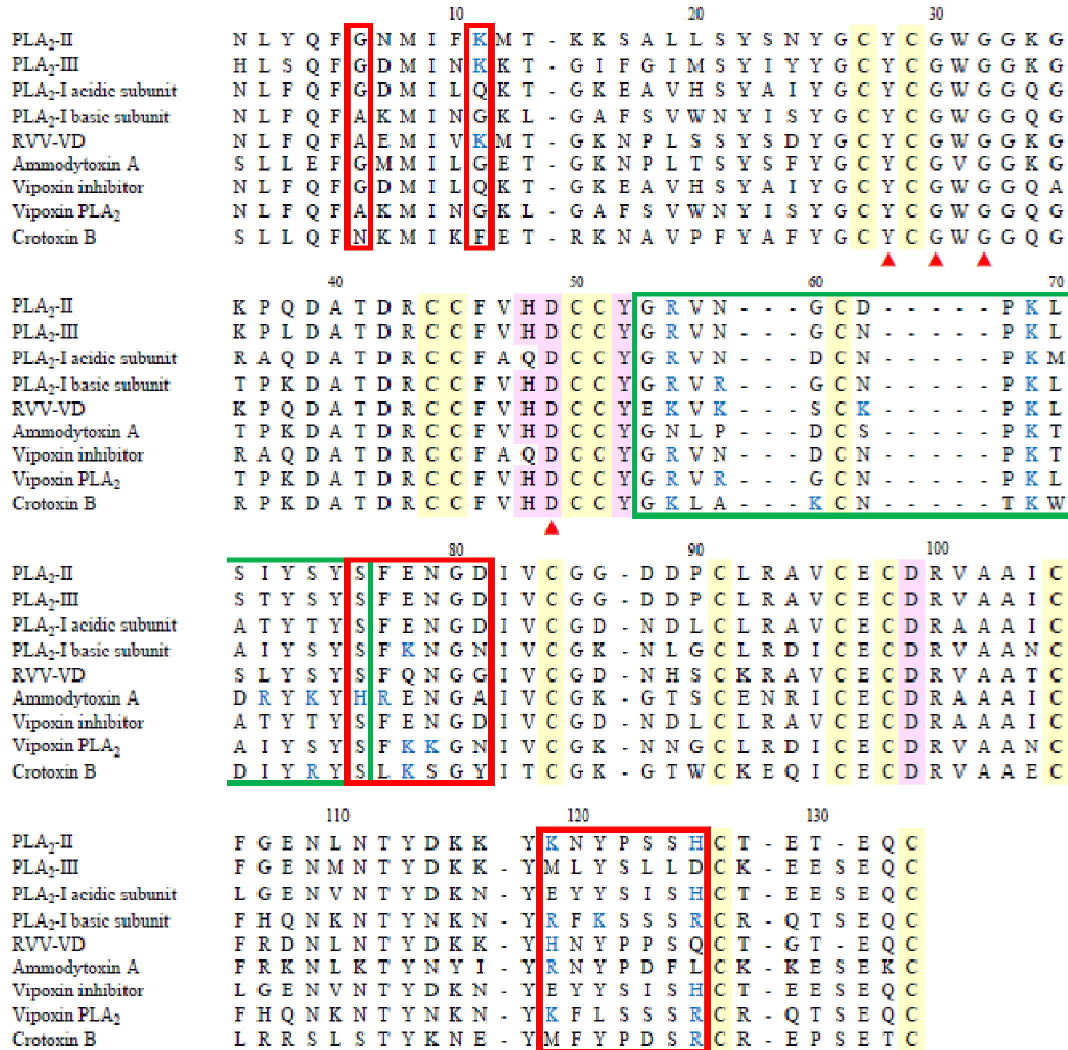

Figure 3. Comparison of primary structures of PLA$_2$s from snake venoms

The common numbering of PLA$_2$ residues (Renetseder et al., 1985) is used. RVV-V sequence is from Carredano et al., 1998, PLA$_2$-I sequence from Komori et al., 1996, Ammodytoxin A sequence from Pungercar et al., 1991, Vipoxin sequence from Mancheva et al., 1984, 1987 and Crotoxin B sequence from Aird et al., 1986. Blanks (-) are introduced for optimal alignment. The amino acids located at the calcium-binding site (Tyr28, Gly30, Gly32 and Asp49) are indicated by closed triangles (▲) and the catalytic network (His48, Asp49, Tyr52 and Asp99) are highlighted with pink. The expected anticoagulant (green) and neurotoxic (red) regions are boxed.

3.3 Molecular Modeling of PLA$_2$s and Comparison of Toxic Sites

As previously reported, PLA$_2$-II and –III hydrolyzed lecithin emulsion to form free fatty acids, and the activity was determined to be 67.03 and 46.92 U/mg, respectively (Komori et al., 1990). Both enzymes prolonged activated partial thromboplastin time (APTT) and possessed myotoxicity; however, these activities were relatively weak compared with PLA$_2$-I. PLA$_2$-I had neurotoxic nature while no lethality was observed in PLA$_2$-II and –III. To clarify these differences, three-dimensional conformation models were constructed (Figure 4).

The molecular models of PLA$_2$-Ib, -II and -III indicated that these enzymes have a similar distribution of α-helix, β-sheet, catalytic site and calcium-binding site (Figure 4A). However, these enzymes differ in the positional relationship and the intermolecular distances of catalytically important residues (His48, Tyr52 and Asp99) (Figure 4B) and these conformational differences could be attributed to the variations in enzymatic activities (PLA$_2$-Ib; 85.27 U/mg, PLA$_2$-II; 67.03 U/mg, PLA$_2$-III; 46.92 U/mg). The catalytic His48, Tyr52 and Asp99 residues of PLA$_2$-Ib might be located in a most suitable position to form the catalytic network.

To clarify the structure-activity relationship of PLA$_2$s, previously reported anticoagulant site and lethal site of molecular models were compared. As shown in Figure 3, the anticoagulant site has been suggested to be in the regions between residues 53 and 76 (Kini and Evans, 1987; Kini, 2005). By the comparison of various PLA$_2$s, they speculated that the strongly anticoagulant enzymes are positively charged in this region. Carredano et al. (1998) also summarized the toxic sites of PLA$_2$s and indicated that the positively charged residues free of intermolecular interactions at this corner of the molecule seem to be a common feature of most anticoagulant PLA$_2$s. The 70-74 sequence of the -wing structure, conserved among the class II secretory PLA$_2$s and contains a tyrosyl residue which participates in the catalytic network has been implicated in the anticoagulation. The anticoagulant activities of *V. aspis* PLA$_2$s were previously reported (Komori et al., 1990). The strong anticoagulancy was observed in the PLA$_2$-I basic subunit when activated partial thromboplastin time (APTT) was measured using citrated human plasma while PLA$_2$-II and –III were less active. The predicted anticoagulant sites of protein models (Figure 5A) indicated that there are three positively charged amino acid residues (Arg54, Arg56 and Lys69) surrounding the active site of PLA$_2$-Ib. On the other hand, the positively charged amino acid residues surrounding the active sites of PLA$_2$-II and –III are Arg54 and Lys69. The strong anticoagulant activity of PLA$_2$-Ib is presumably due to the highly positively charged active site.

Wang et al. (1992) reported that residues 6, 11, 76-81, and 119-125 are important for the neurotoxicity based on the sequence comparison among all the known PLA$_2$s. Their study indicated that all the neurotoxins have a neutral residue 6 (Asn, Ala or Gly) and do not have Lys11, and residues 76-81, 119 and 125 are usually more basic. X-ray crystallographic studies further showed that on substitution of charged residue 6 and Lys11 in group II PLA$_2$s with a neutral residue 6 and non-Lys11, the substrate entrance is narrowed down and the substrate channel at catalytic site is blocked (Scott et al., 1991; Wery et al., 1991). As a result, the non-specific binding of PLA$_2$ toxins to lipids other than the presynaptic membranes lipids is presumably decreased, resulting in increases in the specificity and affinity for binding to the toxin ‘receptor’.

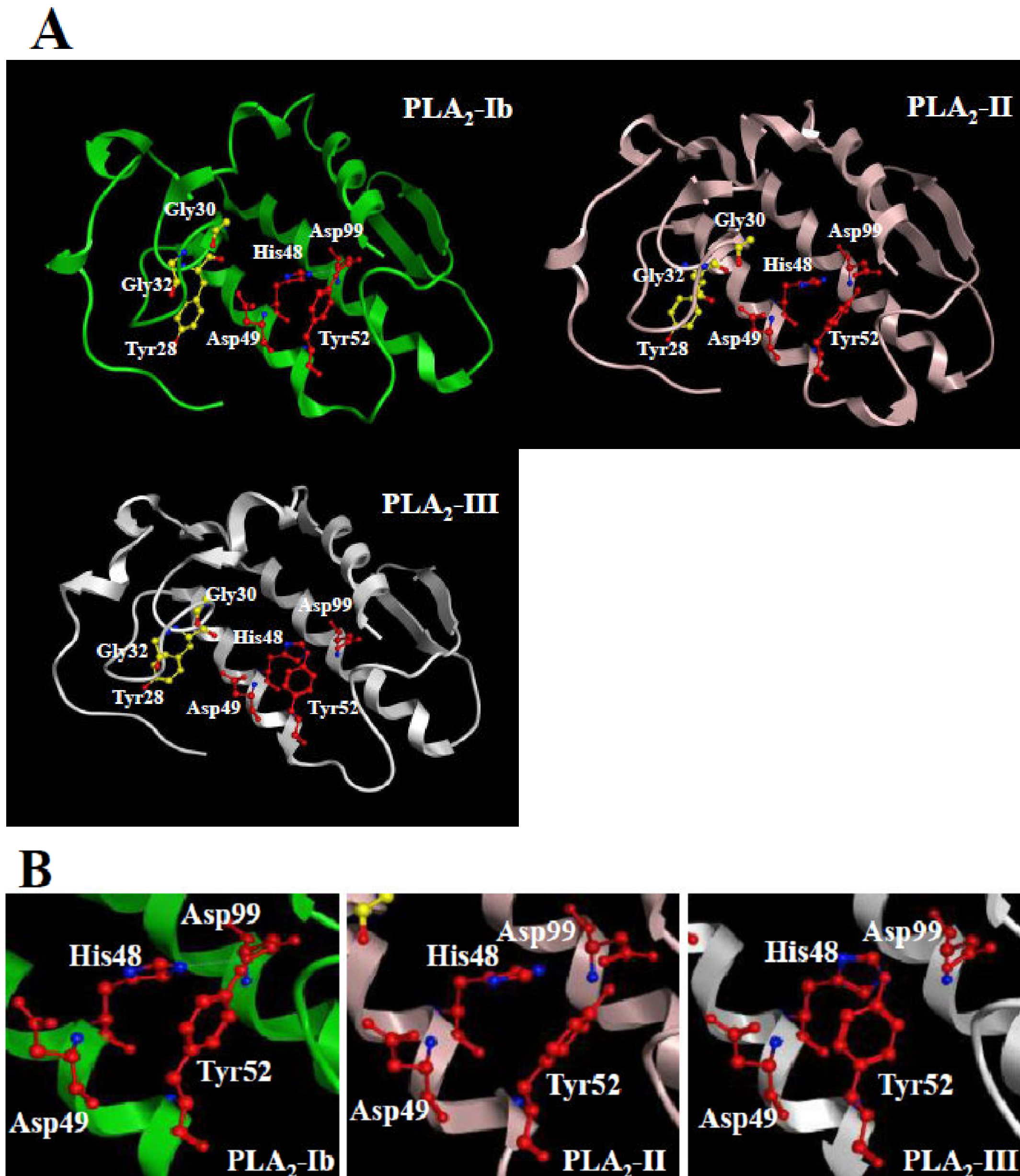

Figure 4. Molecular models of PLA_2-Ib (basic subunit), PLA_2-II and –III

The models of PLA_2-Ib, -II and -III were constructed by using the reported three-dimensional structures of Vipoxin (PDB ID; 1JLT), phospholipase A_2 from Agkistrodon piscivorus piscivorus venom (PDB ID; 1VAP) and Agkistrodon contortrix ***laticinctus*** *venom (PDB ID; 1S8GT) as the templates, respectively. (A) Ribbon representations of PLA_2s. The amino acid residues involved in the catalytic network (His48, Asp49, Tyr52 and Asp99) and calcium-binding site (Tyr28, Gly30, Gly32 and Asp49) are indicated by stick representation (red and yellow, respectively). (B) Comparison of the catalytic sites.*

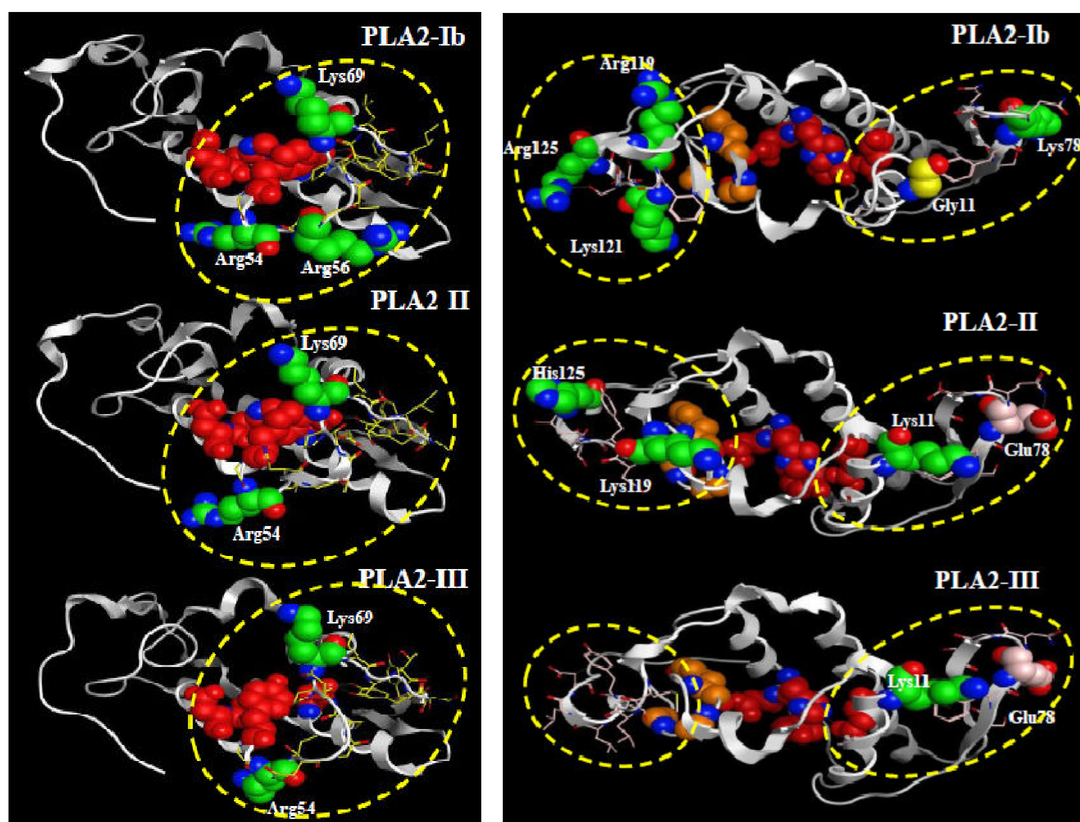

Figure 5. Comparison of predicted biological site of PLA_2-Ib (basic subunit), PLA_2-II and -III

The predicted anticoagulant site (A) and lethal site (B) are circled. The amino acid residues speculated to be included in each active site are shown by stick representation (anticoagulant; yellow, lethal; pink). The amino acid residues involved in the catalytic network (His48, Asp49, Tyr52 and Asp99) and calcium-binding site (Tyr28, Gly30, Gly32 and Asp49) are indicated by ball representation (red and orange, respectively). The charged and neutral amino acid residues expected to be important for the activity are indicated by ball representation (positively charged; green, negatively charged; pink, neutral; yellow).

PLA_2-I has been reported to show lethal activity and its LD_{50} value was estimated to be 0.288 µg/g. On the other hand, PLA_2-II and –III do not have lethal activity (Komori et al., 1990). As shown in Figure 3, PLA_2-II and –III have Lys11, while PLA_2-Ib has a neutral residue (Gly11) at this position. In addition, only PLA_2-Ib has basic Lys78 in the predicted lethal region 76-81 while PLA_2-II and –III have acidic Glu78 in this region. Moreover, the number of basic amino acid residues is three for PLA_2-Ib (Arg119, Lys 121 and Arg125) in the region of 119-125, two for PLA_2-II (Arg119 and Arg125), and none for PLA_2-III. These results indicate that the primary structure of PLA_2-Ib meets the specification requirements for neurotoxin. From the protein models (Figure 5B), it can be seen that the lethal site (residues 6, 11, 76-81, and 119-125) is positioned so as to form the substrate entrance. The region of 119-125 (the left circle in Figure 5B) of PLA_2-Ib is more positively charged compared with PLA_2-II and –III, and neutral Gly11 is located at the opposite side of the substrate entrance (the right circle in Figure 5B). PLA_2-II has a positively charged Lys at position 11, which might cause the repulsion against Lys119 in the region of 119-125, resulting in a substrate entrance wider than that in PLA_2-Ib. As speculated by Wang et al. (1992), as a result of the

increase in the non-specific binding of PLA_2-II to various lipids, the specificity and affinity for binding to the toxin 'receptor' might be decreased. PLA_2-III does not have any basic residues in the region of 119-125, indicating that the affinity of this enzyme to lipids and toxicity are weak.

4. CONCLUSION

Vipera aspis venom contains phospholipase A_2 isozymes and their toxicity is diverse. To clarify the structure-activity relationship of these enzymes, molecular models were constructed, and the proposed toxic sites (anticoagulant and lethal) were compared. Lack or substitution of important amino acid residues for the specific binding to the toxin receptor (such as Arg56 for anticoagulant activity, and Lys11, basic amino acid residues 76-81, 119 and 125 for lethal activity) clearly explained the difference between toxic and weakly toxic PLA_2s.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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