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Characterization, crystallization and preliminary X-ray diffraction analysis of acutohaemolysin, a haemolytic toxin from *Agkistrodon acutus* venom

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Acutohaemolysin, a phospholipase A_2 (PLA_2) from the venom of the snake *Agkistrodon acutus*, has been isolated and purified to homogeneity by anion-exchange chromatography on a DEAE–Sepharose column followed by cation-exchange chromatography on a CM–Sepharose column. It is an alkaline protein with an isoelectric point of 10.5 and is comprised of a single polypeptide chain of 13 938 Da. Its N-terminal amino-acid sequence shows very high similarity to Lys49-type PLA_2 proteins from other snake venoms. Although its PLA_2 enzymatic activity is very low, acutohaemolysin has a strong indirect haemolytic activity and anticoagulant activity. Acutohaemolysin crystals with a diffraction limit of 1.60 Å were obtained by the hanging-drop vapour-diffusion method. The crystals belong to the space group $C2$, with unit-cell parameters a = 45.30, b = 59.55, c = 46.13 Å, β = 117.69°. The asymmetric unit contains one molecule.

1. Introduction

Phospholipase A_2 (PLA_2; E.C. 3.1.1.4; phophatide *sn*-2 acylhydrolase) enzymes hydrolyze the *sn*-2 fatty-acyl bond of phospholipids to produce free fatty acids and lysophospholipids (van Deenen & de Haas, 1963; Dennis, 1983, 1994). These enzymes are abundant in nature, especially in the mammalian pancreas and in animal venoms. PLA_2s are traditionally classified as 'intracellular' or 'extracellular' (Table 1; Clark *et al.*, 1991; Kramer *et al.*, 1991; Dennis, 1994, 1997). Intracellular PLA_2s are often membrane-associated and involved in phospholipid metabolism, signal transduction and other essential cellular processes (Mukherjee *et al.*, 1994). Extracellular PLA_2s are abundant in mammalian pancreatic juices and the venoms of snakes and insects. Based on amino-acid sequences and locations in the cell, PLA_2s can also be classified into nine groups (see Table 1).

PLA_2s from snake venoms are small proteins of 12–15 kDa with a high disulfide-bond content (six or seven) and share a high homology in amino-acid sequences and three-dimensional structures. In addition to their primary enzymatic activity, snake-venom PLA_2s can induce several pharmacological and toxicological effects, including haemorrhage (Gutierrez *et al.*, 1980), myotoxicity (Mebs, 1986; Gutierrez & Lomonte, 1995), haemolysis (Condrea *et al.*, 1981), oedema formation (Lloret & Moreno, 1993), hypotension (Huang, 1984), pre-synaptic (Chang *et al.*, 1977) and post-synaptic neurotoxicity (Bon *et al.*, 1979), cardiotoxicity (Fletcher *et al.*, 1981), convulsions (Fletcher *et al.*, 1980) and the inhibition and/or initiation of platelet aggregation (Yuan *et al.*, 1993; Gerrard *et al.*, 1993). Clearly, not all snake-venom PLA_2s exhibit all these activities; one or more specific effects can be induced by a particular PLA_2. The symptoms induced by the protein may be dependent or independent of the PLA_2 enzymatic activity (Lomonte & Gutierrez, 1989; Chwetzoff *et al.*, 1989; Kini & Evans, 1989). In the case of PLA_2 enzymatic activity dependent induction, the induced effects may be caused by the hydrolysis of critical phospholipids or by the released lysophospholipids and/or fatty acids (Lin-Shiau *et al.*, 1978; Andreasen *et al.*, 1979; Rosenberg, 1986). In the case of PLA_2 enzymatic activity independent induction, the effects may be induced by the physical binding of PLA_2s to their targets (Bevan & Hiestand, 1983; Freedman & Snyder, 1981; Li *et al.*, 1986). For the snake-venom PLA_2s possessing anticoagulant activity, the enzymes appear to inhibit the formation of the prothrombinase complex composed of haemostatic factors Va and X, phospholipids and calcium ions by degrading the phospholipids involved in the complex (Verheij *et al.*, 1980; Kini & Evans, 1988). However, some observations support the notion that the enzymatic activity of PLA_2s is not essential for their anticoagulant effect (Ouyang *et al.*, 1981; Condrea *et al.*, 1981, 1982; Kasturi & Gowda, 1990). Therefore, the anticoagulant mechanism of PLA_2 may be complex and PLA_2s from different sources may have different mechanisms. For those

Table 1
Major groups of phospholipase A_2s.

ND, not determined.

Group	Sources	Location	Disulfides	Ca^{2+}	MW (kDa)
I A	*Elapidae*, *Hydrophidae*	Extracellular	7	m*M*	13–15
I B	Mammalian pancreas	Extracellular	7	m*M*	13–15
II A	*Crotalidae*, *Viperadae*, human synovial fluid/platelets	Extracellular	7	m*M*	13–15
II B	Gaboon viper	Extracellular	6	m*M*	13–5
II C	Rat/mouse testes	Extracellular	8	m*M*	15
III	Bees, lizards	Extracellular	5	m*M*	16–18
IV	Raw 264.7, rat kidney, human U937/platelets	Intracellular	None	<μ*M*	85
V	Human/rat/mouse heart/lung, P388D1 macrophages	Extracellular	6	m*M*	14
VI	P388D1 macrophages CHO cells	Intracellular	ND	None	80–85
VII	Human plasma	Extracellular	ND	None	45
VIII	Bovine brain	Intracellular	ND	None	29
IX	Marine snail	Extracellular	6	<m*M*	14

PLA_2s possessing haemolytic activity, the haemolysis mechanism is still unclear. Some of them have direct haemolytic activity and can induce haemolysis in the absence of phosphatidylcholine (Condrea *et al.*, 1981), while others have indirect haemolytic activity and can induce haemolysis only in the presence of phosphatidylcholine (Angulo *et al.*, 1997). Despite their destructive action at the sarcolemma, most PLA_2s are not haemolytic agents, suggesting that there is a specificity in the membrane targeting or in the interaction between the enzyme and the membrane components. Remarkably, extensive phospholipid hydrolysis does not always lead to a significant increase in the permeability of membranes or cell lysis.

The snake-venom PLA_2s are excellent materials for the study of protein structure–function relationships because of their diverse biological properties. One kind of PLA_2 may have more than one kind of biological activity, different PLA_2s from the same source may display different activities and even for a specific activity the PLA_2s from different sources may show different mechanisms of action. The snake-venom PLA_2s are typical members of a large family of proteins that have diverse biological functions but conserved three-dimensional structures. Because of their varying pharmacological activities, snake-venom PLA_2s are good models for drug design. Many PLA_2 three-dimensional structures have previously been determined, but few of them are at resolutions higher than 1.8 Å. Additional PLA_2 structures at high resolution will be helpful in the design of drugs to counteract their pharmacological activities.

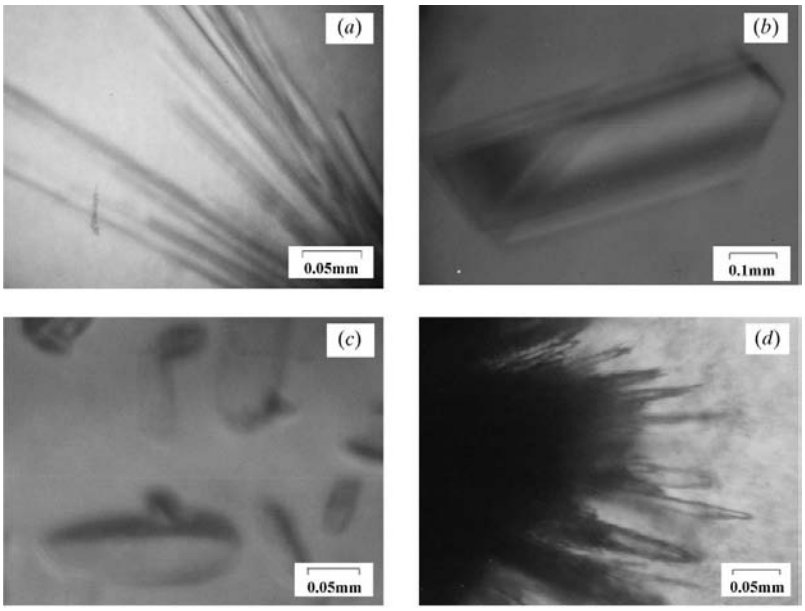

Figure 1
Crystals of acutohaemolysin. (*a*) Form *A* crystals; (*b*) form *B* crystals; (*c*) form *C* crystals; (*d*) form *D* crystals.

The purpose of this paper is to provide details of the purification, N-terminal sequencing, characterization and crystallization of acutohaemolysin, an alkaline PLA_2 from the venom of *A. acutus* with strong haemolytic and anticoagulant activities. The acutohaemolysin crystals are suitable for X-ray diffraction at high resolution.

2. Experimental procedures

2.1. Materials

The dried crude venom of *A. acutus* was obtained from the southern mountain region in the province of Anhui, China. DEAE–Sepharose, CM–Sepharose and ampholytes (pH 3–10) were obtained from Pharmacia (Uppsala, Sweden). SDS, acrylamide, *N*,*N*-methylene bisacrylamide, TEMED, PEG 8000 and PEG 4000 were purchased from Fluka Co. (Switzerland). 2-(*N*-morpholino)-ethanesulfonic acid (MES) was purchased from Sigma (USA). Phosphatidycholine and protein standards were produced by the Shanghai Dongfeng Biochemical Technology Co. (Shanghai, China). Other reagents and chemicals were of analytical grade.

2.2. Methods

2.2.1. Purification. Crude venom (1.5 g) was dissolved in 30 ml of starting buffer (0.02 *M* Tris–HCl pH 8.0) and centrifuged at 10 000*g* to remove insoluble materials. The pH value was adjusted to 8.0 by adding 4 ml of 0.2 *M* Tris solution and the protein solution was applied to a DEAE–Sepharose column pre-equilibrated with starting buffer. The proteins were eluted at a flow rate of 1.5 ml min^{-1} with 0.02 *M* Tris–HCl buffer (pH 8.0) with 0–0.2 *M* (total volume 900 ml) and 0.2–0.5 *M* (total volume 200 ml) linear NaCl concentration gradients. The fractions containing PLA_2 activity were pooled, concentrated, dialyzed against distilled water and applied to a column packed with CM–Sepharose. The column was pre-equilibrated with 0.02 *M* sodium phosphate buffer (pH 6.0) and was eluted with the same buffer with a pH gradient from 6.0 to 8.0 and a 0–0.5 *M* NaCl concentration gradient (Fig. 1). The fractions containing the last protein peak were pooled, concentrated, desalted and stored at 253 K for further characterization.

2.2.2. Determination of molecular weight and isoelectric point. The molecular weight of acutohaemolysin was determined by electrospray ionization mass spectrometry (ESI–MS) and SDS–PAGE with and

without β-mercaptoethanol. The isoelectric point was analyzed by isoelectric focusing (IEF) on an ampholine polyacrylamide slab gel using pH 3–10.5 ampholytes following the instructions of the manufacturer.

2.2.3. Partial sequencing of N-terminal amino-acid residues. The protein obtained from the CM–Sepharose chromatography was further purified by capillary high-performance liquid chromatography (CHPLC; Perkin–Elmer) using a reverse-phase C18 column (150 × 0.5 mm). Partial sequencing of N-terminal amino-acid residues was performed by Edman degradation with an Applied Biosystems 476A Protein Sequencer.

2.2.4. Assay of haemolytic activity. Both direct and indirect haemolytic activities were evaluated by the modified method of Jeng *et al.* (1978). 100 mg of egg-yolk phosphatidylcholine was suspended by sonification in 100 ml of 10 m*M* Tris–HCl buffer pH 8.0 containing 10 m*M* $CaCl_2$ and 0.15 *M* NaCl. 10 ml of fresh rabbit blood was centrifuged at 3000*g* for 10 min at 293 K and the erythrocytes were collected. After being washed twice with 30 ml of 0.15 *M* NaCl, the erythrocytes were suspended gently in the phosphatidylcholine suspension. 100 µl of the enzyme solution (1.0 mg ml^{-1} in 0.15 *M* NaCl) was added to 1 ml of the erythrocytes/phosphatidylcholine suspension and incubated at 300 K for 30 min. The reaction mixture was centrifuged at 3000*g* for 10 min and the absorbance of light by the supernatant was recorded at 540 nm. Another assay was performed using an incubation mixture containing all the same reagents with the exception of $CaCl_2$. The enzyme was excluded from the control experiments. Complete haemolysis was achieved by incubation of 1 ml of the erythrocytes/phosphatidycholine suspension with 4 ml of distilled water for 30 min.

The reaction mixture, consisting of 1 ml of the phosphatidylcholine suspension and 0.1 ml of the enzyme solution, was incubated at 300 K for 30 min and the enzyme was then inactivated by incubating the reaction mixture at 373 K for 30 min. 100 µl of the reaction mixture was removed and added to 1 ml of the erythrocytes suspension (in 0.02 *M* Tris–HCl buffer pH 8.0 containing 0.15 *M* NaCl) and incubated at 300 K for 30 min. The reaction mixture was centrifuged at 3000*g* for 10 min and the absorbance of the supernatant was recorded at 540 nm. 0.15 *M* NaCl solution was used instead of the enzyme solution in the control experiments.

The direct haemolytic activity was assayed according to the method described above in the absence of phosphatidylcholine.

2.2.5. Assay of anticoagulant activity. The one-step prothrombin times were measured according to the method of Quick (1938). The assay was performed with 0.1 ml of citrated human plasma, 0.1 ml of rabbit brain thromboplastin (dissolved in 0.15 *M* NaCl) and different volumes (0, 10, 20, 50 and 100 µl) of the enzyme solution (1.5 mg ml^{-1}), adjusting the final volume to 0.3 ml with 0.15 *M* NaCl. The reaction mixture was pre-incubated at 298 K for 1.5 h, clotting was initiated by adding 0.1 ml of 25 m*M* $CaCl_2$ and the clotting time was recorded.

2.2.6. Crystallization and preliminary X-ray diffraction analysis. The purified acutohaemolysin showed a single protein band on an SDS–PAGE gel. The enzyme was concentrated to 30 mg ml^{-1} and was desalted by dialysis. The crystals were grown by the hanging-drop vapour-diffusion method using tissue-culture plates and siliconized glass cover slips (McPherson, 1982). For the initial crystallization screen, 98 crystallization conditions were employed using the Hampton Crystal Screen based on the sparse-matrix sampling method of Jancarik & Kim (1991). The screen was carried out at room temperature (about 300 K). The hanging drops were produced by mixing 2 µl of the protein solution with 2 µl of the reservoir solution. Four crystal forms were obtained one week later (Fig. 1). Form *A* crystals were grown with a reservoir solution consisting of 30%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5 and 0.2 *M* ammonium sulfate. Form *B* crystals were grown with a reservoir solution consisting of 20%(*v*/*v*) 2-propanol, 20%(*w*/*v*) PEG 4000 and 0.1 *M* sodium citrate pH 5.6. Form *C* crystals were grown with a reservoir solution consisting of 2.0 *M* ammonium sulfate and 0.1 *M* sodium acetate pH 4.6. Form *D* crystals were grown from 30%(*w*/*v*) PEG–MME 5000, 0.2 *M* ammonium sulfate and 0.1 *M* MES pH 6.5. Both form *A* and form *D* crystals were needle-shaped. Form *B* crystals were twinned. Form *C* crystals were too small to be useful. None of these crystals were suitable for X-ray diffraction analysis. To refine the crystallization conditions of form *A*, form *C* and form *D* crystals, the pH values and precipitant concentrations were screened in finer grids, but no improvement was achieved. Better form *B* crystals were obtained by lowering the concentration of protein solution from 30 to 20 mg ml^{-1} and the concentration of PEG 4000 from 20 to 18%(*w*/*v*). The maximum dimensions of the form *B* crystal were 0.8 × 0.8 × 1.0 mm; crystals of this size were obtained in two weeks.

The diffraction data were collected at room temperature from one form *B* crystal using a MAR Research imaging plate (diameter 300 mm) mounted on a X-ray generator with a graphite monochromator and sealed copper-target tube at our laboratory. The working tube voltage and current were 40 kV and 50 mA, respectively. A total of 180 imaging frames were recorded at a 90 mm crystal-to-imaging plate distance, using a 1° oscillation angle and an exposure time of 180 s per imaging frame (Fig. 2). The diffraction data were processed using the *DENZO* and *SCALEPACK* programs (Otwinowski, 1993; Minor, 1993).

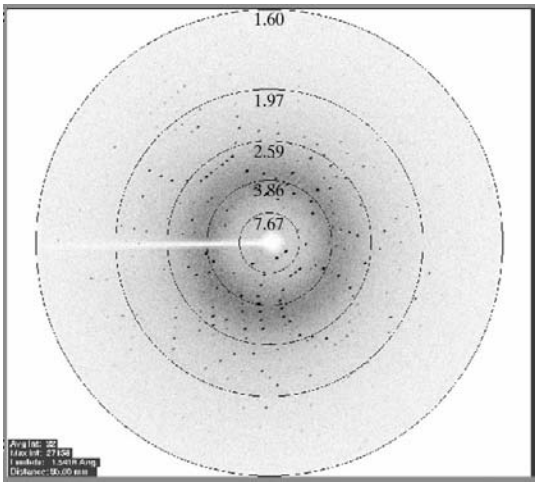

Figure 2
A diffraction pattern of the form *B* crystal of acutohaemolysin.

3. Results and discussion

Acutohaemolysin, a new protein isolated and purified from the venom of *A. acutus*, showed one protein band on a SDS–PAGE under reducing or non-reducing conditions with a molecular weight of 13 938 Da determined by ESI–MS (Fig. 3). It moved as a single band on an IEF gel with an isoelectric point of 10.5 (data not shown). Like other Lys49-type PLA_2s, acutohaemolysin contains no calcium ion and has almost no PLA_2 enzymatic activity (data not shown). Its N-terminal 27 residues are identical to those of a homologous protein (a Lys49-type PLA_2) which was cloned from the same snake venom (Fig. 4). These two facts together suggest that acutohaemolysin is a Lys49-type PLA_2.

Table 2
Diffraction data-collection and reduction statistics.

Values in parentheses are for the highest resolution shell (1.64–1.60 Å).

Space group	*C*2
Unit-cell parameters	
a (Å)	45.298
b (Å)	59.549
c (Å)	46.128
β (°)	117.689
Number of reflections	20380
Number of independent reflections	13205
Resolution limits (Å)	30.0–1.60
$R_{\rm merge}$† (%)	5.1 (22.6)
Completeness‡ (%)	91.8 (77.3)

† $R_{\rm merge} = \sum_h \sum_i |\langle I(h)\rangle - I(h)_i| / \sum_h \sum_i I(h)_i$, where $I(h)_i$ is the intensity of the ith observation of reflection h and $\langle I(h)\rangle$ is the mean intensity of reflection h. ‡ The completeness is the ratio of the number of the observed reflections to the number of possible reflections.

Acutohaemolysin has the capability of inducing a distinct indirect haemolytic effect *in vitro*. In the presence of $CaCl_2$, about 47% haemolysis was observed when 100 µg of acutohemolysin was incubated together with the erythrocyte/phosphatidylcholine suspension; however, 50% haemolysis was observed under similar conditions but in the absence of $CaCl_2$, suggesting that its haemolytic activity is independent of external calcium ions. The haemolytic activity of acutohaemolysin seems to be independent or almost independent of its enzymatic activity. After being heated at 373 K for 30 min to inactivate the enzyme, the reaction mixture did not cause haemolysis, suggesting that it was not the products of the reaction that caused haemolysis. When phosphatidylcholine was excluded from the reaction mixture, no haemolysis was observed, indicating that acutohaemolysin has no direct haemolytic activity. These two experimental observations also suggest that the haemolytic effect of acutohaemolysin is at least partly independent of its enzymatic activity.

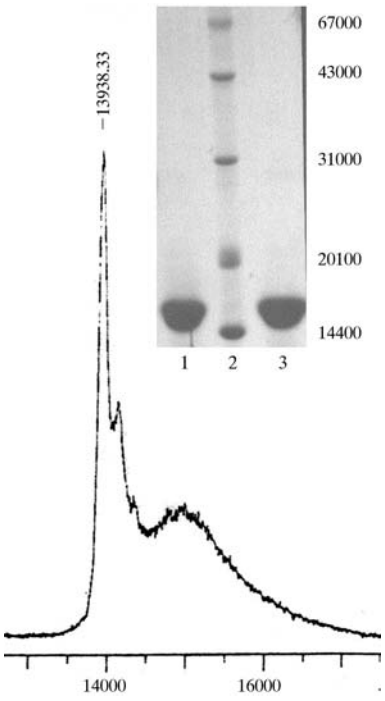

Figure 3
Electrospray mass spectrum of acutohaemolysin. The insert shows the SDS–PAGE of acutohaemolysin. Lane 1, acutohaemolysin under reducing conditions; lane 2, protein standards; lane 3, acutohaemolysin under non-reducing conditions.

Like most other alkaline PLA_2s (Condrea *et al.*, 1981; Martin *et al.*, 1975; Verheij *et al.*, 1980; Ownby *et al.*, 1999), acutohaemolysin has a strong anticoagulant activity. The one-step prothrombin time was doubled when the concentration of the enzyme was 40 µg ml^{-1}. The observation that the anticoagulant activity of acutohaemolysin does not parallel its enzymatic activity suggests that the anticoagulant activity of acutohaemolysin has little or no relation to its enzymatic activity; binding to its target rather than hydrolysis may account for the anticoagulant effect. After acutohaemolysin was pre-heated at 373 K for 30 min, no anticoagulant activity could be detected for the enzyme.

It is possible that there are some regions, *e.g.* the pharmacological site (Ownby *et al.*, 1999; Kini & Evans, 1989) on the enzyme surface, which can direct the enzyme to bind its targets. After the enzyme has bound to its targets, its enzymatic activity is distinctly enhanced under these conditions (Scott *et al.*, 1990; Dennis, 1994). Therefore, the haemolytic and/or coagulant activities of acutohaemolysin may still be dependent on its enzymatic activity. In order to solve this contradiction, much research towards characterizing the enzyme needs to be performed, in particular the determination of the three-dimensional structure at high resolution. The acutohaemolysin crystals obtained by the hanging-drop vapour-diffusion method belong to the monoclinic system, with unit-cell parameters a = 45.30, b = 59.55, c = 46.13 Å, β = 117.69°. The space group is determined to be monoclinic *C*2 owing to the absence of (*hkl*) reflections with $h + k = 2n + 1$. Considering the molecular mass, unit-cell parameters and the packing, one acutohaemolysin molecule is calculated to be present in each asymmetric unit, corresponding to a volume-to-mass ratio of 1.98 Å^3 Da^{-1} (Matthews, 1968). The data-collection and reduction statistics are listed in Table 2.

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                        10        20
Acutohaemolysin     SLFELGKMIWQETGKNPVKN-YGLYGCN
LYS49 PLA2          SLFELGKMIWQETGKNPVKN-YGLYGCN
ACLMT               SLLELGKMILQETGKNAITS-YGSYGCN
APPK49              SVLELGKMILQETGKNAITS-YGSYGCN
BASPER-II           SLFELGKMILQETGKNPAKS-YGAYGCN
BTHTX-I             SLFELGKMILQETGKNPAKS-YGAYGCN
TMK49               SVIELGKMIFQETGKNPVKN-YGLYGCN
TFBP-I              SLVQLWKMIFQETGKEAAKK-YGLYGCN
TFBP-II             SLVQLWKMIFQETGKEAAKK-YGLYGCN
TGR-K49             SVIELGKMIFQETGKNPATS-YGLYGCN
D.acutus K49        SLIELGKMIFQETGKNPVKK-YGLYGCN
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Figure 4
Sequence of the N-terminal amino-acid residues of acutohaemolysin aligned with the N-terminal sequences of Lys49-type PLA_2s from other snake venoms: Lys49-type PLA_2 (Fan *et al.*, 1999), ACLMT (Selistre de Araujo *et al.*, 1996), AppK49 (Maraganore & Heinrikson, 1986), Basper II (Francis *et al.*, 1991), TMK49 (Liu *et al.*, 1991), TFBP I and II (Liu *et al.*, 1990), TGRK49 (Nakashima *et al.*, 1995) and *D. acutus* K49 (Wang *et al.*, 1996). The bold characters represent the conserved residues among snake-venom PLA_2s.

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