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AMINO ACID SEQUENCE OF A NEUROTOXIC PHOSPHOLIPASE A_2 ENZYME FROM COMMON DEATH ADDER *(ACANTHOPHIS ANTARCTICUS)* VENOM

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ABSTRACT

The amino acid sequence of the first neurotoxic phospholipase A_2, acanthoxin A1, purified from the venom of the Common death adder (*Acanthophis antarcticus*) was determined. Acanthoxin A1 shows high homology with other Australian elapid PLA_2 neurotoxins, in particular Acanthin -I and -II, also from Death adder, Pseudexin A from the Red-bellied black snake (*Pseudechis porphyriacus*), and Pa-12a and Pa-9c from the King brown snake (*Pseudechis australis*). Acanthoxin A1 is a single-chain 118 amino acid residue PLA_2, including 14 half cystine residues and the essential residues forming the ubiquitous calcium binding pocket and catalytic site. Critical analysis of the residues hypothesized to be important for neurotoxicity is presented.

INTRODUCTION

A large number of phospholipase A_2 enzymes (PLA_2, EC 3.1.1.4) from the venomous snake families *Elapidae, Hydrophiidae, Crotalidae* and *Viperidae* have been purified and sequenced (Yang, 1994). All the sequences show a high degree of homology, despite the fact that they are capable of different pharmacological activities, such as neurotoxicity, myotoxicity, cardiotoxicity, anticoagulant effects, and oedema-stimulating activities (Kini and Evans, 1989). PLA_2 enzymes isolated from Australian snake venoms are widely known for their neurotoxic activities (Sutherland, 1990); however, identification of the residues responsible for presynaptic neurotoxicity remains controversial.

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Recently, neurotoxic PLA_2 enzymes were isolated from the venom of the Common death adder (*Acanthophis antarcticus*) (van der Weyden *et al.*, 1997). The major PLA_2 from the venom of the Common death adder, acanthoxin A (ActxA), was found to exist in two isoforms, A1 and A2 (ActxA1 and ActxA2, respectively), which can be separated by RP-HPLC (Hains *et al.*, 1999). N-terminal analysis of these two isoforms shows a difference of only 3 residues in the first 32 amino acids (positions 1, 3 and 10) (van der Weyden *et al.*, 1997). The complete sequence of acanthoxin A1 is reported here and discussed in terms of the residues hypothesized to be important for presynaptic neurotoxicity.

MATERIALS AND METHODS

Neurotoxicity Studies

Three male adult QS mice (30-35g) were intraperitoneally injected with 200µL of ActxA (at doses of 80µg, 160µg and 400µg) suspended in 0.1% (w/v) BSA in 0.9% (w/v) saline, and monitored for 24 hours. The positive control was 100µL of whole *A. antarcticus* venom (12.5µg); the negative control was 100µL of 0.1% BSA in 0.9% (w/v) saline. The toxicity of purified ActxA1 and ActxA2 has since been tested (Hains *et al.*, 1999).

Enzymatic Digestions

ActxA was purified from the venom of the Common death adder, as previously described (van der Weyden *et al.*, 1997). All digestions were performed on the performic acid oxidized derivative of ActxA. Tryptic digestions (Promega) were carried out in 50mM ammonium bicarbonate, pH 7.8, at an enzyme:substrate ratio of 1:20 (w/w), at 37°C for 18 hours. The reaction was terminated by the addition of TFA (10% v/v of reaction volume) and the mixture stored at -80°C. Endoproteinase Arg-C (Boehringer-Mannheim) digestions were carried out in 90mM Tris-HCl, pH 7.6, 85mM $CaCl_2$, 5mM DTT, 0.5mM EDTA, at an enzyme:substrate ratio of 1:20 (w/w), at 37°C for 18 or 30 hours. The reaction was terminated by freezing or loading immediately onto an HPLC column. Endoproteinase Glu-C digestions (Boehringer-Mannheim) were carried out in 100mM ammonium acetate, pH 4.0, at an enzyme:substrate ratio of 1:50 (w/w), at room temperature for 1 week. The reaction was terminated by freezing or placing immediately onto an HPLC column.

Chemical Cleavages

BNPS-skatole chemical cleavages were performed on unmodified ActxA. ActxA bound to PVDF was covered in glacial acetic acid:water (1:1 v/v) with a dissolved crystal of cresol, followed by addition of BNPS-skatole, dissolved in acetic acid (approximately 1mg in 50µL in a total volume of 150µL). The reaction proceeded for 48 hours in the dark at room temperature. β-Mercaptoethanol was added to the reaction, and incubated for a further 24 hours. The sample was diluted to 500µL and extracted with ethyl acetate to remove excess reagent and break down products. The aqueous solution and PVDF were freeze dried. The PVDF was wetted with 50% (v/v) methanol and suspended in 0.1% (v/v) TFA/water overnight before being washed and air-dried ready for sequencing.

Purification of Peptides

The proteolytic peptide fragments were separated by RP-HPLC with a Pharmacia Sephasil C18 column (2.1mm x 100mm) connected to a Pharmacia SMART system. The column was equilibrated in 0.1% (v/v) TFA water at a flow rate of 100µL/min. Elution was performed using a linear gradient of 0.1% (v/v) TFA, 80% (v/v) acetonitrile in water over 70 min. The eluant was monitored at 214, 255 and 280nm.

Edman Degradation and Amino Acid Sequencing

Edman degradation and amino acid analysis was performed on the peptides attached to either PVDF or polybrene-treated, precycled glass-fibre disks, using a gas-phase sequencer (PE Applied Biosystems Procise 494 Protein Sequencer, 4 cartridge model).

RESULTS

Neurotoxicity Studies

When ActxA was administered intraperitoneally to adult mice they exhibited classical signs of neurotoxic poisoning; including respiratory distress and hind limb paralysis, followed by respiratory failure and death.

Protein Sequencing

The full amino acid sequence of ActxA1 is shown in Figure 1. This figure includes N-terminal sequencing, tryptic, Arg-C, Glu-C and BNPS-skatole peptides, to fully elucidate the sequence of ActxA1.

Tryptic Digestion

Tryptic digestion of ActxA gave multiple peptides, only one of which was uniquely informative and is included in Figure 1. The sequence of this peptide (Trypsin 11) was GGSGKPVDELR (indicating a chymotryptic-type cleavage). This sequence corresponds to residues 33-43 of ActxA1.

Endoproteinase Arg-C Digestion

The 18-hour digestion of ActxA produced 4 main peptides, separated by RP-HPLC, as monitored at 214nm. One peptide (Arg-C_18hr 30) revealed two sequences (discernible by their quantitative differences): APYNKNNIGIGSKTRCQ, residues 101-117, and CFAKAPYNKNNI GIGSKTRCQ, residues 97-117. Another peptide produced the N- and C-terminal sequences, as well as the minor sequence: CGPKMTLYSW, corresponding to residues 60-69. The remaining peptides produced only low, background levels of amino acids, suggestive that the peptides had not properly attached to the PVDF membrane. Cysteine was identified by the lack of an amino acid in that cycle and by homology to other PLA_2 enzymes. As ActxA was oxidized with performic acid cysteine was present as cysteic acid, the PTH derivatives of which are not detected in our system.

The 30-hour digestion of ActxA again produced 4 main peptides, which were adsorbed to polybrene-treated, precycled glass-fibre disks following purification, in preference to PVDF. One peak consisted of the N-terminus, plus a minor sequence of CGPKMTLYS (Arg-C_30hr 37), corresponding to residues 60-68. An earlier eluting (more hydrophilic) peak also contained the N-terminus, plus a

minor sequence: CCQIHDNCYGEAE KKG (Arg-C_30hr 18), corresponding to residues 44-59 (indicating that Arg-C will cleave after a glycine if left to digest for a long period).

Endoproteinase Glu-C Digestion

The endoproteinase Glu-C digestion of ActxA produced 2 major peaks, separated by RP-HPLC, as monitored at 214nm. The earlier eluting peak (Glu-C 19) gave the sequence LDRCCQIHDNCYGEAEK (corresponding to residues 41-57). The latter (more hydrophobic) peak (Glu-C 39) contained to two unresolved peptides: the major sequence being GFVCDCD AAAAKC, residues 86-97, and the minor sequence AEKKGCGPKMTLYSW, residues 55-69.

BNPS-Skatole Cleavage

BNPS-skatole cleavage of unmodified ActxA was expected to produce 4 peptides, as judged from the amino acid composition data (van der Weyden *et al.*, 1997). The resulting peptides were not separated, but rather the whole reaction mixture was sequenced. As anticipated, four peptides were detected. From knowledge of much of the sequence of ActxA1 we were able to discern 4 individual sequences as follows: (i) the N-terminus, (ii) LSYVNYG..., corresponding to residues 20+, (iii) GGSGKPVD... corresponding to residues 32+, and (iv) KDCANDVPVCNSKSGCEGFV, corresponding to residues 70-88. This latter sequence (iv) was the final section of ActxA1 that remained unknown.

Hydrophobicity Profile

The hydrophobicity profile for ActxA1 was calculated (Figure 3) using AntheProt (Geourjon and Deleage, 1995) with the criteria as defined by Kyte and Doolittle (1982), where a positive value indicates an increase in relative hydrophobicity.

DISCUSSION

Irrespective of their origin, mammalian pancreas or snake venom, the primary structure of PLA_2s show a high degree of homology (Davidson and Dennis, 1990; Dennis, 1994). The sequence of ActxA1 was used as the input of a FASTA search (Pearson, W. R., 1990) of the OWL database (Bleasby *et al.*, 1994). The 5 highest scoring PLA_2 enzymes, aligned with ActxA1 using Clustal W 1.74 (Thompson *et al.*, 1994) are shown in Figure 2. Acanthin-I and -II from *A. antarcticus* (Chow *et al.*, 1998), Pseudexin A from *P. porphyriacus* (Vaughan *et al.*, 1981; Moon and Rys, 1984; Schmidt and Middlebrook, 1989) and Pa-9c and Pa-12a from *P. australis* (Takasaki *et al.*, 1990a, b) all share a high level of homology to ActxA1. ActxA1 showed the highest level of identity (96%) to Acanthin-II, also from *A. antarcticus*. ActxA1 also showed a high level of identity to Acanthin-I (92%), Pseudexin A (77%), Pa-12a (73%) and Pa-9c (70%).

Given the high level of identity between ActxA1 and the Acanthins it is important to consider the possibility that ActxA1 is in fact the same protein as either Acanthin-I or Acanthin-II. Indeed, the PLA_2 enzymes do share similar molecular masses and isoelectric points; however, this is not uncommon for PLA_2 enzymes from

the same venom (Takasaki *et al.*, 1990a, b). Significantly, the sequences of the Acanthins differ from ActxA1 in a number of areas (Figure 2) with ActxA1 possessing amino acids that neither Acanthin-I nor -II contain. Additionally, Acanthin-I contains an additional amino acid. Also, it remains to be elucidated if the Acanthins are neurotoxic, whereas ActxA1 clearly is. As such, the authors feel that ActxA1 and the Acanthins are in fact unique PLA_2 enzymes present in the venom of *A. antarcticus*. Recent LC-MS studies of Death adder venom (Sung, 1998) has shown that at least 7 PLA_2 enzymes are present in this venom.

ActxA1 contains the 14 cysteine residues typical of Group IA PLA_2 enzymes (Davidson and Dennis, 1990; Dennis, 1994, 1997) that form 7 disulfides bonds as demonstrated by mass spectrometry (not shown). The sequence of ActxA1 also contains the 4 residues which form the essential calcium-binding pocket: Y28, G30, G32 and D49 (Dijkstra *et al.*, 1981a, b; Bekkers *et al.*, 1991; Arni and Ward, 1996). ActxA1 also contains the essential catalytic unit residues: H48 and D99 (Verheij *et al.*, 1980). The proposed catalytic mechanism is that a water molecule, which is close to the nitrogen at position 1 of the imidazole ring of the D99-H48 pair, acts as the nucleophile (Verheij *et al.*, 1980).

While residues important for enzymatic activity are widely agreed upon, those responsible for neurotoxicity remain as yet to be fully elucidated. Comparison studies of hydropathy profiles between presynaptically acting PLA_2 enzymes and non-neurotoxic enzymes led Kini and Iwanaga (1986) to suggest that the hydrophobic region around residues 80-110 is important for the presynaptic neurotoxicity. As shown in Figure 3, the neurotoxic ActxA1 has a positive hydrophobicity value around residues 90-110 (using the homology numbering of Renetseder *et al.*, 1985). While these results tend to support the hypothesis of Kini and Iwanaga (1986), it should be realised that this is not the case for all neurotoxic PLA_2s. For example, Pa-11 and Pa-13 from *P. australis*, appear to contradict this theory as they have very similar hydrophobic residues at positions 90-110, yet only Pa-11 is toxic (Tsai *et al.*, 1987; Takasaki *et al.*, 1990a, b).

Alternatively, it has been proposed that the charge scores of certain residues of elapid PLA_2s correlate with toxicity, ie. presynaptic toxins have a higher score of charge (3+ or 4+) than the non-toxic PLA_2 enzymes (-1 to +1) over specific regions of the protein (Tsai *et al.*, 1987; Rosenberg *et al.*, 1989). Similarly, Kondo *et al.*, (1982) stressed the importance of specific basic residues being associated with high or low toxicity. ActxA1 would seem to support this hypothesis as it is a basic PLA_2 (pI = 8.5). However, chemical modification studies have shown that charge alone cannot account for differences in toxicity (Condrea *et al.*, 1983; Barrington *et al.*, 1984). In addition, this theory does not account for the presence of potent acidic neurotoxins in the venoms from *Vipera russelli* (Russell's viper, Huang and Lee, 1984), *Bothrops alternatus* (Half Moon viper) (Nisenbom *et al.*, 1988), and *Pseudechis australis* (King brown snake) (Takasaki *et al.*, 1990a, b).

Takasaki *et al.*, (1990b) proposed that the domain responsible for presynaptic toxicity in elapid snakes consists of 7 hydrophilic residues (R43, K46, D50, E54, K58, D90 and E94); PLA_2 enzymes which lack a few of them, especially D50, K58 or

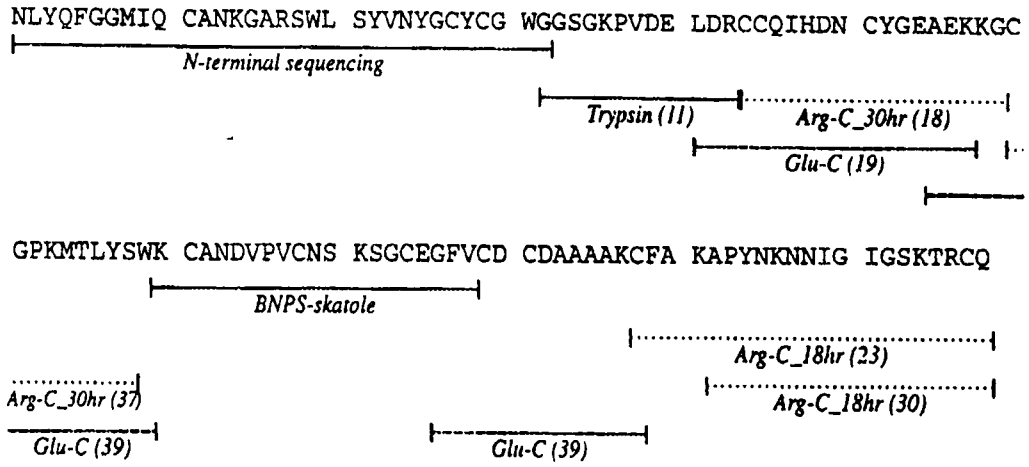

Figure 1: Complete amino acid sequence of ActxA1. The complete amino acid sequence of ActxA1 is shown along with the informative overlapping BNPS-skatole, trypsin, endoproteinase Arg-C and Glu-C peptide data.

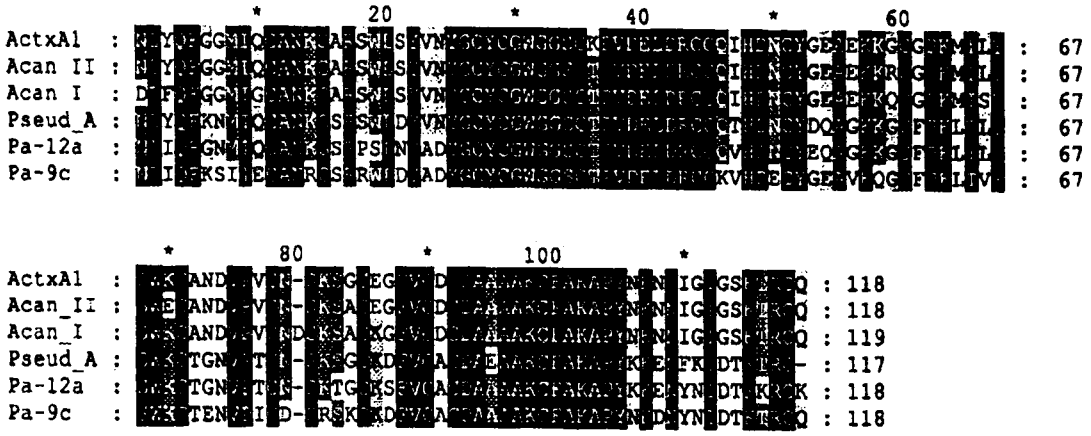

Figure 2: Alignment of ActxA1 with homologous PLA_2 enzymes. ActxA1 is aligned with Acathin-I (Acan_I), Acanthin-II (Acan_II), Pseudexin A (Pseud_A), Pa-12a and Pa-9c using Clustal W (Thompson *et al.*, 1994) and output with GeneDoc. Conserved residues are shaded as follows: 100% blue, 80%, dark grey, 60% grey.

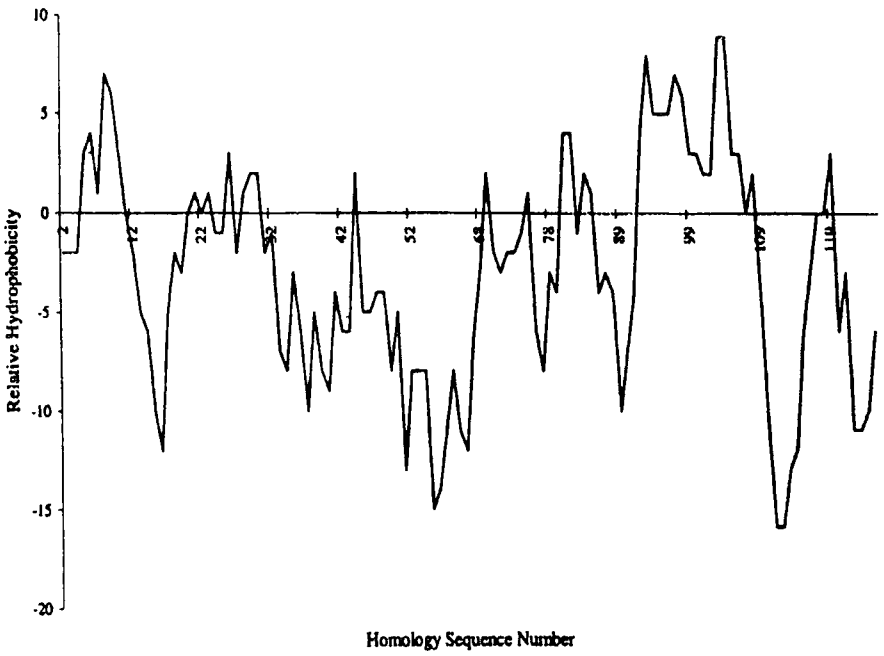

Figure 3: Hydrophobicity plot of ActxA1. The sequence of ActxA1 was plotted on a relative hydrophobicity scale using the algorithm of Kyte and Doolittle (1982) as implemented by (Geourjon and Deleage, 1995) in AntheProt. The sequence has been numbered according to the homology numbering of Renetseder *et al.* (1985).

D90, are only weak neurotoxins. ActxA1 contains 5 of the 7 hydrophilic residues with the exceptions being K46 (replaced by Q) and D90 (replaced by A). Perhaps the substitutions at these two residues could explain the only moderate level of toxicity (LD_{100} = 3.2mg/kg s.c.) possessed by ActxA (purified ActxA1 was lethal at 6.4mg/kg s.c.). (Hains *et al.*, 1999).

Curin-Serbec *et al.* (1994) suggest the controversy over the exact location of the neurotoxic site on PLA_2s may be due to the fact that the amino acids responsible for toxicity are not in exactly the same positions in these molecules. Considering the great diversity among the quaternary structures of the β-neurotoxins, such as the single-chain PLA_2s (notexin, ammodytoxin; Halpert and Eaker, 1975; Krizaj *et al.*, 1989), non-covalently linked multi-chain PLA_2s (crotoxin, taipoxin, textilotoxin; Fohlman *et al.*, 1976; Faure and Bon, 1988; Pearson, J. A. *et al.*, 1993) and the covalently linked β-bungarotoxins (Kondo *et al.*, 1978), this suggestion appears highly probable.

We have also investigated the phenomenon of presynaptic toxicity using a three-dimensional model of ActxA1 (Hains *et al.*, 1999). By comparing the electrostatic surface potential of ActxA1 to several toxic and non-toxic PLA_2 enzymes,

we were able to postulate that negatively charged residues on the non-catalytic surface of PLA_2 enzymes are important for toxicity. This hypothesis is supported by considerable evidence available (see Hains *et al.*, (1999) and references therein). Nevertheless, the defining elements of presynaptic toxicity remain controversial and will remain so until a consensus mode of action is agreed upon. It is for this reason that we need to continue the purification and characterization of PLA_2 enzymes from a range of snakes in which they have yet to be fully characterized, as without sufficient data the mode of presynaptic action will remain contentious.

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