The Molecular Biology of Venom Genes from Death Adder and King Brown Snake

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CERTIFICATE OF AUTHORSHIP/ORIGINALITY

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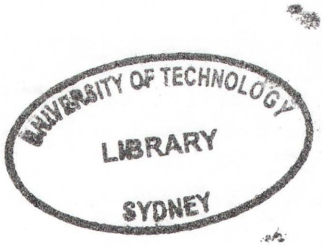

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Abstract

A complex mixture of post-synaptic and pre-synaptic neurotoxins have been identified in both King Brown snake and Death Adder venom. However, since the experiments were conducted using pooled venom samples from different snakes, it could be argued that the large number of homologous toxins previously reported were due to individual (intra-species) variation. Furthermore, previous studies on King Brown snake and Death Adder toxins were mainly at a protein level and there have been few studies at a genotypic level. Thus, a series of experiments were conducted to ascertain whether individual King Brown snakes and Death Adders express an array of toxins, or whether the results were due to the use of pooled venom samples.

In order to isolate all venom genes and their homologues from the genome of both snakes, individual snakes from both species were sacrificed and cDNA libraries were prepared from their excised venom glands. The venom gland cDNA libraries were then screened exhaustively for the presence of any post-synaptic and pre-synaptic neurotoxin genes i.e. the Phospholipase A_2 gene and its homologues, as well as the alpha-neurotoxin genes (both long and short chain α-neurotoxins genes). While the results from King Brown snake cDNA library supported the hypothesis that individual snakes express a number of homologues of PLA_2 enzymes, some of which might be toxins, the actual number of venom genes and the variation between each homologue were lower than previously reported values. Multiple copies of PLA_2 genes were isolated supporting the contention that there was gene duplication of these venom genes. Regions of hyper-variability were also observed within the PLA_2 genes, further supporting the idea that these neurotoxin genes evolved at an accelerated rate. However, with respect to Death Adder cDNA library, the results indicated that there was only a single copy of the PLA_2 γενε, ανδ συππορτεδ τηε νυλλ ηψποτηεσισ τηατ ϖαριατιον μαψβε δυε εντιρελψ το ποολεδ ϖενομ σαμπλεσ.

Υνφορτυνατελψ, νο ρεσυλτσ ωερε οβταινεδ φορ α-neurotoxin genes despite numerous attempts at isolating the long and short chain α-neurotoxin genes. Thus no conclusions could be drawn regarding the evolution and complexity of post-synaptic neurotoxins.

Chapter One

Introduction – The Death Adder and The King Brown snake

1.1 Uniquely Australian

The island continent of Australia was separated from the other continents approximately 30 million years ago when the continents began to drift apart from the massive land mass of Gondwana. Since then, the flora and fauna in Australia have enjoyed relative isolation from competition from species of other continents and have developed many adaptations to the climate and conditions unique to Australia. Two of the native fauna which have captured the interest of this project are the King Brown snake (*Pseudechis australis*) and the Death Adder (*Acanthophis antarticus*). Both are members of the *Elapidae*, a family of venomous snakes with infamous members such as the cobras (*Naja spp.*) and the mambas (*Dendroaspis spp*). The elapid family is particularly interesting in Australia as it has become the dominant family of snakes in Australia.

1.2 The Death Adder

Death Adders (genus *Acanthophis*) are characterised by a broad, triangular head and a short stout body. A characteristic curved soft spine is found at the tip of the tail which has become the distinguishing feature of this genus. Traditionally, there are three species of Death Adder currently recognised in Australia, the Common Death Adder (*A. antarticus*), the Northern Death Adder (*A. praelongus*) and the Desert Death Adder (*A. pyrrhus*) with a number of subspecies within each group (Cogger, 1992). Amongst the members of the elapidae, the Death Adders (genus *Acanthophis*) is perhaps the most intriguing in that it seems to have undergone convergent evolution with the *viperidae*, hence the name adders.

For the purposes of this project, the focus is on the Common Death Adder. This species prefers to live in undisturbed bushlands, using ground cover for shelter. They have been observed to be nocturnal, and spend most of the time half buried in the ground litter waiting in an ambush position for prey. This snake has also developed the caudal luring technique to assist in attracting prey to itself. This technique involves twitching or curling their tail to perhaps simulate the appearance of a worm to lure a hungry animal close to striking distance. While caudal luring is not unique to Death Adders, it is the only reported species within Australia that uses this technique.

A bite from a death adder is toxic, the symptoms of envenomation include pain, and local

swelling at the initial stages, but within 20 hours, there is an onset of paralysis. Personal accounts by Hoser (1995) reported symptoms such as suffer difficulty in movement including breathing leading to a reduction of the total intake of air by 10% of normal conditions (Hoser, 1995, Lalloo *et al.*, 1996, Swindells *et al.*, 2006). Fortunately, recovery is rapid and within two days the subject reported complete recovery. These symptoms are consistent with envenomation by neurotoxins, where the neurotransmitters at the synapses are blocked either in a pre-synaptic or post-synaptic manner leading to loss of muscular control (Swindells *et al.*, 2006). As illustrated by the subject, the loss of muscular control is widespread leading to loss of locomotion and breathing (Van der Weyden *et al.*, 2000). Under normal hunting conditions, the prey would be quickly incapacitated and probably dies from asphyxiation.

1.3 Death Adder Venom Components

Because of the symptoms of neuroparalysis, it was thought that the neurotoxicity of death adder venom was due to the presence of alpha neurotoxins (α-neurotoxins). This was confirmed by Kim and Tamiya in 1980 who isolated small proteins of approximately 60 to 70 amino acid residues (Kim and Tamiya, 1980a, Kim and Tamiya, 1980b). They found that the proteins (Aa-b and Aa-c) were abundant components in the venom and blocked neuromuscular transmission in a post-synaptic manner. Another larger post-synaptic neurotoxin, Acanthophin D consisting of 74 amino acid residues, was isolated by Sheumack *et al.*, from Death Adder (Sheumack *et al.*, 1990) but no further work was done to further study the venom components in Death Adder until 1997, when at University of Technology, Sydney (UTS), Van der Weyden isolated a phospholipase A_2 (PLA_2) enzyme from crude death adder venom and clearly demonstrated that the PLA_2 enzyme was also neurotoxic but acted in a pre-synaptic manner. In other words, it blocked the release of neurotranmitters across the synaptic junction. She also indicated that PLA_2 enzymes could also be a major component in the crude venom of death adder. Following on from her work, Sung (1998) isolated another possible seven isoforms of PLA_2 enzymes from crude death adder samples using liquid chromatography and was able to confirm that PLA_2 enzymes were an important component within the death adder venom. The presence of multiple PLA_2 enzymes showed that death adder venom is in fact a very complex mixture of toxins and more importantly (at least from an evolutionary point of view) is that the venom genes of death adder may have undergone (and is still undergoing) gene duplication and accelerated evolution. While gene

duplication and accelerated evolution of venom genes have been reported in many venomous snakes (Bharati *et al.*, 2003, Chen *et al.*, 2004, Chijiwa *et al.*, 2003) this was the first time it was noted in death adder. Further discussions on the phenomenon of gene duplication and accelerated evolution are covered in later sections of this chapter. There was, however, another explanation for the multiple isoforms of neurotoxins present in the venom, it could be explained by the use of pooled crude venom samples obtained from various snakes, where the phenotype variations amongst the various members could accounted for the presence of multiple genes.

1.4 The King Brown Snake

King Brown snakes (*Pseudechis australis*) or Mulga snake belong to the family of black snakes and the family includes Butler's snake (*P. butleri*), Collette's snake (*P. colletti*), spotted black snake (*P. guttatus*) and red bellied black snake (*P. porphyriacus*). King Brown snakes have a few features which also makes them as interesting as Death Adders. Although the average King Brown snakes has been reported to be around 1.5m to 2m in length, individual specimen up to 3m has been sighted. The size alone, in fact, makes King Brown snakes the heaviest venomous snakes in Australia. It also has the largest venom output per bite, although it not the most venomous (Kuch *et al.*, 2005). The title of the most venomous snake goes to the Inland Taipan or Fierce snake. Despite being a member of the Black snake family, King Brown snakes are not black in colour but rather uniformly brown with some specimen appearing olive green colouration. They prefer dry eucalyptus forests, plains and woodlands as a habitat, and are found throughout the drier regions of Australia. King Brown snakes have been observed to have increased activity at night time, but they could also be active during the day and are aggressive hunters. Their diet includes reptiles, mammals, frogs and birds and will readily prey on other snakes.

Similar to the Death Adder, the bite from a King Brown snake is venomous and considered potentially dangerous. King Brown snake envenomation has been described as haemotoxic (Doery and Pearson 1961), myotoxic (Leonardi *et al.*, 1979) and haemolytic (Sharp *et al.*, 1989). The reported clinical effects which are commonly associated with King Brown envenomation include immediate local pain which can be dull or acutely painful, which may (sometimes) develop into oedema. The systemic effects which follow include headaches, nausea, vomiting, abdominal pains, leading eventually to impaired consciousness and convulsions have also been

noted. Victims have also reported acute renal failure and rhabdomyolysis.

1.5 King Brown Snake Venom Components

Unlike the Death Adder venom (which has largely remained uncharacterised), the venom composition of King Brown snakes is quite well documented. As early as 1961, venom components which were found to be haemolytic were isolated from the crude venom of King Brown snakes (Doery and Pearson 1961). However, the haemolysins were not further characterised. Subsequent research conducted by Leonardi *et al.*, (1979) isolated another toxin, Mulgatoxin which was reported to have mytoxic activity. Then, from 1985 to 1990, a series of neurotoxins were also discovered in King Brown crude venom. The neurotoxins are Pa 1 G, Pa 3, Pa 5, Pa 9C, Pa 10a, Pa 11, Pa 12 A, Pa 12 C, Pa 13 and Pa 15 which are PLA2 enzymes. Another two neurotoxins were discovered, named Pa a and Pa 1D, these are α-neurotoxins (Rowan *et al.*, 1989, Nishida *et al.*, 1985a, Nishida *et al.*, 1985b, Takasaki and Tamiya, 1985, Takasaki *et al.*, 1990a and Takasaki *et al.*, 1990b). These findings definitively showed that King Brown envenomation is a combination of myotoxicity, haemotoxicity and neurotoxicity. Unfortunately, all studies were conducted from pooled venom samples and the large number of neurotoxin isoforms noted above could be due to intra-species variation.

1.6 Habitat destruction and loss of a natural resource

Death adder and King Brown snake are two native venomous snakes in Australia which have adapted to the harsh climate of Australia and have developed rich and complex venom as means of hunting and defence. This rich complexity of biomolecules is an excellent resource for researchers interested in designing or studying drugs, biological probes and other bioactive molecules (Kini, 2006, Cury and Picolo, 2006). As noted earlier, both snakes have a wide distribution over Australia, but recent climate changes in Australia, drought and more importantly, the expansion of cities and farmlands have removed much of the habitat previously suitable to these snakes. In fact, the populations of these snakes have been in decline and although they were both considered common in 1995, Death Adder and King Brown snakes have now been enlisted as endangered. Thus, one of the purposes of this project is to provide further characterisation of the venom from these very interesting animals before it becomes no longer possible to study these animals further.

In the earlier sections, phospholipase A_2 enzymes and α-neurotoxins were both identified as very important components in Death Adder and King Brown venom, and are thought to be responsible for the neurotoxicity from envenomation. In the following sections, the detailed structures of these two proteins are discussed.

1.7 Phospholipase A_2 enzyme

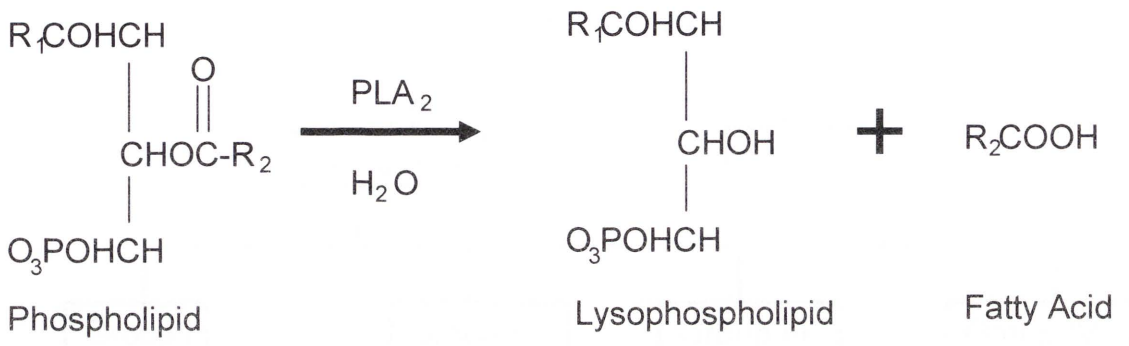
PLA_2 enzymes comprise approximately 5% to 25% of the total protein content in crude venom, depending on the species which contributed the venom (Mirtschin *et al.*, 1990) and is amongst the most abundant bioactive molecule found in the venom. More importantly, PLA_2 enzymes are not only restricted to snake venom, but are also present in many different types of mammalian tissue and play important roles in many cellular processes. For example, PLA_2 enzymes have been isolated from mammalian pancreatic tissue and play an important role in lipid metabolism (Kini, 1997). PLA_2 enzymes are also involved in the regulation of phospholipid turnover in the plasma membrane as well as the production of eicosanoids (Terao *et al.*, 1997). It has also been reported that these enzymes accumulate at inflammatory sites such as synovial fluids (Terao *et al.*, 1990) and may be involved in regulation of the inflammatory response. High levels of PLA_2 enzymes were also found circulating in patients with multisystem organ failure (Vadas, 1997) as well as amongst sufferers of Alzheimer's disease (Ross *et al.*, 1998). It is the ubiquitous nature of PLA_2 enzymes that has made this enzyme become one of the most well characterised enzymes in biology today and the possible roles it may play in various diseases have fuelled the need for further research into isolation and determination of the structure and function of different PLA_2 enzymes.

1.8 The PLA_2 Reaction

By definition, an enzyme is recognised as a PLA_2 enzyme (E.C. 3.1.1.4 phosphatide sn-2 acylhydrolase) if it can hydrolyse phospholipids at the sn-2 position on the glycerol backbone of a phospholipid to release a lysophospholipid and a fatty acid. PLA_2 substrates include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and many other naturally occurring phospholipids. Figure 1.1 below shows the site of action and the catalytic reaction (Waite, 1987; Kini *et al.*, 1997). This unique reaction of PLA_2 enzymes could be exploited in

enzymatic assays to detect the presence of PLA_2 enzymes by the creation of mixed micelles (containing phospholipids) as substrates and the detection of the fatty acids and lysophospholipid products that would be produced if PLA_2 activity were present in a novel enzyme.

Figure 1.1 : The Catalytic Reaction Of PLA_2 On Phospholipids



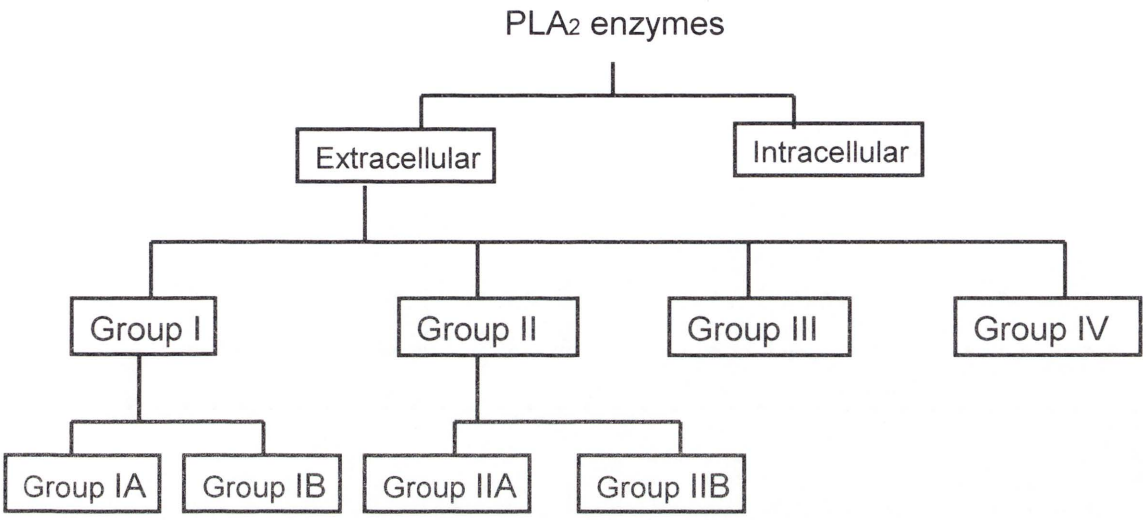
1.9 Classification

PLA_2 enzymes can be isolated from many different sources. Some PLA_2 enzymes, such as those isolated from snake venom or pancreatic tissue are secretory enzymes while other types of PLA_2 enzymes are found as intracellular enzymes. PLA_2 enzymes can also be monomers, dimers (both homodimeric or heterodimeric), and multimeric units such as taipoxin and textilotoxin. Since there are many types of PLA_2 enzymes, scientists have found it convenient to organise the various types into groups or classes, based on primary structure and sequence homology and for the purposes of this project, the most common classification of PLA_2 enzymes is adopted.

PLA_2 enzymes have been traditionally classified into two broad categories based on their size and sequence homology (Arni *et al.*, 1996):- intracellular and extracellular PLA_2 enzymes. The intracellular PLA_2 enzymes are typically 80 kDa to 400 kDa in size and share little sequence homology with extracellular PLA_2 enzymes. They can be calcium dependent or calcium independent in their catalytic activity (Kini, 1997) and are often membrane associated (Arni, *et al.*, 1996). Intracellular PLA_2 enzymes are not homologous to the neurotoxic snake venom PLA_2 enzymes and share little in common with them. Thus, these intracellular PLA_2 enzymes will not be discussed further. The other group, however, is more interesting. Extracellular or secretory PLA_2 enzymes are considered low molecular weight enzymes (12 to 15 kDa) and this group is further divided into four groups (groups I to IV). Groups I and II are subdivided into Groups IA

and IB and Group IIA and IIB based on sequence homology. Unlike intracellular PLA_2 enzymes, all members of the extracellular PLA_2 enzymes are calcium dependent. For the purposes of this project, this classification system is used. Figure 1.2 is a diagrammatic representation of this classification (Kini, 1997, Arni *et al.*, 1996, Jeyaseelan *et al.*, 2000)

Figure 1.2 - Classification of PLA_2 enzymes



1.9.1 Group I

Group I PLA_2 enzymes are typically 115 to 120 amino acid residues in length for the monomeric unit and are 13 kDa to 15 kDa in size. All members of this group (IA and IB) have seven disulfide bonds, six of these bonds are also conserved in Group II PLA_2 enzymes. The unique disulfide bond for this group is formed by cysteines (Cys) 11 and 77. Group IA PLA_2 enzymes are isolated from *Elapidae* and *Hydrophiidae* (sea snake) venoms and all members of this group have a unique 'elapid' loop, which is a two to three amino acid residue insertion in the region of residues 52 - 65 (Arni *et al.*, 1996, Danse *et al.*, 1997). Group IB PLA_2 enzymes differ from Group IA enzymes in that they are isolated from mammalian pancreatic tissue and have a five amino acid extension to the elapid loop, forming the 'pancreatic' loop (Arni *et al.*, 1996, Dennis 1994).

1.9.2 Group II

Group II PLA_2 enzymes are slightly larger in size with 120 to 125 amino acids in the monomeric unit (Kini, 1997), they also have seven disulfide bonds. However, instead of the Cys-11 to Cys-77 disulfide bond, Group II PLA_2 enzymes have a five to seven amino acid residue C-terminal extension which contains a cysteine that forms a disulfide bond with Cys-51 on the main body of the enzyme (Arni *et al.*, 1996; Kini, 1997). Group IIA and Group IIB are further distinguished by the amino acid residue in position 49 which coordinates the binding of calcium ion to the protein (Scott, 1997). Group IIA have an aspartate (Asp) at position 49 and group IIB have a lysine (Lys) or serine (Ser) substitution. It was believed that Asp-49 was essential for the binding of Ca^{2+} to the active site. However, it has been shown that in the presence of a suitable substrate, Lys-49 PLA_2 enzymes can also bind Ca^{2+} (Waite, 1987). The crotalids and viperids are the main sources for the isolation of Group II PLA_2 enzymes.

1.9.3 Group III

Group III PLA_2 enzymes have low primary sequence homology with groups I and II and only four to five disulfide bonds are present (Waite, 1987; Scott, 1997). However, the active site where catalysis of the substrate takes place shares similar features with the active sites of groups I and II PLA_2 enzymes (Arni *et al.*, 1996). For example, the Ca^{2+} binding machinery is the same (Scott, 1997). Group III PLA_2 enzymes are isolated from bee and the gila monster venom (Kini *et al.*, 1997). All members in this group are glycoproteins and are 130 to 135 amino acid residues in length.

1.9.4 Group IV

Group IV PLA_2 enzymes are isolated from Conus magus, the marine invertebrate coneshell. The primary sequence differs significantly from groups I, II and III, and the enzyme is composed of two chains. The longer chain is 77 amino acids in length and is joined to the shorter 42 amino acids chain by a disulfide bond (Kini *et al.*, 1997).

1.9.5 Subgroups of PLA_2 enzymes

A more refined classification was proposed by Danse *et al.*, (1997) where Group I was further divided into five subgroups (IA, IA', IB, IB' and IB") and Group II was subdivided into six

subgroups (IIA, IIA', IIA", IIB, IIB' and IIB") based on amino acid sequence homology and the number of disulfide bonds. Table 1.2 is a summary Danse's proposed classification.

Despite the amazing variety of PLA2 enzymes available, the important characteristics of the secretory PLA2 enzymes are that they are all 13 to 15 kDa in size, ranging from 115 to 125 amino acids residues in length and contain 6 to 8 disulfide bonds with 7 disulfide bonds being the most common motif by far. As for the purposes of this project, only the PLA2 enzymes which occur in elapid snakes are of interest, that is, this project is concerned with Group 1A PLA2 enzymes, which may or may not be neurotoxic.

Table 1.1 : Danse's Classification Of Group I And II PLA_2

Group	Number of Disulfide Bonds	Source of PLA_2
IA	7 disulfide bonds in the monomeric unit	Elapids except *N. scutatus* and *O. scutellatus*
IA'	6 disulfide bonds, and one disulfide bond between the A chain and the dendrotoxin-like B chain	*β-bungarotoxins* and its isoforms
IB	7 disulfide bonds in the monomeric unit	mammalian pancreatic PLA_2
IB'	8 disulfide bonds 7 disulfide bonds and 1 free cysteine residue (normally forms a disulfide bond with another subunit)	γ chain of taipoxin D chain of textilotoxin
IB"	7 disulfide bonds in the monomeric unit. Contains a pancreatic loop.	*N. scutatus* and *O. scutellatus*
IIA IIA' IIA"	7 disulfide bonds in the monomeric unit. Group IIA are Asp-49. Group IIA' are Ser-49 or Lys-49.	*Viperinae, Crotalinae*
IIB IIB"	6 disulfide bonds in the monomeric unit	*Bitis gabonica* and *B. nasicornis* human and rat tissue
IIB'	8 disulfide bonds in the monomeric unit	rodent brain

110 The Phospholipase A_2 Fold

Although there are significant differences in primary amino acid sequences of groups I and II PLA_2 enzymes, they adopt secondary and tertiary structures which are conserved. This similarity or common motif is known as the 'PLA_2 fold' (Scott, 1997; Arni *et al.*, 1996) which is

characterised by two antiparallel disulfide linked α-helices (Arni *et al.*, 1996), labelled A and B in Figure 1.2 below, and a third N-terminal α-helix (labelled C) which lies on top of the two α-helices.

Figure 1.3 : Three Dimensional Ribbon Representation Of PLA_2

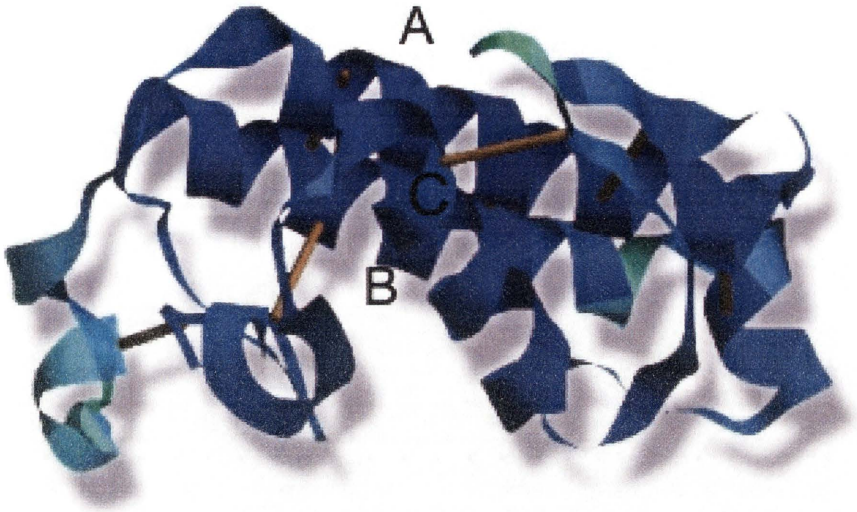


Figure 1.3: 3 Dimensional ribbon representation of Bovine PLA_2 showing the typical PLA_2 fold which consists of two α-helices (labeled A and B) and a n-terminal α-helix (labeled C). (www.isis.rl.ac.uk/isis2004/highlights/Vacklin.htm)

In the PLA_2 fold, the hydrophilic amino acid side chains are exposed to the solvent while the hydrophobic residues are directed towards the inner core (Arni *et al.*, 1996). Histidine (His)-48, Asp-49, Asp-99, Tyrosine (Tyr)-52, Proline (Pro)-58, Asparagine (Asn)-71, Alanine (Ala)-1, Tyr-73 and Glutamate (Gln)-4 together form a hydrogen bonding network in the catalytic/active site of the PLA_2. The presence of Asp-99 is believed to be critical in the binding site for proper catalytic activity (Sekar *et al.*, 1997).

Other than the small size and unique PLA_2 fold, PLA_2 enzymes are unique also in that they contain 14 cysteines (or 7 disulfide bonds) in their primary sequence. The positions and functions of the 7 highly conserved disulfide bonds common to groups I and II PLA2 enzymes are shown in Table 2 below :-

Table 1.2: Location And Function Of The Conserved Disulfide Bridges In PLA_2 enzymes

Location	Function
Cys 27 - Cys 126	anchors Calcium binding loop and C-terminal
Cys 29 - Cys 45	stabilises the Calcium binding loop
Cys 44 - Cys 113	holds the antiparallel α-helices
Cys 51 - Cys 106	holds the antiparallel α-helices
Cys 61 - Cys 99	stabilises another minor α-helix
Cys 89 - Cys 104	holds a β-sheet structure to α-helix
Cys 11 - Cys 80 (Group I) or Cys 51-Cys 133 (Group II)	anchors the N terminal α-helix to the protein body stabilises the C-terminus

Assembled from Danse *et al.*, 1997 and Scott, 1997

It has been generally assumed that the seven highly conserved disulfide bonds (together with intrachain hydrogen bonds) are needed to ensure proper folding of the protein and to maintain structural integrity of the PLA_2 enzyme. However, Zhu *et al.*, (1995) proposed that disulfide bonds are not critical to the proper folding of the enzyme except for the Cys 89 - Cys 104 pair and that only the Cys 11 - Cys 80 pair is important in lending structural integrity and conformational stability to the protein. Zhu *et al.*, (1995) also stated that Cys 27 - Cys 126 disulfide bridge actually destabilises the protein.

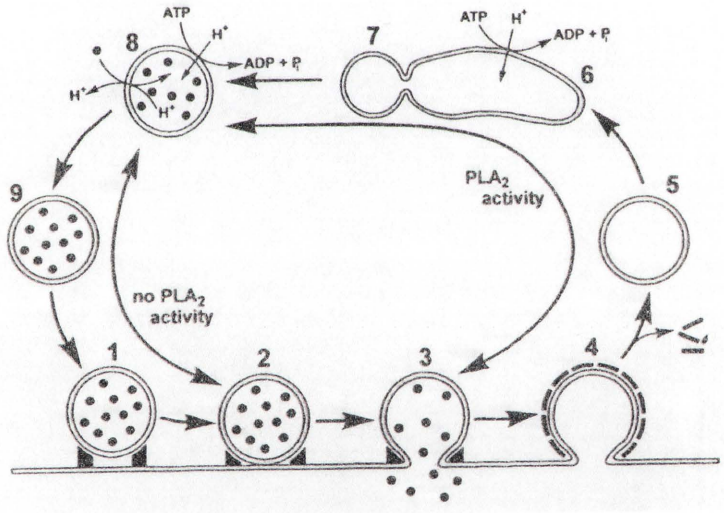
1.11 Toxicity of PLA_2 enzymes

As mentioned in the introductory sections, PLA_2 enzymes are also associated with a diverse range of physiological effects such as pre-synaptic neurotoxicity, local and systematic myotoxicity, cardiotoxicity, anti-coagulation, platelet aggregation initiation and inhibition, haemorrhage, convulsions and oedema inducing effects (Kini *et al.*, 1997, Sharpe *et al.*, 1989, Leonardi *et al.*, 1979). Specific tissue damage has also been reported, possibly due to the presence of PLA_2 acceptors such as M-type monomeric PLA_2 acceptors on human kidney cells (Gubensek *et al.*, 1997).

1.11.1 Neurotoxicity

PLA_2 enzymes which act as pre-synaptic neurotoxins such as those isolated from Death Adder and King Brown snake venoms are also know as β-neurotoxins. These neurotoxins are also found in other native Australian snakes such as Notexin which was first isolated from the common Tiger snake Notechis scutatus (Halpert and Eaker, 1975), and the trimer Taipoxin isolated from Taipan (Oxyuranus scutellatus), (Lind and Eaker 1982). It has been proposed that the region between residues 59 to 92 may be responsible for neurotoxicity (Dufton and Hider, 1983) and is independent of the PLA_2 activity or the active site for PLA_2 activity. The mode of action has been described as a triphasic change in the neuromuscular tissue:- There is an initial transient inhibitory phase or a suppression in transmission; followed by a facilitatory phase (a phase of enhanced transmission) and a late inhibitory phase which is irreversible (Gubensek *et al.*, 1997). The mechanism of this reaction is not well understood but may involve the β-neurotoxin interfering with the synaptic vesicle cycle and stopping the uptake of choline into nerve cells. Figure 1.4 below offers a possible explanation for the triphasic response.

Figure 1.4 : The Synaptic Vesicle Cycle



The exocytosis of neurotransmitters (indicated on stages 1 to 3 and 9) into the synaptic cleft is not inhibited, but it is believed that β-neurotoxins may interfere with the subsequent endocytosis and regeneration of neurotransmitters in the synapse (stages 4 to 8). Adapted from Gubensek *et al.*, 1997, page 259.

1.11.2 Myotoxicity

Another type of toxicity commonly associated with venom PLA_2 enzymes is myotoxicity which involves the disruption of the plasma membrane, mitochondria and myofibrils in muscle cells (Harris and Maltin, 1982). Furthermore, dilation in the sarcoplasmic reticulum and hypercontractions are observed. This eventually leads to local tissue necrosis (Gopalakrishnakone *et al.*, 1997). Myotoxicity may be localized or systematic depending on the type of venom being injected. For example, severe muscle damage due to myotoxicity is a symptom of envenomation by the King Brown snake (*P. australia*) (Fry, 1998). In addition, viperid and crotalid envenomation also typically show signs of myotoxic damage but, neurotoxicity is still the most significant physiological effect for the Australian elapids (Tsai, 1997). However, while it is an interesting phenomenon, myotoxicity is beyond the scope of this project.

1.11.3 Haemotoxicity

Other than neurotoxicity and myotoxicity, some PLA_2 enzymes are haemotoxic and can cause coagulation defects. The effects of PLA_2 may be classified into:- PLA_2 enzymes which initiate aggregation of platelets; PLA_2 which inhibit platelet aggregation; and PLA_2 enzymes which may initiate or inhibit aggregation (Kini and Evans, 1997). More importantly, even PLA_2 enzymes which cause platelet aggregation can have anti-coagulant effects. Systematic initiation of coagulation and platelet aggregation lowers the level of fibrin in the blood, leading to an inability to coagulate (Mirtschin, 1990). The region between residues 53 and 76 was proposed to be responsible for anti-coagulant activity in Class I PLA_2 enzymes (Carredano *et al.*, 1998, Kini and Evans, 1989). Among Australian Elapids, the venom PLA_2 enzymes from the Copperheads (*Austrelaps spp*) are most commonly associated with anti-coagulation (Fry *et al.*, 1998). As noted in the introductory sections, King Brown snake venom is reported to be haemotoxic but the components causing haemotoxicity are not well characterised.

1.12 Toxicity and PLA_2 activity

The β-neurotoxins are secretory PLA_2 enzymes but not all secretory PLA_2 enzymes are neurotoxins. There has been some speculations as to whether there is any correlation between enzyme activity and toxicity. Chang (1985) first suggested that neurotoxicity was due to the binding of the toxin molecule to the target receptors and was independent of PLA_2 activity.

However, some researchers have reported that if the enzyme activity is altogether eliminated, toxicity is also reduced significantly in many cases (Choumet *et al.*, 1991), thus offering evidence that PLA_2 activity and toxicity are linked. However, it was found that notexin (which is one of the most potent β-neurotoxins) has a relatively low enzyme activity, thus demonstrating that toxicity and enzyme activity are not directly related and furthermore, chemical modification of the PLA_2 enzyme at tyrosine 77 and tyrosine 110 resulted in a reduction in lethality of the toxin but no reduction in PLA_2 activity (Scott *et al.*, 1992, Mollier *et al.*, 1989, Yang and Chang 1991). In an effort to resolve this contradiction, a model was proposed by Kini and Evans (1989) which suggested that the venom PLA_2 enzyme possesses a catalytic site (for PLA_2 activity) and an additional pharmacological site which has an affinity for ligands found on the targeted cell or tissue (i.e. a target site). It was proposed that different venom PLA_2 enzymes would have different pharmacological sites with different sites being responsible for different physiological effects such as neurotoxivity, myotoxicity or haemotoxicity. It was further proposed that different isoforms of venom PLA_2 enzymes would have different pharmacological site affinities for the target ligand thus resulting in different binding strengths. Under this model, the most important determinant for toxicity then was the interaction between the target site and the pharmacological site. Thus, while notexin might have a low enzyme activity, its high affinity for the target tissue meant a lower concentration of notexin would achieve the same toxic result as higher concentrations of other venom PLA_2 enzymes which did not bind as strongly to the target tissue. However, by eliminating PLA_2 activity altogether, toxicity is reduced because even though the toxin molecule might bind to the target receptor, it is catalytically inert.

This issue, however, is still not at rest. Experiments conducted by Chang *et al.*, (1996) on notexin demonstrated a reduction in both enzyme activity and lethality by chemical modification of the residue lysine at positions 82 and 115. Subsequently, another experiment where arginine residues at position 43 and 79 were also chemically inactivated again demonstrated a loss in both PLA_2 activity and neurotoxicity (Chang *et al.*, 2004). Finally, in 2003, Chioato and Ward conducted a series of site directed mutagenesis experiments and analysed the data with a new computer assisted protocol. They found that the residues involved in myotoxicity and membrane damaging activities were the same ones (Chioato and Ward, 2003) and thus supporting the model proposed by Kini that there were biologically active "hotspots" on the surface of the molecules. Undoubtedly, further research will be conducted in this area to resolve this controversy.

1.13 The venom PLA_2 genes

The discussion so far has been focused entirely on venom PLA_2 enzymes at the protein level, and if a database search were conducted, it would reveal that a great deal of information has already been made available on venom PLA_2 enzymes. However, if a search were conducted on the gene level for Australian snake venom genes, the results are very different. Table 1.3 shows a exhaustive list of the snakes from which various venom PLA_2 genes have been found.

Although a number of venom PLA_2 genes have been identified from snakes such as the Copperhead (Jeyaseelan *et al* 2000), Tiger Snake, Taipan and Eastern Brown snake, other members are still absent, such as Death Adders. The contrast between information available on venom PLA_2 genes of snakes which are not native to Autralia and native snakes is stark and illustrates the general lack of interest by researchers of native Australian snakes especially at a molecular level.

Table 1.3 :- Overseas and native snakes which have been identified with positive PLA_2 gene

Non Australian Snakes	Source
Aipysurus eydouxii	Li *et al.*, 2005
Aipysurus laevis	Ducancel *et al.*, 1988b
Bothriechis schlegelii	Chen *et al.*, 2004
Bothrops asper	Pescatoriet *et al.*, 1998
B. erythromelas	de Albuquerque Modesto *et al.*, 2006
B. insularis	Junqueira-de-Azevedo and Ho, 2002
B. jararacussu	Andriao-Escarso *et al.*, 2002
Bitis arietans	Oliver *et al.*, 2005
Bitis gabonica	Francischetti *et al.*, 2003
Bungarus fasciatus	Zha and Zhang, 2001
B. multicinctus	Danse, 1990
Calloselasma rhodostoma	Tsai *et al.*, 2000
Cerrophidion godmani	Chen *et al.*, 2004
Crotalus atrox	Tsai *et al.*, 2000
C. viridis viridis	Tsai *et al.*, 2003
Daboia russellii russellii	Madhukumar *et al.*, 2006

Daboia russellii siamensis	Yong-Hong *et al.*, 2003
Gloydius halys	Pan *et al.*, 1997
Lachesis muta muta	Fortes-Dias *et al.*, 2003
Lapemis hardwickii	Wei *et al.*, 1999
Laticauda colubrina	Fujima *et al.*, 2002
L. laticaudata	Fujima *et al.*, 2002
L. semifasciata	Tamiya and Fujima 2000
N. naja naja	Pan *et al.*, 1994
Ophiophagus hannah	Lee and Zhang, 2000
Protobothrops elegans	Chijiwa *et al.*, 2006
Python reticulatus	Thwin *et al.*, 2000
Sistrurus miliarius streckeri	Chen *et al.*, 2004
Trimeresurus flavoviridis	Chijiwa *et al.*, 2003
Trimeresurus jerdoni	Lu *et al.*, 2002
Australian snakes	Source
Austrelaps superbus	Singh *et al.*, 2000
Notechis scutatus scutatus	Ducancel *et al.*, 1988a
Oxyuranus scutellatus scutellatus	St Pierre *et al.*, 2005
Oxyuranus microlepidotus	St Pierre *et al.*, 2005
Pseudechis australis	St Pierre *et al.*, 2005
Pseudonaja textilis	Armugam *et al* 2004
Tropidechis carinatus	St Pierre *et al.*, 2005

1.14 The Alpha Neurotoxins

The second group of neurotoxins found in Death Adder and King Brown venoms are the alpha neurotoxins (α-neurotoxins) which are a very well characterised group (in other snakes) of small peptides of approximately 6 to 8 kDa in size. They have a high affinity for N-acetylcholine receptors (nAChR)s located at the post-synaptic membranes of skeletal muscles and thus act as post-synaptic neurotoxins. The reversible binding of α-neurotoxins to the receptor prevents the binding of the neurotransmitter acetylcholine and the generation of the excitatory response in muscle, leading to a flaccid paralysis (Lang *et al.*, 2005). Due to their high affinity for receptors

and their small size, α-neurotoxins make excellent biological probes and are used in the study of ligand to substrate binding studies as well as many electrophysiology experiments. For example, it was the initial isolation and discovery of the α-neurotoxin α-bungarotoxin that made it posssible for the detailed examination of the nicotinic acetylcholine receptor (AChR) due to the high affinity of α-bungarotoxin to the receptor (Hucho *et al.*, 1996). The structure of potassium channels and muscarinic receptors were also similarly deduced by studying the binding of a selective potassium channel blockers to the channels themselves (Fry, *et al.*, 1998, Gubensek *et al.*, 1997). Furthermore, the three dimensional fold of the α-neurotoxin is a very heat resistant and stable structure and it has been proposed that this scaffold can be utilized in biotechnology (Kini, 2006).

1.15 Classification

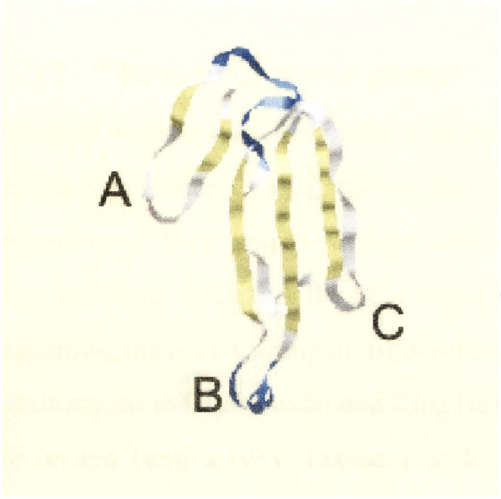
Traditionally, α-neurotoxins have been classified into two groups (Menez 1998, Tsetlin 1999) based on their size and structural differences. These are the short and long chain α-neurotoxins with the examples of Aa C and Aa b (from Death Adder) as members of the short and long α-neurotoxin groups respectively (Kim and Tamiya, 1981a, Kim and Tamiya, 1981b) and Pa-ID (isolated from King Brown) as a member of the long chain α-neurotoxins group (Takasaki 1989). More recently, a third group of α-neurotoxins, possibly unique to Pseudonaja textilis have been proposed (Jeyaseelan 2000). The short chain α-neurotoxins are approximately 60 residues in length, the long chain α-neurotoxins 70 residues in length and the third group of α-neurotoxins are slightly larger, being 80 residues in length with significant protein homology found amongst members within the same group. Despite differences in the primary and secondary structures, all three groups have eight conserved cysteine residues which form four cysteine bridges and fold the molecule into a similar tertiary structure called the "three finger" fold.

1.16 Structure and Function of Three Finger proteins

In fact, α-neurotoxins are part of a large family of proteins which share a similar protein fold. Other notable members of this family include the cardiotoxins, the fasiculins and the muscarinic toxins (e.g. MTX2). This fold has been described as the extension of three major loops (marked A, B and C in figure 1.4) emerging from a hydrophobic globular core (Tsetlin, 1999) (see figure 1.5 below) while the secondary structure of the three extensions are exclusively composed of β-

sheets. A prominent feature of the α-neurotoxins conformation is the formation of disulphide bonds found in Cys-30 and Cys-34, and this disulphide bond takes part in the formation of an extra loop in the molecule (Menez, 1998).

Figure 1.5 - Ribbon representation of α-neurotoxin



A ribbon diagram showing the putative 3 dimensional structure of a α-neurotoxin isolated from cobra (*Naja* spp.) venom. (www.srs.ac.uk/px/manual9.6/home_files/cvants.gi)

All three groups have potent postsynaptic neurotoxicity and bind readily to acetylcholine receptors non-covalently, relying on hydrophobic interactions and hydrogen bonds. In addition to having an affinity for skeletal muscle nAChRs, long chain α-neurotoxins have also demonstrated an affinity for neuronal nAChRs (Endo *et al.*, 1991, Servent *et al.*, 1997) and this affinity may be attributed to the structural difference of an extra loop present in long chain α-neurotoxins (Servent *et al.*, 1997). Site mutagenesis studies, NMR and X-ray crystallography have demonstrated that the 3 loops play an important part in the biological activity of α-neurotoxins (Tsetlin *et al.*, 1982, Tremeau *et al.*, 1995). Mutations of the hydrophobic residues found on the loops of the α-neurotoxins erabutin-A support the hypothesis that these residues contribute to the binding affinity of the α-neurotoxins to its ligand (Tremeau *et al.*, 1995, Tselin, 1999), while the middle loop may have a significant role in the neurotoxicity of α-neurotoxins. In fact, specific residues found on the tips of the extensions, in particular residues Trp-29, Arg-37 and Gly-38 were found to be essential for neurotoxicity (Tsetlin *et al.*, 1979). In cardiotoxins (another toxin isolated from venom which contains the three finger fold), the first loop (residues 2 to 14) is considered important in lytic activity with residues Leu6 to Tyr 11 playing an important role in

the activity. Mutation of these residues have been shown to reduce lytic activity in the toxin (Kumar *et al.*, 1997). While the middle loop has been proposed to act as a chaperone to the binding of the toxin to its ligand (Kumar *et al*, 1997). Menez (1998) also observed that the fold can be differentially twisted and accommodate different orientations between different members.

1.17 The α-neurotoxin genes

Despite the abundant information on protein sequence and structure, there are relatively few nucleotide sequences available for the α-neurotoxins of Australian venomous snakes (while β-neurotoxins from non Australian snakes are plentiful). The NCBI database for nucleotide sequences revealed that the only significant contribution thus far is from Jeyasaleen (2000) regarding the SNTX group of α-neurotoxins. Furthermore, while the amino acid sequences of α-neurotoxins in Death Adder and King Brown are available, no nucleotide sequences are available (Kim and Tamiya 1981, Takasaki, 1989, Tyler, 1997). A list of the α-neurotoxins identified in King Brown snakes and Death adder, together with the novel group of α-neurotoxins from tiger snakes are shown in Table 1.4.

Similar to PLA_2, multiple isoforms of these α-neurotoxins may also exist in Death Adder and King Brown due to gene duplication and accelerated evolution. This is supported by the presence of five novel α-neurotoxin genes, SNTX1,2, 3, 6 and 7, isolated from a genomic DNA library prepared from Eastern Brown snake (*Pseudonaja textilis*) (Jeyaseelan 2000). However, there are significant differences in the amino acid sequence between SNTX and long and short chain α-neurotoxins. In fact, only the three finger fold and neurotoxicity have been conserved. Furthermore, homologues to SNTX have not been identified in other Australian venomous snakes such as the King Brown or Death Adder. Thus it could be argued that gene duplication may be restricted to the SNTX group of toxins only. It would be interesting to establish whether muliple isoforms of α-neurotoxins also exist for long and short α-neurotoxins in Death Adder and King Brown snake as well as to characterise this family of toxin genes and to determine if SNTX like genes exist in both King Brown snake and Death Adder.

Table1.4 :- α-neurotoxins from Australian snakes

	Short α-Neurotoxins	Long α-Neurotoxins	Group 3
Death Adder	Aa-c (Kim and Tamiya 1981a)	Aa-b (Kim and Tamiya 1981b) Aa-e (Tyler *et al.*, 1997)	
King Brown	Pa-A (Takasaki, 1989)	Pa-ID (Takasaki and Tamiya, 1985)	
Tiger Snake			Sntx 1-3, 6 and 7 (Jeyaseelan 2000)

1.18 Accelerated Evolution

All genes evolve over time due to random mutations and this rate of change is constant. However, under some circumstances which have not yet been determined, some genes undergo an accelerated rate of mutation and this is termed accelerated evolution (Gubensek *et al.*, 1997). Often coupled with accelerated evolution is the concept of gene duplication which simply means a gene is duplicated in its entirety into multiple copies which are simultaneously expressed. However, each new copy would be different to the original in a few places due to accelerated evolution. Most importantly, neither gene duplication nor accelerated evolution are random events, there are undetermined mechanisms which actively cause genes to produce multiple copies of themselves and undergo changes at multiple sites, resulting in the production of new proteins which are isoforms of the original. The phenomenon has been reported in both the *Viperinae* and the *Crotalinae*. Nakashima *et al.*, (1993) isolated and sequenced PLA_2 isozyme genes from the Habu snake (*Trimeresurus flaviridis*) and found six different genes which encoded for six different PLA_2 enzymes and demonstrated that at least for the crotalids, a single snake does express a host of different PLA_2 enzymes. In addition, they also found that the introns of the six genes were remarkably conserved and that the protein coding exons underwent accelerated substitutions. They concluded that the venom PLA_2 genes in *T. flaviridis* have evolved so as to bring about accelerated evolution.

Further evidence that multiple venom PLA_2 genes exist in a single snake was provided by Moura-da-Silva *et al.*, (1995) who found that venom PLA_2 gene duplications may have occurred in

Bothrops jararacussu. However, they warned that the evidence is speculative and further investigation is warranted. Tsai (1997) went on to state that venom PLA_2 gene duplication and accelerated evolution in the exons of venom PLA_2 genes are common for all members of the *Crotalinae* and *Viperinae*. As for the *Elapidae*, Jeyaseelan (2000) isolated four isoforms of a novel group of α-neurotoxin, and observed that these isoforms had also undergone accelerated evolution and gene duplication. Thus, it would be quite plausible that the multiple death adder PLA_2 enzymes and King Brown PLA_2 enzymes were a product of multiple PLA_2 genes which have undergone accelerated evolution and gene duplication. However, this remains for now a speculation.

1.19 Aims and Summary

This introductory chapter has hopefully identified several areas of interest in the molecular biology of Death Adder and King Brown snake venom, especially with respect to PLA_2 enzymes and α-neurotoxins. PLA_2 enzymes are an important group of enzymes with interesting physiological effects and potential pharmaceutical benefits. While current research projects in the field of native Australian snakes have yielded data which are predominantly protein, there is hardly any data on the molecular biology of these important venom genes. Thus, the aim of this project is to determine the gene sequences of these PLA_2 genes (and also, to a lesser extent, α-neurotoxin genes). Another area of interest is to obtain some preliminary evidence to support the hypothesis that the processes of accelerated evolution and gene duplication have occurred in Death Adder and King Brown snake venom genes since it is almost taken as dogma that these processes have occurred but as yet there is no definitive proof to support this idea. This will also resolve the question as to whether using pooled samples of crude venom in studying toxinology at the protein level could result in misleading results later.

However, in order to obtain definitive proof of accelerated evolution, the rate of mutation (dn) in the genes of interest must be shown to be greater than the normal rate of mutation (ds) or base rate of mutation. One method to establish that dn/ds ratio is greater than 1 would be to obtain the entire genomic sequences of the genes of interest (both exons and introns) for direct comparative study. In particular, the introns which (in theory) are not subjected to any pressure from natural selection would be ideal as an indicator of the base rate of gene mutation. Furthermore,

housekeeping genes such as GAPDH should also be included as an additional control to obtain the rate of mutation for non-venom genes. Unfortunately, this goes beyond the limited resources available; thus any evidence for accelerated evolution that could be discovered in this project is at best indicative.

CHAPTER TWO

Construction of complementary DNA libraries from Death Adder and King Brown Snake venom glands

2.0 Introduction

Death Adder and King Brown snakes are protected species and only a limited supply of tissue samples could be made available for this project. While there are a number of different techniques available for isolation and determination of various venom genes from the venom glands of these snakes such as RACE PCR and RT PCR, the construction and screening of cDNA libraries from the appropriate venom producing tissue (the venom glands) is the method of choice as this method requires the least amount of venom gland tissue. In this chapter, the methods for preparation of the materials for extraction of messenger RNA to the construction and verification of the libraries are discussed.

2.1 Preparation of DEPC water

DEPC (Diethypyrocarbonate) treated water was prepared by addition of DEPC to a final concentration of 0.1% (v/v) to water. The solution was then vigorously mixed by shaking and left to incubate overnight at 37°C with the bottle cap loose for the decomposition of DEPC, any remaining DEPC is then removed by autoclaving.

2.2 Precautions taken in messenger RNA extraction

Since RNA is rapidly destroyed by RNases present in the air, RNA isolation experiments were carried out with 0.1% (v/v) DEPC treated water at all times. Where possible, the experiments took place in a laminar flow hood to reduce contamination and the equipment used were sterilized by double autoclaving or previously cleaned with a solution of 0.1M sodium hydroxide solution to remove residual RNases on the surface. It should be noted here that steam in autoclaving process can sometimes introduce RNases, thus only deionised water was used in the autoclaving machine.

2.3 The Modified Chomczynski and Sacchi Method

Total RNA extraction was based on the single step method using acid guanidium thiocyanate and phenol-chloroform, as described by Chomczynski and Sacchi in 1987, with a few modifications detailed below. In the original method, the recommended denaturing solution was 4M guanidium thiocyanate, buffered by 25 mM sodium citrate at pH 7, with 0.5% sarcosyl and 0.1 M β-mercaptoethanol. The modified experiments used a denaturing solution which consisted of 4M

guanidium thiocyanate, buffered by 0.1M Tris-HCl at pH 7.5 with the addition of 0.1% (v/v) β-mercaptoethanol just prior to extraction. This method required previously homogenised tissue samples (approximately 100 mg) which were added to 1 ml of chilled guanidium thiocyanate solution on an ice bath, then immediately followed by the addition of 50 µl of 2M sodium acetate (at pH 4.0); 500 µl of water saturated phenol (acidified to pH 4) and 100 µl of chloroform-isoamyl alcohol, at a ratio of 49:1 (v/v). The addition of each ingredient was done in sequence with thorough mixing required for each ingredient. Following the final mixing, the solution was then chilled on ice for 15 minutes and centrifuged at 10000g for 20 minutes in a cold room (4°C). Total RNA would be retained in the top aqueous layer while DNA and proteins would be extracted from the top layer and be trapped at the interface.

While the original method only required a single step extraction, it was found that in order to obtain RNA of high purity, several extra rounds of extraction were necessary, thus, the modified method also included an additional two rounds of extraction. The first round required the addition of 500 µl of phenol and 100 µl of chloroform-isoamyl alcohol mix to the decanted aqueous solution, followed by mixing and then placement in an ice bath for 15 minutes. The solution was then centrifuged again for 20 minutes at 10000g in a cold room (4°C). This was then followed by another round of extraction. The top aqueous layer was retained and an equal volume of chloroform-isoamyl alcohol was added to remove residual DNA. The mixture is again thoroughly mixed and left on ice for 15 minutes then centrifuged for 20 minutes in a cold room. After the two extra rounds of extraction, the total RNA can then be recovered by addition of 500ul of isopropanol and the solution is incubated at 4°C for at least 1 hour to allow the RNA to precipitate out of solution. A pellet of total RNA could be obtained after centrifugation again for 20 minutes in a cold room. This pellet was washed by resuspension in 100 µl of 4M guanidium-thiocyanate solution (without the addition of β-mercaptoethanol), and again precipitated by addition of 150 ul of isopropanol. Another hour of incubation was necessary for the precipitation to occur and finally the pellet was washed with 100 ul of ice cold 70% ethanol and stored at -80 °C until needed.

2.4 The RNeasy Midi Kit method

This alternate method was also trialed to compare the purity and yield of the RNA. The method is based on the selective binding properties of a silica-gel based membrane which binds RNA. This method removes the need for chloroform-phenol extraction and is considerably faster.

All steps were carried out without deviation from the instruction manual. Briefly, the steps were the addition of approximately 100mg of snake venom gland tissue to 3.8 ml of RLT solution with β-mercaptoethanol at a concentration of 1μl per ml of RLT solution (RLT solution contains guanidium isothiocyanate in unknown quantity, presumable between 4 - 6 M and was the primary denaturing agent). The tissue was homogenised by a mechanical rotor and the cell lysate was then centrifuged for 5 minutes at 4000g. The supernatant was then collected and 3.8 ml of 70% ethanol was added to the supernatant and mixed by vortexing. The entire sample was then loaded onto the RNeasy midi spin column and centrifuged at 4000g for five minutes. Since the maximum load of the column was 3.8 ml, two runs were required. The flow through which contained proteins and low molecular weight RNA such as transfer RNA and 5 S RNA were not retained by the column and was discarded. The column which contained the bound RNA was then washed with 3.8 ml of RW1 (also contains guanidium isothiocyanate) solution by centrifugation for 5 mins at 4000g. Then the column is washed again with 2.5 ml of RPE solution (constituents were not mentioned in the manual but RPE contains at least 75% (v/v) ethanol) by centrifugation for two minutes at 4000g. A further 2.5 ml of RPE was then added to wash the column of remaining contaminants, and the column was spun dried by centrifugation for five minutes at 4000g. The bound RNA could be recovered and eluted from the column by addition of RNase free water.

2.5 Protocol for Agarose Gel electrophoresis of RNA

The quality and quantity of RNA extracted were examined by loading a sample of the total RNA onto a 1% (w/v) agarose gel for visual inspection. A preparation was judged to be acceptable if th 18s and 28s ribosomal RNA were visible, with the 28s rRNA being approximately 1.5 to 2 times brighter than the 18s band. A slight smearing of mRNA should also be visible.

The agarose gel was prepared as follows :- 0.3g of agarose (Seakem) was dissolved in 30 ml of

1x MOPS (O.2 M MOPS, 0.05M sodium acetate and 0.01M EDTA, adjusted to pH6.5 with sodium hydroxide) buffer by heating. After the solution was cooled, but before the agarose has solidified, 1.2 ml of formaldehyde was added to the gel. Each well was loaded with approximately 10 µl of RNA sample (previously heated to 72°C with loading buffer in a 1 to 1 ratio) and electrophoresis was carried out at 90 volts. Any RNA present was stained by the chelating agent - ethidium bromide which was present in the loading buffer. The loading buffer consisted of the denaturing agents 50% (v/v) foramide and 10% (v/v) formaldehyde, 7% (v/v) glycerol in 1x MOPS buffer and ethidium bromide (5 µl of EtBr was added for every 1.4 ml of loading buffer). The running buffer was 1x MOPS buffer. DNA and RNA molecules were visualised by placing the gel over a UV lamp.

2.6 Method for Purification of mRNA from total RNA

As ribosomal RNA subunits may interfere with subsequent reactions, an attempt was made to purify the messenger RNA from the total RNA. This was achieved by using the PolyAtract mRNA Isolation system (Promega). The basis of this procedure was to rely on the hybridization of a biotinylated oligo (dT) to the poly A tail of mature messenger RNA. After the hybridization procedure, streptavidin conjugated magnetic beads were used to trap the biotin and the beads were then retained by a magnet. The bound mRNA could then be recovered later from the beads.

There was no deviation from the protocol as set out in the user manual. The steps taken are briefly mentioned below. The procedure initially required a large amounts of total RNA (1-5 mg) in a final volume of 2.43 ml of RNase free water. The RNA samples (snake liver RNA) were then heated to 65°C for 10 minutes and 10 µl of biotinylated oligo (dT) was then added to the RNA sample together with 60 µl of 20x SSC solution (NaCl and sodium citrate, at a pH of 7.2). The solution was then allowed to cool to room temperature. Streptavidin conjugated beads were washed (three times in 0.5x SSC solution) and added to the RNA sample, then incubated for 10 minutes at room temperature to allow the binding to occur. The beads were then captured by using a magnetic stand and the supernatant discarded. The remaining beads were then washed again (4 times with 0.1x SSC solution) and finally the mRNA was eluted from the beads by addition of 1.0 ml of RNase free water.

2.7 Complementary DNA Library construction protocol

While it is possible to rely on RT-PCR and RACE PCR only to ascertain the presence of PLA_2, and α-neurotoxins genes in Death Adders and King Brown, the unstable nature of RNA meant that there must be a supply of fresh venom glands (and countless numbers of snakes) if the project were to rely solely on the above methods. DNA library, however, is a means to preserve the RNA in a stable structure (as double stranded cDNA) and produce an almost limitless supply of template for analysis.

DNA library construction was a multiple steps process, involving (1) the production of first and second strand cDNA from total RNA; (2) digestion of cDNA products to produce complementary ends to the vector; (3) size fractionation and purification; (4) ligation to the vector; (5) packaging reaction; (6) titration of unamplified library; and (7) amplification and titration of the library.

Total RNA was obtained using the modified Chomzynski and Sacchi method mentioned above from Death Adder venom gland and King Brown venom gland and was used in the production of first and second strand cDNA. The method involved the addition of 1 μl of SMART III oligonucleotide and 1 μl of CDS III Primer to 3 μl of total RNA. The mixture was then incubated at 72°C for 2 minutes to denature the secondary structure of mRNA molecules which would inhibit extension of the first strand. After 2 minutes, the mixture was cooled on ice for an additional 2 minutes and the first strand synthesis reaction mix was added. The mix contained the following:-

2.0 μl 5x first strand buffer

1.0 μl DTT (20 mM)

1.0 μl dNTP mix (10 mM)

1.0 μl MMLV reverse transcriptase (200 units)

The mixture was then incubated at 42°C for 1 hour in the hybaid thermocycler, and the reaction was terminated by plunging the reaction tube into ice.

Unlike the standard RT-PCR, which used only 1 primer (the oligo d(T)) in the production of the first strand, this method which was essentially RT-PCR relied on 2 primers since all mRNA

molecules were to be transcribed in reverse orientation. The CDS III primer possessed a poly-T region and annealed to the poly-A tail (3' end) of all mRNA molecules present in the sample. Furthermore the 5' region of this primer possessed a Sfi IB site for later insertion into the phage vector (bearing in mind the 5' end of the primer was actually the 3' tail of the gene). After the CDS III primer has annealed to the 3' end of the RNA molecule, the primer was extended by the reverse transcriptase until the 5' end of the gene where a triple cytosine was added into the 5' end of the gene. This terminal cytosine addition activity was vitally important and was found to be very temperature sensitive. The triple cytosine then acted as an anchor and allowed the SMART III oligonucleotide (which had a triple guanosine at the 3' end) to anneal to the 5' end of the gene, thus allowing the entire mRNA molecule to be reverse transcribed. The SMART III oligonucleotide which then acted as the 5' end of the gene contained a Sfi IA site for later insertion into the vector for directional cloning.

The production of the second strand cDNA was by PCR, and the following reaction mix was used :-

2 µl First strand cDNA (either Death Adder or King Brown)
80 µl Deionised water
10 µl 10x Advantage 2 PCR buffer (with $MgCl_2$ concentration optimised)
2 µl 50x dNTP mix
2 µl 5' PCR primer (forward primer) (10pmoles)
2 µl CDS III primer (reverse primer) (10 pmoles)
2 µl 50x Advantage 2 Polymerase Mix

The reaction mix was then amplified by several rounds of PCR, with the 5' PCR primer annealing to the region corresponding to the SMART III oligonucleotide, thus acting as the forward primer and the CDS III acting as the reverse primer. The initial denaturing temperature was 95°C, followed by several cycles at 95°C for 15 seconds and 68°C for 6 minutes. The number of cycles was initially set at 18. This was necessary because over amplification could lead to preferential selection of abundant genes while rare genes would be masked. After 18 cycles, samples were removed from the reaction mix and analysed on a 10% agarose gel to determine whether

adequate amplification has been reached. If not, then additional cycles were introduced to the reaction mix in 2 cycle increment until the requisite amount of cDNA was produced.

After the production of double stranded cDNA, the cDNA was then prepared for insertion. This required the reaction mix to be firstly digested by proteinase K to inactivate the DNA polymerase then digested again by Sfi I to produce the complementary ends. The steps involved were as follows (there was no diversion from the manual). The procedure required the addition of 2 µl of proteinase K [20 mg/ml] to 50 µl of the reaction mix and an incubation for 20 minutes at 45°C. Thereafter, the mix was diluted by 50 µl of deionised water and the DNA was extracted from the solution by the addition of 100 µl of phenol:chloroform: isoamyl alcohol mix (ratio of 25:24:1). The phenol used was at pH 6.8 (unlike the phenol used in RNA extraction). The mixture was then centrifuged and the top aqueous layer was collected. The cDNA was recovered by precipitation from solution using 10 µl of 3 M sodium acetate, 1.3 µl of glycogen [20 mg/ml] and 260 µl of room temperature 96% ethanol. A pellet was obtained after 20 minutes of centrifugation at 14000 rpm and the pellet was washed with 100 µl of 80% ethanol, air dried and finally resuspended in 79 µl of deionised water.

Following the precipitation step, the cDNA was now ready for Sfi I digestion. The following reaction mix was prepared :-

79 µl cDNA
10 µl 10x Sfi Buffer
10 µl Sfi Enzyme
1 µl 100x Bovine serum albumin

The reaction mix was incubated for 2 hours at 50°C in a water bath. At the cessation of this reaction, the Sfi sites previously introduced into the gene via the primers would have been cut and was now ready for insertion into the vector system.

Prior to insertion into the vector, various components other than the cDNA had to be removed, such as the Sfi enzymes and more importantly, the remaining unbound primers and other DNA

contaminants. These were removed by using the CHROMA SPIN-400 system. Chroma Spin columns are essentially size fractionation columns which separated molecules by size, with larger molecules having a shorter retention time in the column. As per the instructions as set out in the user manual, the storage buffer was removed and the entire sample of DNA added to the column. While the protocol suggested recovery of the DNA sample by gravity flow method, this method was found to cause significant dilutions to the cDNA sample, resulting in an eventual loss of sample. Thus, the protocol suggested in the Chroma-Spin user manual was adopted instead. The Spin column was centrifuged for 3 minutes at 1500g to recover the cDNA, while smaller oligonucleotides would remain trapped in the column.

Purified cDNA was then ligated to the λTripIEx2 vector. In this stage, significant departure from the protocol was necessary as the 10x buffer and the T4 ligase provided by Clontech were faulty, a 5x buffer from Biorad was found to be a suitable substitute. Thus, the following reaction mixes were prepared :-

cDNA	1.5 µl	1.0	0.5
Vector [500 ng/ml]	1.0 µl	1.0	1.0
5x Ligation buffer	1.0 µl	1.0	1.0
ATP [10 mM]	0.5 µl	0.5	0.5
T4 DNA ligase (200 units/µl)	1.0 µl	1.0	1.0
Deionised water		0.5	1.0

A series of three reactions with a different cDNA to vector ratio were set up as specified in the user manual. The reaction tubes were then incubated overnight at 4°C (the protocol specified 16°C) but the lower temperature proved to be more efficient for ligation.

The packaging reaction was carried out next, using the Gigapack III Gold packaging extract (Stratagene) and there was no deviation from the protocol. The entire experimental cDNA (5 µl) sample was added to freshly thawed packaging extract and gently mixed. The tube was then incubated for 2 hours at room temperature (note that the highest efficiency occurred between 90 minutes and 2 hours) followed by the sequential addition of 500 µl of SM buffer (0.1M NaCl,

0.1M MgSO4, 0.05M Tris-HCl, pH at 7.5, 0.001% gelatin) and 20 ml of chloroform. The supernatant containing the unamplified library was then ready for titering.

Phage titres were performed as instructed by the user manual with no deviations. A 90 mm LB/$MgSO_4$ plate (prepared prior to this experiment) was warmed and dried in an incubator at 37°C in preparation. Then, 1 µl of the unamplified library was diluted 1 in 5 by 1x λ dilution buffer (0.1M NaCl, 0.1M $MgSO_4$, 0.05M Tris-HCl, pH at 7.5) and the diluted phage mixture was then used to infect a 200 µl culture of XLI-blue E.coli cells grown previously to an optical density of 2.0 as measured at a wavelength (λ of 600nm. The phage was then left to incubate at 37°C for 15 minutes with the bacteria to allow for adequate adsorption to the bacteria. Finally, the phage and bacteria were then mixed with 2 ml of molten (warm) LB/$MgSO_4$ (containing 100 µl of 100mM IPTG and 20 µl of 50 mM X-Gal) soft agar and poured onto the 90mm LB/$MgSO_4$ plate and left to grow overnight in a 37 °C air incubator.

Lastly, the unamplified cDNA library was amplified for stability, and the instructions as set out in the user manual for amplification were followed. This time, 150mm LB/$MgSO_4$ plates were warmed and dried as above. A sufficient volume of unamplified library was used to infect 10 ml of bacterial culture to yield 1 x 105 pfu per 150mm plate. For the Death Adder library, twenty plates were made, but there was only sufficient initial titre to produce 4 plates for the King Brown library. After the plaques have been grown to confluence over the soft agar, 12 ml of 1x λ dilution buffer was poured onto the plate to liberate the phage. The plates were stored at 4oC overnight and the supernatant (the amplified library) collected the next day after gently shaking for 1 hour. The λ-phage lysate were then pooled and any cell debris removed by addition of 10 ml of chloroform. The lysate was centrifuged for 10 minutes at 4000g and the supernatant collected and stored as 500 µl aliquots. The titre for the amplified library was also obtained using the same method as delineated above with the exception that the amplified library was initially diluted 1 in 20 with 1x λ dilution buffer.

2.8 Method for Verification of proper gene insertions

In order to demonstrate that different mRNA molecules have been inserted into the λ phage vector, and thus were incorporated into the libraries, 10 random plaques from each of the two

cDNA libraries were picked and used as template in a PCR experiment with primers being complementary to the region flanking the insertion site. The primer pairs used were the SMART III oligonucleotide and CDS III 3' PCR primer and the reaction mix were as follows:-

1µl cDNA (either Death Adder or King Brown) template

1µl SMART III oligonucleotide at a final concentration of 10 pmoles per reaction

1µl CDS III 3' PCR at a final concentration of 10 pmoles per reaction

1µl 50x dNTP mix (10mM)

5µl 10x Redtaq PCR buffer (with $MgCl_2$ concentration optimised)

1µl Redtaq Polymerase Mix at a final concentration of 1 unit per reaction

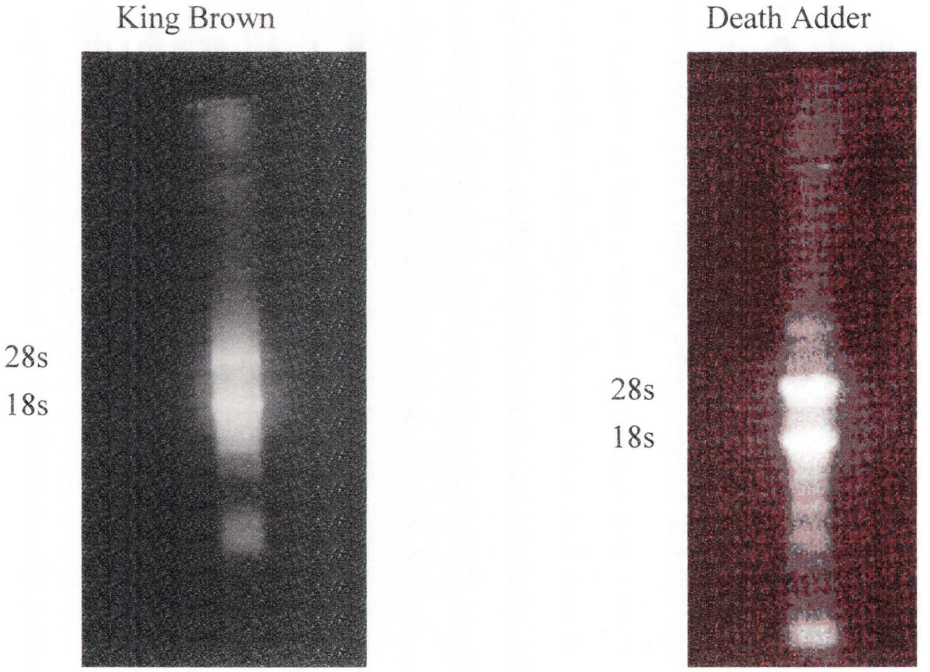
40µl Deionised water

The denaturing temperature was set at 95°C (30 seconds), annealing temperature optimised at 60°C (30 seconds) and extension temperature at 72°C (2 mins). A total of 30 cycles were repeated to maximise the yield. PCR products were analysed by gel electrophoresis on a 10% agarose gel, and visualised ethidium bromide staining (as previously described).

2.9 Results for the messenger RNA extraction

Total RNA was extracted from approximately 50-100 mg of venom gland tissue from both Death Adder and King Brown snake using the modified Chomczynski and Sacchi method. In figure 2.1 below, an aliquot (5µl) from each of the extracts was loaded onto 1% (w/v) agarose gel in MOPS buffer and analysed by gel electrophoresis. In both total RNA profiles, the 28s and 18s ribosomal RNA subunits were clearly visible with the smaller 18s rRNA subunit yielding a stronger band in the Death Adder sample, and in the King Brown sample both subunits formed equally bright bands. The transfer RNA could also be seen in both samples, resulting in the formation of a band at the bottom of the gel. Furthermore, in the King Brown sample and to a lesser extent in the Death Adder sample, a number of other bands could also be clearly seen. These bands are presumed to be the abundant messenger RNA which has also intercalated with the ethidium bromide dye, used to visualise the nucleic acids. The quality of the RNA extraction could be determined by the brightness and the sharp definition of the ribosomal subunit bands and the presence of the abundant messenger RNA bands. In both samples, there was a notable absence of contaminating DNA, which if present, would form a bright smear at the top end of the gel.

Figure 2.1 - Quality of messenger RNA



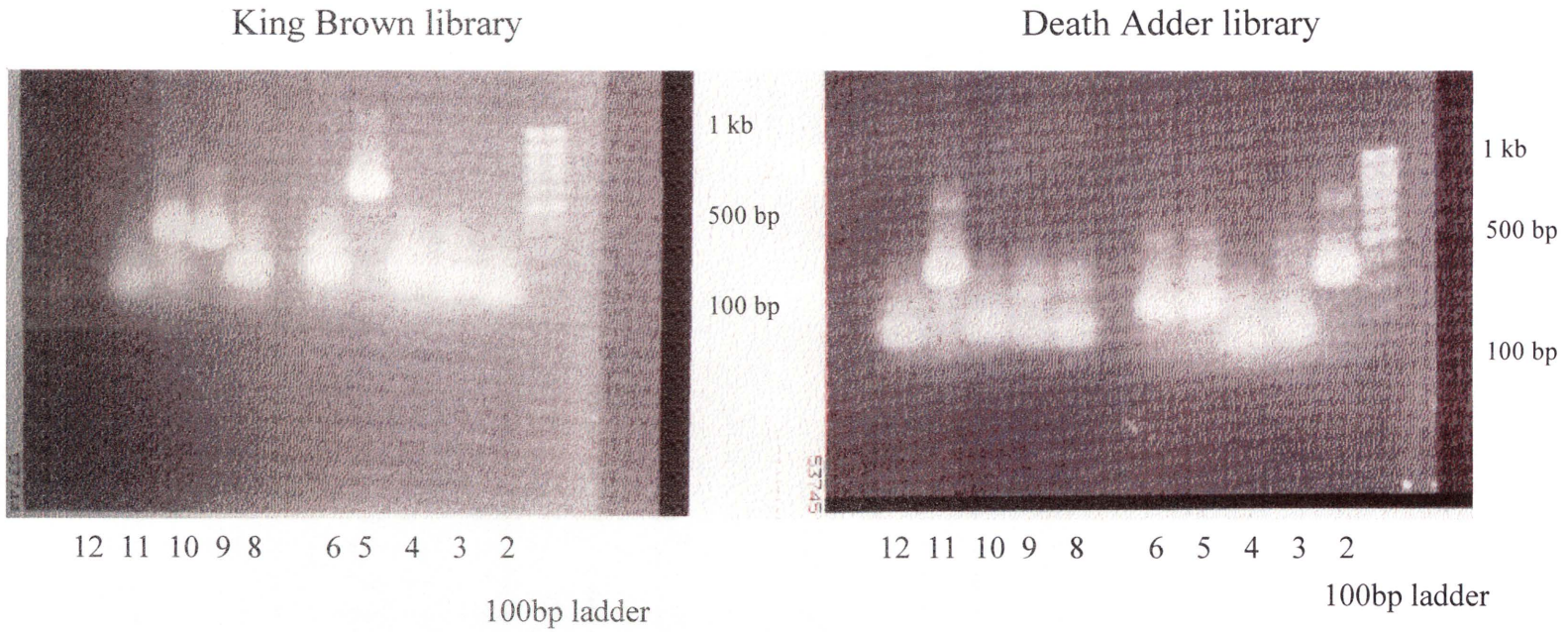
The above two figures are 10% agarose gels with formaldehyde used in RNA specific experiments, showing samples of the total RNA extracted from the venom glands of King Brown snake and Death Adder. Both samples show strong bands of 28s and 18s ribosomal units with a detectable smear of messenger RNA and little degradation was observed.

An alternate method was also used, which was the RNeasy Midi Kit from Qiagen. This kit also yielded high quality RNA but at a significantly lower concentration (and overall yield was also lower). The results from the agarose gel electrophoresis are not shown. Since the modified Chomczynski and Sacchi method provided total RNA at high yields and high quality, this was the method of choice.

2.10 Screening the library for insertion of random genes for additional verification

The successful creation of the unamplified library could be tested by selecting random plaques and using a PCR to determine if cDNA inserts had in fact been incorporated into the vector (the primers used in this PCR were complementary to the flanking regions of the insertion site). If the purification step was not done properly then the inserts would contain very small fragments of primers and oligonucleotides only and when visualised on an agarose gel, they would appear as small bands of 100 bp or less. Thus, both the unamplified libraries from Death Adder cDNA library and King Brown cDNA libraries were screened and the agarose gel results shown in diagram 2.2. The markers in lane 1 are 100 bp markers, and a number of plaques did contain inserts which were possibly only oligonucleotides. However, it could also be argued that these were in fact very small genes as the oligonucleotides could not be digested by SfI enzyme and thus were not complementary to the vector (an oligonucleotide would have to have the 5' end digested by the Sfi A enzyme and the 3' end digested by the Sfi B enzyme in order to be ligated successfully into the λ phage vector). More importantly, the presence of distinct and larger bands in lanes 5, 9 and 10 in the Death Adder cDNA library and lanes 2, 5, 6 and 10 of the King Brown library clearly indicated that a number of different genes were inserted into the libraries. Finally, the relatively short extension time (2 mins) in the PCR screening process eliminated genes which were larger than 2 kb in length. However, this is strictly limited to the subsequent PCR screening stage, as the PCR for the RNA transcription into DNA and subsequent incorporation was considerably longer (6 mins extension was used) and technically, genes up to 6 kb in length could have been transcribed.

Figure 2.2 Verification of gene insertions into vector



The figure shows two 10% agarose gels with PCR products from 10 randomly selected plaques from the unamplified cDNA libraries of King Brown snake and Death Adder. The different gene inserts are shown in lanes 2-6 and 8-12 with products ranging between 600 bp to less than 100 bp. Larger gene inserts could have been present but these were not detected due to the short extension times of the PCR conditions.

2.11 Conclusions

The results from the RNA extractions clearly demonstrated that modified Chomczynski and Sacchi method had produced high quality total RNA from the venom glands which were subsequently incorporated into the cDNA libraries. This initial step is critical to the entire project as even the best cDNA library preparation subsequently cannot compensate for the initial poor quality of RNA. Fortunately, the lack of obvious signs of RNA degradation, and the clearly visible presence of messenger RNA in both samples suggest that there can be confidence in the RNA samples and the subsequent cDNA libraries.

Even with good quality RNA, there was a possibility that the reverse transcription and subsequent ligation of the PCR products into the λ vector were unsuccessful. The results in the screening of random plaques from both libraries clearly showed that both libraries contained various gene inserts and demonstrated that the cDNA had been successfully incorporated into the respective libraries, and was representative of the RNA present in venom glands at the time of extraction. The libraries could then serve as templates for the isolation and determination of various neurotoxin genes present in the venom gland tissue from both Death Adder and King Brown snakes.

Chapter 3

Screening for PLA_2 and α-Neurotoxin Genes in King Brown Snake Venom Glands

3.1 Introduction to screening procedure

After the successful establishment of the King Brown snake venom gland cDNA library, this set of experiments is concerned with the screening of the library for the genes of interest. The more common method of cDNA library screening involves "panning" a large number of plaques on a large surface and then screening each plaque for a positive gene insert by relying on a radioactive labelled DNA probe to hybridise with the gene of interest. Plaques containing the relevant genes could then be identified by placing a photosensitive film over the surface of the colonies where radioactive labelled plaques would leave an impression on the film. By referring to the spots on the developed film, the positively labelled plaques could then be isolated and sequenced directly. However, this method has been criticised as labour intensive and prone to large number of false positive results (Israel 1993) and an alternative system developed by Israel (1993).This system is more specific, yielding less number of false positive clones and does not involve radioactive labelled probes. Details of this system are set out in section 3.3 below. However, it is important to note that the efficacy of any screening system is dependent on the affinity of the DNA probes to the genes of interest, many false positive results could be directly attributed to the DNA probes binding improperly to the irrelevant genes. Thus the determination and design of the correct probes also deserves some discussion.

3.2 DNA Primers for Screening of PLA_2 genes

As previously noted in the introductory chapter, despite the large number of protein sequences available on the various databases, there is very little data on venom PLA_2 gene sequences from Australian snakes and in this case, the nucleotide sequence of the venom PLA_2 gene, notexin was selected as the basis for primer design as it is believed it would possess a primary structure most similar to other venom PLA_2 genes from Australian snakes. The reason for this assumption is because notexin is isolated from another venomous Australian snake, *Notechis scutatus* (Tiger Snake) and it is believed that despite divergent evolutionary forces, there would still exist significant homology between notexin gene and other PLA_2 genes in other snakes, such that a primer used to hybridise with notexin would also bind specifically to other PLA_2 genes.

Thus, gene specific primers to PLA_2 genes were designed based on the nucleotide sequence of notexin isolated from *Notechis scutatus* (Tiger snake). The Copperhead (*Austrelaps superbus*),

Taipan (*Oxyuranus scutellatus*) and King Brown were also other possible candidates for primer design as a large number of PLA_2 genes have been isolated from these snakes. Unfortunately, the nucleotide sequences for PLA_2 in Taipan and King Brown snake were yet to be determined at the time of this project. However, PLA_2 gene sequences from *A. superbus* could be used as template for further primer design should the current primers fail to obtain any sequences. However, these PLA_2 genes are associated with inhibition of platelet aggregation rather than neurotoxicity (Jeyaseelan *et al.*, 2000), thus making them less desirable. Other PLA_2 genes have also been found in the Australian venomous snake *Pseudonaja textilis* (Gong *et al.*, 1999) and could be used as potential templates, however, with the greatest respect to the authors for their contribution, the nucleotide sequences were direct submission to databases, and it was not known whether these were venom genes. Nonetheless, as the aim of the project was to isolate potential venom PLA_2 genes, these nucleotide sequences were deemed suitable.

The gene specific primers were :-

NsPLA2 forward :- 5'-CCCTCTTAGGAGCCGCC-3', located at -38 to -21 (region of the signal peptide);

NsPLA2 reverse :- 5'-GGCAATGTGTCTCGG-3', located at +439 to 454 (the C-terminal of the mature protein);

All primers were analysed by the Primer Finder program for elimination of the formation of secondary structure regions and primer dimerisations as well as optimal temperature range for PCR (i.e between 50oC to 68oC). These primers span the entire open reading frame of the gene, and in theory, a PCR should be able to isolate the entire gene sequence including the signal peptide region. The signal peptide region was chosen for the forward primer as the signal peptide is amongst the most conserved regions in PLA_2 genes, other regions such as the catalytic site for PLA_2 activity and the seven disulfide bonds are also highly conserved but as these regions are either, respectively, located within the gene or dispersed throughout various regions of the gene, they were not deemed as optimal regions. The reverse primer region was chosen at the region which produced the least amount of dimerisation and secondary structure formation. The position where the primers were designed (as well as their intended binding sites) are shown in Figure 3.1.1 which has listed the nucleotide sequence of notexin together with the signal peptide region. The mature protein is coded at the region of 103bp to 456bp.

3.3 DNA Primers for α-neurotoxin genes

With respect to the isolation of α-neurotoxins genes from the King Brown snake cDNA library, a number of gene specific primer sets (forward and reverse primers) for α-neurotoxins were designed. These primers were based on the nucleotide sequence of previously published α-neurotoxins genes by Jeyeesalan *et al.*, (2000). Futhermore, gene specific but degenerate primers based on the amino acid sequences of α-neurotoxins isolated from King Brown snake and Death Adder snakes (Kim and Tamiya 1981a, Kim and Tamiya 1981b, Takasaki and Tamiya, 1985) were also constructed in order to isolate both short chain and long chain α-neurotoxins. These primers were necessary as the primary structure of the three groups of α-neurotoxins were significantly different from each other, as shown in figure 3.1.2 which is the clustal alignment of the amino acid sequences from representative members from all three groups of α-neurotoxins. Finally, gene specific forward primers were coupled with either a poly-thymine reverse primer and subsequently with a gene specific reverse primer to the vector backbone to facilitate potential binding in the subsequent RACE-PCR.

In order to reduce the degeneracy levels in the above degenerate primers, the codon usage table for *Pseudechis australis* was applied to the degenerate primers. In areas where there were ambiguities as to the choice of two or more nucleotides coding for an amino acid, the nucleotides recommended by the codon usage table was selected. No codon usage tables were available for *Acanthophis spp.* at the time of this project but due to the conserved nature of the genes involved, it is believed that the codon usage table for *Pseudechis australis* would have been adequate.

Figure 3.1.1 - Primers used for Screening PLA2 genes

Based on *Notechis scutatus* PLA_2 gene

```
  1 ttgcagctca ccactgacaa aatgtatcct gctcacctc tggtcctgtt gacagtttgt
 61 gtctcccctct tagaagcctc cagcattcct gcgcggcctc tcaacctcta tcagttcggc
121 aacatgattc aatgtgccaa ccatggcagg agacctactt tggcttatgc ggactacggt
181 tgctactgcg gcgcaggagg tagcgggaca ccggtggatg agttggatag gtgctgcaaa
241 gcacatgatg actgctatgg tgaagccgga aaaaaaggat gctaccccac gttgacgttg
301 tatagttggc aatgtattga aaaaacaccc acctgcaatt caaaaacggg atgtgaacgt
361 tctgtgtgtg attgtgatgc cacagcagcc aagtgctttg ccaaagcccc ttacaacaag
421 aagaactaca atatcgacac cgagaaacgt tgccaatgat atttgagagg cttcagcgca
481 aggactgtgg cagttactca cctgcgcgtg gcaattctct ggacgggcct ctattataca
541 tataaaaata gaaaattata tatatataat tattaaaaaa caaaaggaac catttcctga
601 acaataaagt gaggtgccga t
```

The above nucleotide sequence is the sequence for the β-neurotoxin gene Notexin, isolated from the Tiger snake. The coding sequence for the gene begins at +103 to +456. Forward primers were based on the putative signal peptide region at position +39 and reverse primer at the 3' end of the gene, ending at position +456. The forward and reverse primer locations are underlined. Thus, the primers should encompass the entire gene.

Figure 3.1.2 - Alignment of various α-neurotoxin amino acid sequences

```
Short chain α-neurotoxins
>short Ntx              mqccnqqssq pkttttcpgg vsscykktwr dhrgtiierg cgcprvkpgi rliccktdec nn
>PA A                   mtccnqqssq pkttticagg esscykktws dhrgsrterg cgcphvkpgi kltccktdec nn

Long chain α-neurotoxins
>PAL D                  ltcykgrdrs setcrseqel cc-tktwcdqw cqdrgprlem gctatcprrm pgldftcctt dncnpvpt
>Long Ntx               vicyrgynnp qt-cppgenv cftrt-wcdaf cssrgkvvel gcaatcpivk synevkccst dkcnpfpvrpr rpp
>Acanthophin D          vicyrkytnn vktcpdgenv cy-tkmwcdgf ctsrgkvvel gcaatcpirk pgnevkccst nkcnhppkrkk rrp

Pseudonaja textilis α-neurotoxins
>Ntx1                   mktllltlvm vtimcldlgy tltcykgyhd tvvckpheti cyeyfipath gnailargcg tscpggirpv ccrtdlcnk
>Ntx2                   mktllltlvm vtimcldlgy tltcykgyhd tvvckpheti cyrylipath gnaipargcg tscpggnhpv ccstdlcnk
>a-ntx                  mktllltlvm vtimcldlgy tltcykslsg tvvckpheti cyrrlipath gnaiidrgcs tscpggnrpv ccstdlcnk
```

The above alignment of the three groups of α-neurotoxins are the short α-neurotoxins isolated from King Brown snake (PA-A) and Death Adder (short Ntx); Long chain α-neurotoxins from King Brown (PAL-D) and two others from Death Adder (Long Ntx and Acanthophin D) and finally a group of novel proteins isolated from *P. textilis* (Ntx1, Ntx2 and a-ntx) (Jeyeesalan, 2000). While the secondary structure, three finger fold and cysteine bridges are conserved, there is very little sequence homology between members of different groups.

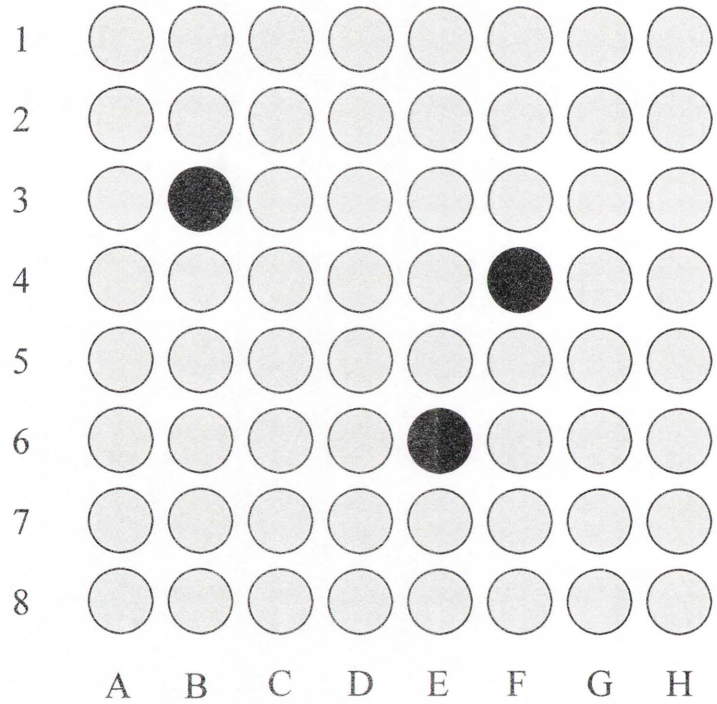
3.4 Screening Procedure

Briefly, the method as suggested by Israel (1993) involved the following steps :-

An overnight culture of E.coli (strain XL1-blue) was centrifuged to form a pellet and resuspended in a 1 ml solution of nutrient broth (LB) and SM buffer in a 1:1 ratio. The correct titre of cDNA library of interest was then used to infect the culture to produce approximately 1000 pfu/ul, (1 μl was added per 5ml of culture). The infected culture was then incubated at room temperature for 20 minutes to facilitate the binding of the phage to the bacteria. The next step involved a further dilution by addition of 20 ml of nutrient broth (with 10mM $MgSO_4$ added as a supplement) to the culture, followed by plating the culture onto a 8x8 well plate (100 μl per well), two additional wells were prepared for subsequent phage titre and negative control. The plate was amplified for 6 hours in a 37°C incubator, and shaken at 200 rpm.

After 6 hours, the phage titre was ascertained and a PCR was performed to determine which wells contained the genes of interest. Samples (2 μl aliquots) from each of the 8 wells in a row were collected and pooled to form one template, yielding 8 row templates. Similarly, samples from each of the 8 wells per column were pooled to form one template, yielding 8 column templates. The 16 samples were then used as templates in PCR, with an additional negative control (no library template). If the genes of interest were present in any wells, then the corresponding row template and column template would produce a positive banding pattern. The illustration in figure 3.2 is provided as an example of this screening procedure. In this example, the 3 wells which contained the genes are highlighted in black while the wells without the genes of interest remain grey. As these wells occur in 3B, 4F and 6E, PCR products are expected in rows 3, 4 and 6 and columns B, E and F.

Figure 3.2 Example of a hypothetical screen



An example of the 64 well PCR screening method. In this example, wells 3B, 4F and 6E (highlighted in black) contain potential genes of interest.

When the samples from each column and row are pooled, rows 3, 4, and 6 and columns B, E and F should yield positive PCR products.

The PCR conditions and reaction were as follows :-

1μl Sample template

1μl Forward Primer (NsPLA2-F) at a final concentration of 10 pmoles per reaction

1μl Reverse Primer (NsPLA2-R) at a final concentration of 10 pmoles per reaction

1μl 50x dNTP mix (10mM)

5μl 10x Redtaq PCR buffer (with $MgCl_2$ concentration optimised)

1μl Redtaq Polymerase Mix at a final concentration of 1 unit per reaction

40μl Deionised water

The primer annealing temperature was previously optimised at 50°C for 30 seconds, denaturation temperature at 95°C for 30 seconds and extension temperature at 72°C for 1 minute. The number of PCR cycles was 30.

After the first round of analysis, samples from positive well were collected and used to reinfect fresh culture, but at a much lower innoculum. Israel recommended the second screening to produce 250 pfu/well, and the screening was to be repeated as above. A tertiary screening was also recommended with the initial innoculum to produce only 25 pfu/ml. These rounds of screening should theoretically lead to almost all the wells eventually containing the genes of interest as each round only positive wells are selected.

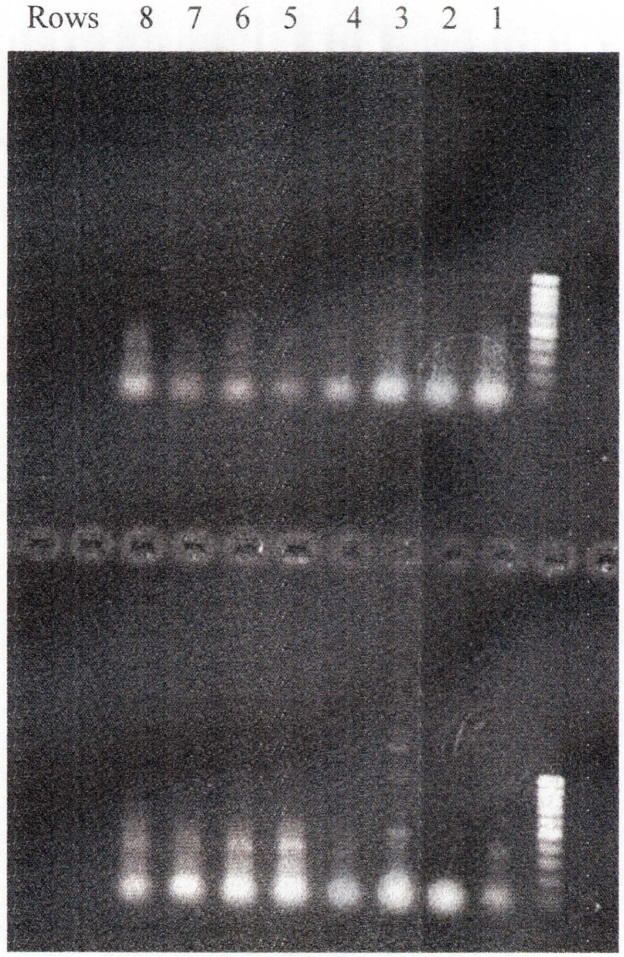
In addition, Israel recommended checking that the primers were binding to the correct gene by hybridizing the PCR product (from the first screening) with labelled primers. While this was a viable method, for the purposes of this experiment, it did not entirely remove the possibility of a false positive result (bearing in mind the gene sequences were mostly unknown, thus designing internal primers were quite impossible), thus the PCR products produced in the screening were instead inserted into pGEM-T, amplified and sequenced to confirm the result. It was acknowledged that this added considerably more time to the screening protocol.

3.5 Results for the first screen

The PCR results from the initial screening procedure were analysed by electrophoresis on a 1% agarose gel on TAE buffer. The bands were visualized by ethidium bromide staining and illuminated under UV light and shown in figure 3.3. The expected PCR product was approximately 400 bp and prominent bands around that size could be observed in rows 1 to 3, 6 to 8 with row 8 giving the strongest signal. Columns A, E, F and H also gave rise to prominent bands with columns E and F showing the strongest signals. Thus, wells at position E8 and F8 were selected for further screening. Interestingly, another signal was also detected at column C, around 500 bp. As this band was significantly larger than the expected product and also no corresponding bands of similar size was observed in the rows, this was most likely a false positive result and was ignored.

Despite the initial promising results, aliquots of E8 and F8 could not be further screened as the bacteriophage would not adhere to the host cells. This could be due to a number of factors such as the fastidious nature of the bacteriophage, the long duration between the initial and the subsequent screening as the PCR had to be prepared or even the buffers used which may have lacked co-factors necessary to facilitate binding. As further screening was not possible, this method was abandoned for another PCR screening method.

Figure 3.3 - Screening Results for King Brown library (PLA_2 genes) showing positive wells



This is a 1% (w/v) Agarose gel in TAE buffer showing the PCR products from rows 1-8 and columns A-H from the initial screening plate. The standards used were 100 bp ladder (Promega).

Possible bands of PLA_2 genes were observed in rows 1, 3, 6-8 and columns A, E, F and H where faint bands of the approximate size (400 bp) were visible.

3.6 The Problem of Preferential Amplification in PCR

The most straightforward way to look for a gene is to use PCR to amplify the gene of interest and directly sequence the PCR product by automated sequencing. While this is routinely done when only a single gene is to be determined, in instances where there could be a number of unknown homologues of the gene of interest in existence, direct PCR methods are unsuitable due to the problem of preferential amplification of some genes. A typical enzyme kinetics graph is shown in figure 3.4, which could also be used to describe the relationship between the enzyme polymerase and its substrate - DNA. The typical polymerase chain reaction follows a sigmoid curve as shown, with an initial lag period of approximately 1- 10 cycles where very little product is produced. This is because the limiting factor for the reaction is the amount of substrate available to interact with the enzyme. The next is an exponential phase where the PCR product is doubled with every cycle until it reaches around 30 cycles, where the products no longer double, but is amplified at a lower rate. Around 30 cycles, the limiting factor has changed to the enzyme while the substrate has become abundant. This is a unique feature of PCR where the product is also the substrate. The position of the curve is dependent on the initial copy number of the genes, of course, with high copy number of genes shifting the curve to the left while low copy number genes shifting the curve to the right.

This is not a problem when only a single gene is being isolated, however, when there are homologues of the genes in existence and the aim is to locate all of them, preferential amplification means that rare homologues would not be amplified. This can best be explained by some hypothetical numbers. For example, if the sample contained two PLA_2 genes which would both hybridise with the primers, however gene A is 10 times more common than gene B. Then at the initial lag phase where the polymerase enzyme is in excess, all copies of gene A and gene B would be amplified (i.e. the chances of both genes being amplified are 100%) and the ratio of the two genes remain 10:1. This ratio is also preserved at the exponential phase. However, at the final phase, where the number of enzymes become the limiting factor, the ratio of genes start to change. Assuming cycle 30 is the beginning of the final stage, and at cycle 30, the ration of gene A to B were 10:1 but the substrate to enzyme ratio has changed to 10:1 as well. Then in cycle 30, the chances of gene B being amplified has suddenly dropped to 10%. At cycle 31, the chances of gene B being amplified has dropped to 1% while gene A dominates at 99%. This effect becomes more pronounced with each subsequent cycle until the presence of gene B is almost entirely

concealed. This is one of the reasons why most cDNA library screening methods rely on "panning" methods and not PCR alone to screen for genes. There are other reasons why "panning" methods might be advantageous, such as if PCR is not possible because only a partial sequence is known, or the gene is very large.

However, it is suggested that if the PCR can be limited to the cycles before preferential amplification occurs, then all the genes should in theory still be present. In this project, the protocol involved initially ascertaining the optimum number of PCR cycles then the PCR product is ligated to a sequencing vector. The vector is then plated out and screened for genes of interest. This has the advantage of ensuring that all the vectors have the genes of interest without having to screen through large number of false positives and at the same time, the ratio of genes is maintained so that rare genes are not concealed. Fortunately, this method is suitable for PLA_2 genes which are small genes of only 400 bp in length. Genes which are larger would have a significantly lower rate of successful ligation to the sequencing vector. Furthermore, where only partial sequences are available then this method would have to be modified extensively.

3.7 The Protocol for Optimisation

The protocol for PCR follows the standard reaction and is set out below :-

1μl Sample template

1μl Forward Primer (NsPLA2-F) at a final concentration of 10 pmoles per reaction

1μl Reverse Primer (NsPLA2-R) at a final concentration of 10 pmoles per reaction

1μl 50x dNTP mix (10mM)

5μl 10x Redtaq PCR buffer (with $MgCl_2$ concentration optimised)

1μl Redtaq Polymerase Mix at a final concentration of 1 unit per reaction

40μl Deionised water

The primer annealing temperature remained at 50°C, for 30 seconds, denaturation temperature at 95°C for 30 seconds and extension temperature at 72°C for 1 minute.

However, instead of preparing only a single reaction mix, twenty reactions were prepared and between cycles 10 to 30, a reaction mix was removed each cycle and frozen to halt the reaction. The PCR products from each cycle were then analysed by electrophoresis on a 1.0% (w/v) agarose gel with TAE buffer and visualized by ethidium bromide staining.

After determination of the optimum cycle for PCR without preferential amplication, the PCR was repeated in a 250 µl reaction mix, the resultant PCR product was analysed by gel electrophoresis on a 1.0% (w/v) agarose gel in TAE buffer and the bands which occurred at the correct range of 400bp were excised.

3.8 Elution of the PCR product

The PCR product which contained the PLA_2 gene of interest was extracted from the excised agarose gel using the Agarose Gel purification kit (Qiagen) with no deviation from the user manual. Briefly, 300 µl of QG solution was added to every 100 mg of diced agarose gel and the entire mixture was warmed in a waterbath at 50°C for 10 minutes or until the agarose gel had completely dissolved. The solution was then loaded onto a spin column and centrifuged for 1 min at 13,000 rpm to remove all traces of the QG solution. A silica membrane contained within the column was used to entrap the PCR product, which was subsequently washed with a wash solution. The PCR product was then recovered by elution from the column using 30 µl of sterile water.

3.9 Ligation to pGEM-T vector

After obtaining purified PCR product of the correct size (and presumably the gene of interest), the PCR product was ligated to the sequencing vector pGEM-T using the pGEM-T easy vector system (Promega). The ligation reaction was as follows :-

7 µl of PCR product

2 µl of pGEM-T vector

10 µl of x2 ligation buffer

1 μl of T4 DNA ligase

The ligation reaction was incubated overnight at 4°C for optimum results.

3.10 Precipitation and Transformation

The DNA from the above reaction mix was recovered by precipitation by addition of 1/10 volume (2 μl) of 3.0M sodium acetate (at pH 5.5) and 2 times volume (40 μl) of 100% ice cold ethanol in sequential order to the reaction mix. The solution was then mixed by vortexing and left to incubate at -20°C for 1 hour. The precipitated DNA was recovered by centrifugation for 15 minutes at 4°C and the pellet of DNA washed by addition of 50 μl of 70% ethanol. It was noted that omission of this wash step often led to "sparking" at the on the next step of electroporation.

The DNA pellet was dried and resuspended in 10 μl of deionised water before being mixed with 45μl of electrocompetent DH5α cells. The DH5α cells were then transformed by electroporation using a Gene Pulsar (BioRad) with no deviation from the user manual. The conditions of electroporation were 1.8 kV, 200 ohms and 25 μF. After electroporation and obtaining a time constant of approximately 4.3 seconds, the solution was resuspended in 500 μl of LB broth and left to incubate at 37°C for 2 hours on a shaker at 200 rpm. An aliquot of 100 ml of this transformed cell suspension was then plated onto LB ampicillin agar (0.17M NaCl, 10% (w/v) tryptone, 5% (w/v) yeast extract, 1% (w/v) agar, pH 7 with 100 μg of ampicillin per ml of agar). Colonies were selected based on the X-gal/IPTG blue white colour selection system where positive colonies were identified by a white colour.

3.11 Plasmid amplification and purification

A number of positive colonies were then selected at random and grown overnight in 5 ml of LB ampicillin broth (as above without the addition of agar). The plasmid was recovered from the cells using the Fastprep plasmid purification system from Eppendorf with no deviation from the user manual. The system required the cells to be initially lysed with a solution of lysozyme prepared in accordance with the user manual and the cell debris removed by centrifugation for 10 minutes. The supernatant was retained and loaded onto a column fitted with a silica disc which retained the plasmids when the column was centrifuged while the supernatant was discarded. The

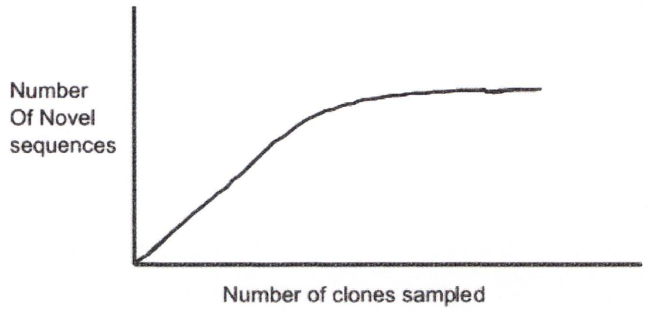
plasmid was then washed and eluted in 50 μl of deionised water.

Plasmid concentration and integrity were monitored by gel electrophoresis using 5 μl of the sample on a 1.0% (w/v) agarose gel in TAE buffer. Plasmid concentration and integrity were judged visually, based on whether distinct bands of the plasmid could be observed (in relaxed and supercoiled conformations). If these bands were clearly visible, then 10 μl of the sample together with 5 μl of deionised water and 1 μl of M13 forward [10 pmoles per reaction] and 1 μl of M13 reverse primer [10 pmoles per reaction] in separate reaction tubes were sent to Australian Genome Research Facility (AGRF) for sequencing analysis. The results from plasmid preparations are not shown.

3.12 How many colonies should be sampled?

The assumption is that there are multiple PLA_2 genes in the King Brown venom gland cDNA library, and the objective of this project is to sequence these PLA_2 genes. Thus, it is important to ascertain how many colonies should be sampled so that there is confidence that all the PLA_2 genes have been determined. Unfortunately, as with most sampling issues, there is no absolute certainty that all PLA_2 genes could be determined from within the sample size. However, the end point of the sampling exercise is reached when the effort it takes to sample no longer can be justified. Diagram 3.1 is a graphical plot of a hypothetical situation where each time a sample is taken and sequenced, a new gene would be discovered. As shown in this graph, at the initial stages, the chances of discovery is very high, but the probability of discovery is gradually reduced, until there is almost no possibility of a new gene being discovered. At this point, the graph is axiomatic and the end point has been reached. In theory, there is always the possibility that a novel gene could still be found, but the effort and resources required to locate this novel gene cannot be justified (under this method of sampling).

Diagram 3.1 - a graphical representation of reach the end point for sampling



Bearing the above sampling system in mind, in this project, the putative positive colonies were sent to AGRF for automated sequencing in batches of ten each time, and the results of the number of novel genes were compared against the number of samples sent until no more novel genes could be found.

3.13 Analysis

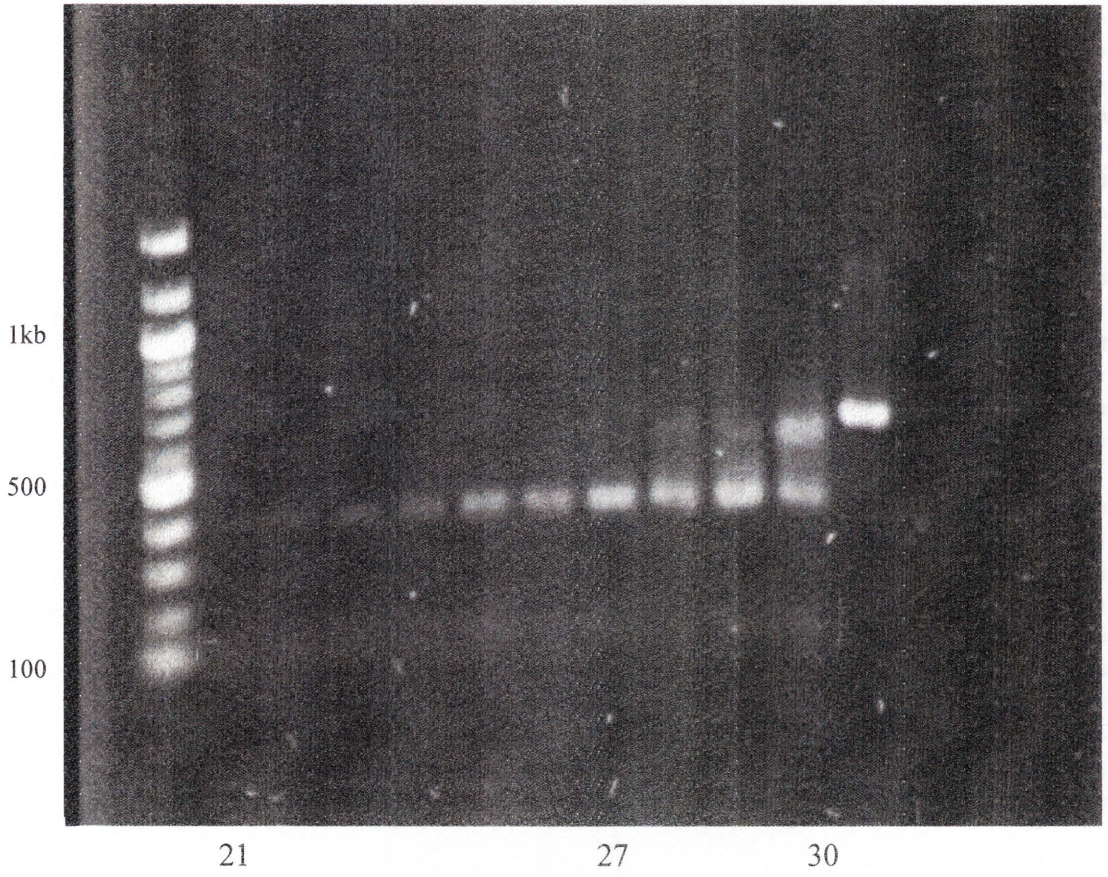
All nucleotide and protein sequence comparisons and alignment were performed using the computer aids generously provided by the School of Molecular and Biomedical Science, Adelaide University. Clustal alignments were visually inspected before they were used for phylogram construction to eliminate any misalignment.

3.14 Results for optimum number of cycles

As stated in section 3.5, in order to ensure that no preferential amplification of common genes had occurred, there was an additional step of determining the optimum number of cycles of PCR and in figure 3.4 below, the results for the optimisation experiment is shown.

The figure shows the results of an electrophoresis of a series of PCR cycles (Cycles 21-30 are shown here) on a 1.0%(w/v) agarose gel using the King Brown venom gland cDNA library as template and the NsPLA2 forward and reverse primer set. The standards used were 100 bp ladder (Promega) and the PCR products were stained by ethidium bromide and visualized under UV light illumination. The expected PCR product was 400 bp in size and a band of approximately this size was visible in cycles 22 to 30. More importantly, since the intensity of the band is directly related to the amount of PCR product present, it was observed that there was a progressive increase in the intensity of the band with each cycle as the product was amplified each time, with cycle 27 showing the strongest band. Cycles 28 to 30 also produced strong bands, however, the results clearly indicated that there were also non-specific products in those reactions, as an additional band approximately 650-700 bp could also be observed. The presence of these non-specific products from PCR were an indication that cycles 28 to 30 were not suitable for subsequent cloning experiments. Finally, the band on the last lane is in relation to another experiment which is not relevant to this project. Based on these results, cycle 27 was selected as the optimum cycle for PCR. Although cycles 10 to 19 were also analysed, the results are not shown as the PCR was still at the lag phase of the reaction and no visible products were observed.

Figure 3.4 - Optimisation for King Brown snake cDNA library based on gene specific PLA_2 primers



This is a 1.0% (W/V) agarose gel in TAE buffer, visualised by ethidium bromide staining. Lane 1 contains the 100 bp ladder as a standard. Lanes 2-11 contain the various PCR products obtained at different cycles of PCR, starting at cycle 21 to 30.

While very faint PCR products were observed In cycle 21, the strongest band was visible in Lane 8 (cycle 27) which shows a bright band With no non-specific binding. Lanes 9, 10 and 11 also contain strong bands but non-specific Binding can also be observed in the form of Smearing located above the band.

The last lane contain an irrelevant sample to This experiment.

After having established that 27 cycles was the optimum number of cycles of PCR to be used for subsequent sequencing experiments, PCR products were then resolved on 1% agarose gels by electrophoresis, excised, and inserted into pGEM-T vector. The vector was then transformed into DG5α cells for amplification, extracted and sequenced.

3.15 Sampling and Sequencing the PLA_2 genes

As mentioned in section 3.11, positive colonies based on XGAL/IPTG selection system were randomly selected and amplified. The plasmids were then extracted and sent to AGRF for automated sequencing. A total of four batches, each containing 10 colonies were eventually sampled to satisfy the requirement that the PLA_2 genes from King Brown venom glands have been exhaustively sequenced. Each batch also contained a positive control (untransformed pGEM-T vector) to demonstrate the fidelity of the amplification and cloning processes.

A total of fifteen novel nucleotide sequences of PLA_2 genes were found in the forty samples, in fact, at sample 21-40 (the third batch), no new sequences were identified which indicated that the chance of isolating a novel PLA_2 genes have been exhausted. That is, the end stage of the screening process had been reached and further resources spent on isolating genes would not be justified.

3.16 Catergorising Groups A, B and C

Initially, the nucleotide sequences were categorized into two groups based on the length of the sequences, resulting in long chain nucleotide sequences with 409 base pairs and short chain nucleotide sequences with 388 base pairs. However, when the sequences were aligned with each other using ClustalW, a phylogram could be constructed which suggested that there could be three groups (Groups A, B and C) based on sequence similarity. The nucleotide sequence alignment and the phylogram for the gene sequences are shown in figures 3.5 and 3.6. As shown in figure 3.5, members of group A consisted of novel sequences 1, 4, 6 and 9 (which all belonged to the long chain group), a second group (Group B) consisted of previously unidentified sequences 11 to 15 (which were all members of the short chain group) with sequence 14 bearing some dissimilarity to other members of that subgroup, and the remaining sequences (sequences 2, 3, 5, 7, 8 and 10) fell into the last group (Group C).

Several additions were responsible for the increase in length for members of groups A and C. The additions were found in position 208 to 210 where there was an addition of two thymines and a single cytosine; At positions 232 to 237, there was an addition of six nucleotides - guanine, thymine, guanine, guanine, cytosine and cytosine. At positions 249 to 257, there was an addition of nine nucleotides, these were adenine, cytosine, cytosine, thymine, adenine, thymine, thymine, cytosine and adenine. A single addition of cytosine was found at position 295; and finally two other additions of thymine and guanine at positions 302 and 303. The additions were conserved amongst all ten sequences and did not contribute to the differences between members of groups A and C.

However, the sequence homology between members of group A and C were more conserved. All differences were due to nucleotide substitutions instead of additions and substitutions were found at positions 7 (either guanine or adenine); position 12 (guanine or thymine); position 24 (guanine or cytosine); position 26 (guanine or cytosine); position 48 (sequence 7 had cytosine, while all others had thymine); position 104 (again sequence 7 differed from the others with adenine, while other members had guanine); position 110 (members of group A had thymine while members of group C had cytosine); position 165 (sequence 7 had adenine, others had thymine); position 174 (group A had guanine, group C had adenine); position 224 (group A had cytosine, group C had thymine); position 226 (same as position 224); position 248 (group A had thymine, group C had cytosine); position 271 (Group A had guanine, group C had adenine); position 298 (group A had cytosine, group C had thymine); position 305 (group A had cytosine, group C had guanine); position 329 (sequence 5 from group C differed from the others with guanine); position 352 (sequence 4 from group A differed from the rest by cytosine); position 354 (sequence 7 differed from the others by thymine); and finally position 355 (sequence 2 differed from the others by adenine). Interestingly, the variability seems to be restricted to the abovementioned positions only and mutations (whether substitution, addition or deletion) were absent from all other positions.

Figure 3.5 – ClustalW alignment of the novel PLA_2 genes from King Brown snake (All Groups)

```
4       TCTTAGAAGCCTCCAGCATTCCTGCCCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
6       TCTTAGGAGCCTCCAGCATTCCTGCCCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
1       TCTTAGAAGCCGCCAGCATTCCTCCCCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
9       TCTTAGGAGCCGCCAGCATTCCTCCGCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
2       TCTTAGGAGCCGCCAGCATTCCTGCGCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
3       TCTTAGGAGCCGCCAGCATTCCTGCGCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
8       TCTTAGGAGCCTCCAGCATTCCTGCGCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
5       TCTTAGAAGCCGCCAGCATTCCTGCGCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
10      TCTTAGAAGCCGCCAGCATTCCTCCGCAGCCTCTCAACCTCTATCAGTTCAAGGAGATGA 60
7       TCTTAGGAGCCGCCAGCATTCCTGCGCAGCCTCTCAACCTCTATCAGCTCAAGGAGATGA 60
12      TCTTAGGAGCCTCCAGCATTCCTGCGCAGCCTCTCAACATCCTTCAGTTCAGGAAGATGA 60
15      TCTTAGGAGCCTCCAGCATTCCTGCGCAGCCTCTCAACATCCTTCAGTTCAGGAAGATGA 60
11      TCTTAGGAGCCGCCAGCATTCCTGCGCAGCCTCTCAACATCCTTCAGTTCAGGAAGATGA 60
13      TCTTAGGAGCCGCCAGCATTCCTGCGCAGCCTCTCAACATCTTTCAGTTCAGGAAGATGA 60
14      TCTTAGGAGCCGCCAGCATTCCTGCGCAGCCTCTCAACCTCATACAATTAAGCAACATGA 60
        ****** **** *********** * *********** **   **  * *   * ****

4       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATATGGTTGCTACT 120
6       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATATGGTTGCTACT 120
1       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATATGGTTGCTACT 120
9       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATATGGTTGCTACT 120
2       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATACGGTTGCTACT 120
3       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATACGGTTGCTACT 120
8       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATACGGTTGCTACT 120
5       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATACGGTTGCTACT 120
10      TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCGGGATACGGTTGCTACT 120
7       TTGAATGTGCCAACAAGGGCACTATAAGTGGGCTGGCTTATGCAGGATACGGTTGCTACT 120
12      TTCAATGTGCTAACAAGGGCAGTCGAGCTGCTTGGCATTATCTGGACTACGGTTGCTACT 120
15      TTCAATGTGCTAACAAGGGTAGTCGAGCTGCTTGGCATTATCTGGACTACGGTTGCTACT 120
11      TTCAATGTGCTAACAAGGGCAGTCGAGCTGCTTGGCATTATCTGGACTACGGTTGCTACT 120
13      TTCAATGTGCTAACAAGGGCAGTCGAGCTGCTTGGCATTATCTGGACTACGGTTGCTACT 120
14      TTAAATGTGCCATACCTGGCAGTTGACCTTTGTTCCACTATCAGGACTACGGTTGCTACT 120
        ** ******* *     ** * *  *  *          ***   *  ** **********
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Continued

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4           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAGCACATG 180
6           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAGCACATG 180
1           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAGCACATG 180
9           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAGCACATG 180
2           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAACACATG 180
3           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAACACATG 180
8           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAACACATG 180
5           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAACACATG 180
10          GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGTGCTGCAAAACACATG 180
7           GCGGCAATGGAGGTCGTGGGACACCGGTAGATGAGTTGGATAGGAGCTGCAAAACACATG 180
12          GCGGCCTGGAGGTCGTGGGACACCTGTAGATGAGTTGGATAGGTGCTGCAAAATACATG 180
15          GCGGCCTGGAGGTCGTGGGACACCTGTAGATGAGTTGGATAGGTGCTGCAAAATACATG 180
11          GCGGCCTGGAGGTCGTGGGACACCTGTAGATGAGTTGGATAGGTGCTGCAAAATACATG 180
13          GCGGCCTGGAGGTCGTGGGACACCTGTAGATGAGTTGGATAGGTGCTGCAAAATACATG 180
14          GCGGCCAGGAGGTCATGGGAAACCTGTAGATAAGTTGGATAGGTGCTGCAAAGCACATG 180
            *****   ******* ***** *** ****** *********** ********  *****

4           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTACGCGATGAGTGGCCCCT 240
6           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTACGCGATGAGTGGCCCCT 240
1           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTACGCGATGAGTGGCCCCT 240
9           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTACGCGATGAGTGGCCCCT 240
2           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTATGTGATGAGTGGCCCCT 240
3           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTATGTGATGAGTGGCCCCT 240
8           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTATGTGATGAGTGGCCCCT 240
5           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTATGTGATGAGTGGCCCCT 240
10          ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTATGTGATGAGTGGCCCCT 240
7           ACGAGTGCTATGGTGAAGCCGAAAAACTTCCTGCATGTAATTATGTGATGAGTGGCCCCT 240
12          ACGACTGCTATATTGAAGCCGGAAAAG---ACGGATGCTACCCCAAGTTGA------CCT 231
15          ACGACTGCTATATTGAAGCCGGAAAGG---ACGGATGCTACCCCAAGTTGA------CCT 231
11          ACGACTGCTATATTGAAGCCGGAAAAG---ACGGATGCTACCCCAAGTTGA------CCT 231
13          ACGACTGCTATATTGAAGCCGGAAAAG---ACGGATGCTACCCCAAGTTGA------CCT 231
14          ACGACTGCTATGGTGAAGCTGGAAAGA---AAGGATGCTACCCAGTGTTGA------CGT 231
            **** ******  ****** * ***       * ***  *      * ***      * *
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4          GCTACAATACCTATTCATATGACTGTGTTGGACACCAACTCACCTGCAAAGGAGACAACG 300
6          GCTACAATACCTATTCATATGACTGTGTTGGACACCAACTCACCTGCAAAGGAGACAACG 300
1          GCTACAATACCTATTCATATGACTGTGTTGGACACCAACTCACCTGCAAAGGAGACAACG 300
9          GCTACAATACCTATTCATATGACTGTGTTGGACACCAACTCACCTGCAAAGGAGACAACG 300
2          GCTACAACACCTATTCATATGACTGTGTTGAACACCAACTCACCTGCAAAGGAGACAATG 300
3          GCTACAACACCTATTCATATGACTGTGTTGAACACCAACTCACCTGCAAAGGAGACAATG 300
8          GCTACAACACCTATTCATATGACTGTGTTGAACACCAACTCACCTGCAAAGGAGACAATG 300
5          GCTACAACACCTATTCATATGACTGTGTTGAACACCAACTCACCTGCAAAGGAGACAATG 300
10         GCTACAACACCTATTCATATGACTGTGTTGAACACCAACTCACCTGCAAAGGAGACAATG 300
7          GCTACAACACCTATTCATATGACTGTGTTGAACACCAACTCACCTGCAAAGGAGACAATG 300
12         GGTATAGT---------TGGGACTGTACTGGAGATGCACCCACCTGCAATCCAAA-ATCG 281
15         GGTATAGT---------TGGCAATGTACTGGAGATGCACCCACCTGCAATCCAAA-ATCG 281
11         GGTATAGT---------TGGGACTGTACTGGAGATGCACCCACCTGCAATCCAAA-ATCG 281
13         GGTATAGT---------TGGGACTGTACTGGAGATGCACCCACCTGCAATCCAAA-ATCG 281
14         TGTATAGT---------TGGGAATGTACTGAAAAAGTACCCATCTGCAATTCAAA-AACG 281
             ** *           *   * ***  ** * *   ** ** ******   * * *  *

4          ATGACTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTCTCGCCAAAG 360
6          ATGACTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTCGCCAAAG 360
1          ATGACTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTCGCCAAAG 360
9          ATGACTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTCGCCAAAG 360
2          ATGAGTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTCACCAAAG 360
3          ATGAGTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTCGCCAAAG 360
8          ATGAGTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTCGCCAAAG 360
5          ATGAGTGTAAAGCCTTTATTTGTAATTGGGACCGCACAGCAGCCATCTGTTTCGCCAAAG 360
10         ATGAGTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTCGCCAAAG 360
7          ATGAGTGTAAAGCCTTTATTTGTAATTGTGACCGCACAGCAGCCATCTGTTTTGCCAAAG 360
12         A--AGTGTAAAGATTTTGTGTGTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAG 339
15         A--AGTGTAAAGATTCTGTGTGTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAG 339
11         A--AGTGTAAAGATTTTGTGTGTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAG 339
13         A--AGTGTAAAGATTTTGTGTGTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAG 339
14         A--AGTGTAAACGTATTGTGTGTGATTGTGACGCCGCAGTAGCCAAGTGCTTTGCCAAAG 339
           *  * ******     * * ***  *** **   * *** *****  **  *  ******
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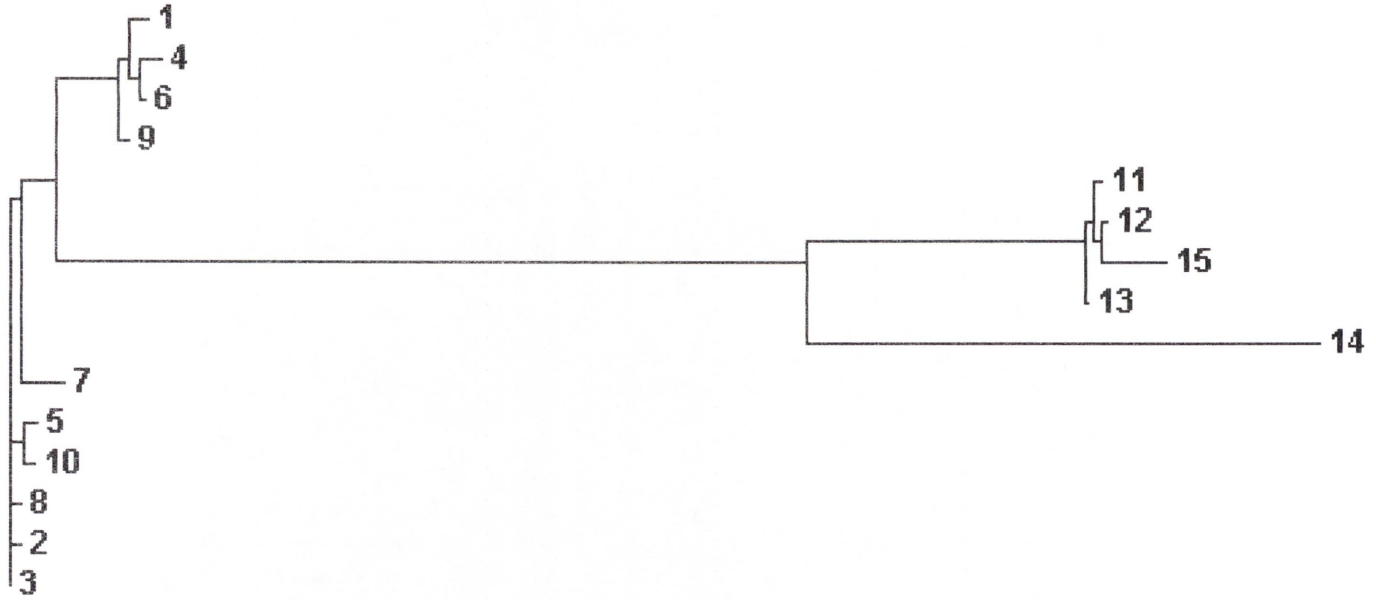
Continued

```
4          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
6          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
1          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
9          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
2          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
3          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
8          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
5          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
10         CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
7          CCCCTTTCAACAAGGAGAACTGGAATATCGACACCGAGACACATTGCCA 409
12         CCCCTTACAACAAGGCGAACTGGAATATCGACACCGAGACACATTGCCA 388
15         CCCCTTACAACAAGGCGAACTGGAATATCGACACCGAGACACATTGCCA 388
11         CCCCTTACAACAAGGCGAACTGGAATATCGACACCGAGACACATTGCCA 388
13         CCCCTTACAACAAGGCGAACTGGAATATCGACACCGAGACACATTGCCA 388
14         CCCCTTACAAGAAGGAGAACTACAATATCGACACCGAGACACATTGCCA 388
           ****** *** **** *****  **************************
```

This is a clustalW alignment of 10 novel PLA_2 gene sequences obtained from screening the previously mentioned King Brown snake venom gland cDNA library.

The sequences are all 413 base pairs in length, and the differences in each sequence due to nucleotide substitutions are highlighted in red.

Figure 3.6 - Phylogram of the Unknown Nucleotide Sequences



The fifteen unknown nucleotide sequences of potential PLA_2 genes which were determined after sequencing forty positive colonies were aligned by ClustalW and a phylogram was then constructed based on the alignment. This phylogram suggests that the sequences could be grouped into 3 groups based on sequence homology.

With respect to members of group B where five novel sequences were isolated, the positions where variations were observed differed from members of groups A and C but again variation was in the form of nucleotide substitutions only. Substitutions were observed at position 12 which divided members into two subgroups - sequences 11 and 13 had guanine and sequences 12 and 15 had thymine; at position 42, sequence 13 had thymine which distiguishes it from the rest and at position 80, sequence 15 had thymine which distinguished it from the other sequences. Other positions where there were variations were position 206 where sequence 15 had guanine; position 243 where sequence 15 again differed from the others by cytosine; at position 245, sequence 15 had adenine and at position 294, sequence 15 had cytosine; it would seem that the nucleotide sequences were more conserved for members of this group. The only exception is sequence 14 which had substitutions in a large number of positions, see figure 3.7. The differences between the various sequences are represented as phylograms in figure 3.8 which provides a more visual representation of the results.

It is also important to state that the positive controls in all four batches of sequencing showed a 100% match to the known sequence of pGEM vector, demonstrating that there was no discernable error during transcription and amplification and any variation in the sequences should be attributed to differences in the genes.

While categorising these new sequences into groups might be convenient, this exercise could not reveal any further clues to their identity and thus more detailed scrutiny of these sequences were needed. Due to the similarity between groups A and C, these two groups were subsequently pooled together and analysed as one group (Group AC). Sequences in group B had significant differences which justified treating the group separately.

Figure 3.7 - Novel King Brown PLA_2 genes (Group B)

Continued

```
11          ACGACTGCTATATTGAAGCCGGAAAAGACGGATGCTACCCCAAGTTGACCTGGTATAGTT 240
13          ACGACTGCTATATTGAAGCCGGAAAAGACGGATGCTACCCCAAGTTGACCTGGTATAGTT 240
12          ACGACTGCTATATTGAAGCCGGAAAAGACGGATGCTACCCCAAGTTGACCTGGTATAGTT 240
15          ACGACTGCTATATTGAAGCCGGAAAGGACGGATGCTACCCCAAGTTGACCTGGTATAGTT 240
14          ACGACTGCTATGGTGAAGCTGGAAAGAAAGGATGCTACCCAGTGTTGACGTTGTATAGTT 240
            ***********  ****** *****  * ***********   ****** * ********

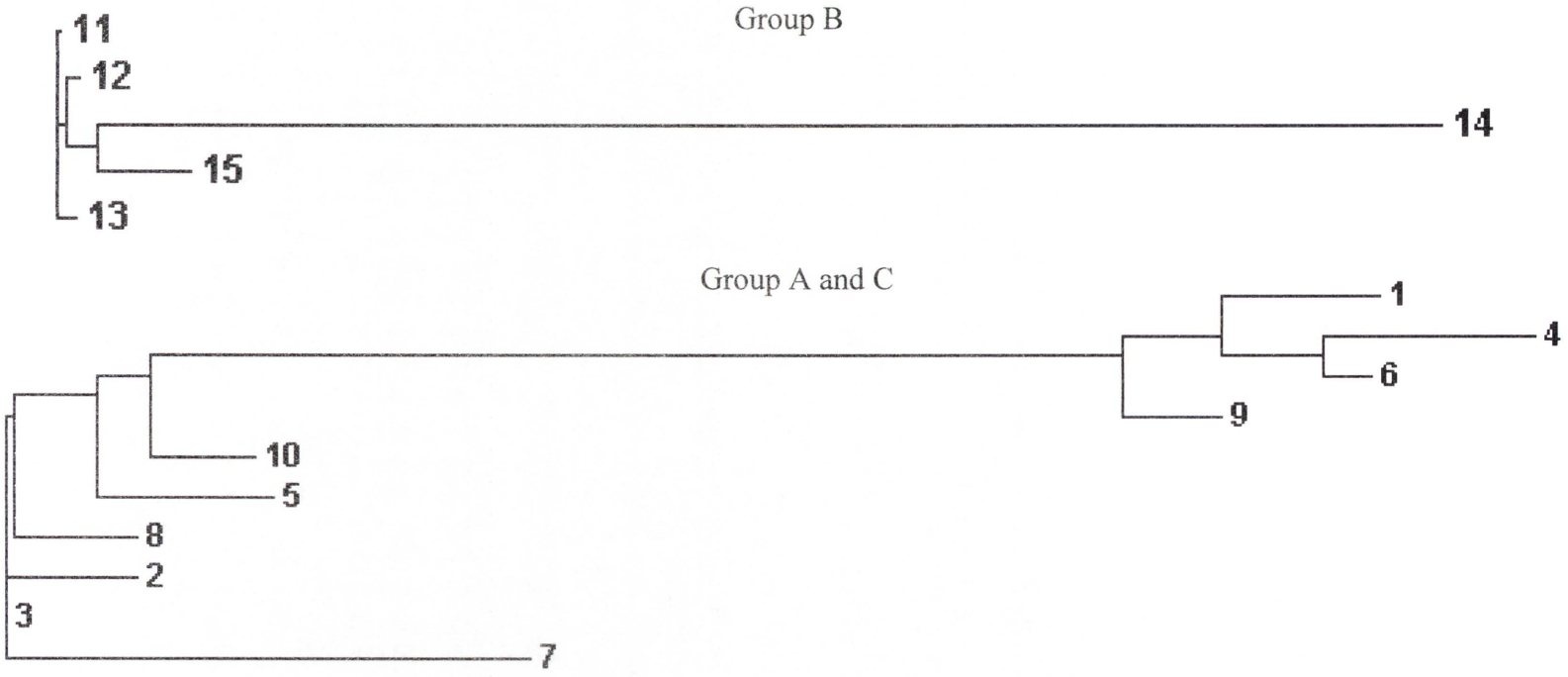
11          GGGACTGTACTGGAGATGCACCCACCTGCAATCCAAAATCGAAGTGTAAAGATTTTGTGT 300
13          GGGACTGTACTGGAGATGCACCCACCTGCAATCCAAAATCGAAGTGTAAAGATTTTGTGT 300
12          GGGACTGTACTGGAGATGCACCCACCTGCAATCCAAAATCGAAGTGTAAAGATTTTGTGT 300
15          GGCAATGTACTGGAGATGCACCCACCTGCAATCCAAAATCGAAGTGTAAAGATTCTGTGT 300
14          GGGAATGTACTGAAAAAGTACCCATCTGCAATTCAAAAACGAAGTGTAAACGTATTGTGT 300
            ** * ******* * * * ***** ******* ***** ***********  *  *****

11          GTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAGCCCCTTACAACAAGGCGAACT 360
13          GTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAGCCCCTTACAACAAGGCGAACT 360
12          GTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAGCCCCTTACAACAAGGCGAACT 360
15          GTGCTTGTGATGCCGCAGCAGCCAAGTGCTTTGCCAAAGCCCCTTACAACAAGGCGAACT 360
14          GTGATTGTGACGCCGCAGTAGCCAAGTGCTTTGCCAAAGCCCCTTACAAGAAGGAGAACT 360
            *** ****** ******* ****************************** **** *****

11          GGAATATCGACACCGAGACACATTGCCA 388
13          GGAATATCGACACCGAGACACATTGCCA 388
12          GGAATATCGACACCGAGACACATTGCCA 388
15          GGAATATCGACACCGAGACACATTGCCA 388
14          ACAATATCGACACCGAGACACATTGCCA 388
              ***************************
```

This is a clustalW alignment of the shorter 5 novel nucleotide sequences. All members of this group (Group B) have 388 nucleotides as opposed to members of Group AC which have 408 nucleotides each. Substitutions are highlighted in red.

Figure 3.8 - Phylograms of Group AC and B sequences



The above phylograms were constructed from clustalW alignments of Group B sequences (Top) and Group A and C sequences (Bottom) separately. By separating the two groups, a different phylogeny could be observed.

3.17 Comparison of the Long Chain PLA_2 nucleotide sequences to other PLA_2 genes

The novel sequences of groups A and C were submitted to various databases (EMBL, GenBank, DDBJ and PDB) using a Blast search for sequence comparison and identity matches. The result was that the highest score was the PLA_2 gene γ Taipoxin 2 isolated from *Oxyuranus scutellatus scutellatus* which had an approximate 86% identity over 245 bp (varying by a few percentiles depending on the actual sequence) to the novel sequences (Welton and Burnell 2004, accession number AY691657). The PLA_2 gene $\tilde{\gamma}$ Taipoxin 2 itself is 459 bp in length, so even though the novel sequences span over significant portions of the gene, it could be argued that there were significant portions which were not homologous. More importantly, $\tilde{\gamma}$ Taipoxin 2 is part of subunit of multimeric PLA_2 gene and the sequence of $\tilde{\gamma}$ Taipoxin 2 have significant differences to PLA_2 genes for monomeric PLA_2 enzymes. The actual highest match was with a PLA_2 gene isolated from *Lapemis hardwickii* (found in the Gulf of Carpentaria) with a score of 88% identity over 282 bp (Zhong *et al* 1999, accession number Q8UW31). The entire PLA_2 gene was 458 bp in length so significant portions of the gene also did not match.

The sequences also showed high homology to the PT- PLA_2 gene precursor from *Pseudonaja textilis* with 83% identity over 274 nucleotide residues as well (Jeyaseelan *et al*, 1999). PT- PLA_2 is only 380 bp in size but if the signal peptide is also included, then it is 460 bp in size. Again significant portions of the sequences did match with the known PLA_2 gene but there were also sections which did not seem to match to PLA_2 genes. Most importantly, the sequences matched with *Notechis scutatus* PLA_2 gene (Ducancel *et al.*, 1988), with a score of 83% over 206 bp. As the gene for *Notechis scutatus* PLA_2 is 353 bp, the novel sequences had significant portions which matched a known PLA_2 gene.

3.18 Comparison of the Short Chain PLA_2 nucleotide sequences to other PLA_2 genes

When the sequences from group B were submitted to the same databases for comparison (EMBL, GenBank, DDBJ and PDB) using a Blast search, the highest score this time (for homology) was the PLA_2 gene ASPLA8 isolated from the Australian venomous snake *Austrelaps superbus* which had an approximate 87% identity over 388 residues (varying by a few percentiles

depending on the actual sequence) to the novel sequences. The PLA_2 gene ASPLA8 itself is 437 bp in length, thus the novel sequences span over significant portions of the gene. Interestingly, ASPLA8 is not a neurotoxin but it is reported to exhibit inhibition of platelet activity (Singh *et al.*, 2000). This result confirms that the isolated genes were indeed potential venom PLA_2 genes.

Most importantly, the sequences also showed high homology to the PLA_2 genes notexin with 84% identity over 388 nucleotide residues as well. As stated above, notexin is only 353 bp in size but since the primers used in these experiments also included the signal peptide region, the result meant that the entire span of the notexin gene was matched with the novel sequences. Notexin is a neurotoxin and this result strongly supports further testing of the novel sequences from the King Brown snake for neurotoxin activity.

To a lesser extent, the four novel sequences also showed significant homology to the neurotoxin PLA_2 gene γ taipoxin 2 from *Oxyuranus scutellatus* with approximately 90% identity over a short nucleotide sequence of 118 residues only (with the exception of sequence 3 which had significantly lower identity due to its variations). However, the result with γ Taipoxin 2 gene should again be viewed with some caution for the same reasons as above.

3.19 Translation into protein sequences and alignment

As the majority of information on PLA_2 enzymes PLA_2 are in the form of amino acid sequences, translation of the nucleotide sequences into putative amino acid sequences for analysis proved to be valuable. The putative amino acid sequences were then aligned with known venom PLA_2 enzymes using clustalW (Figure 3.9). The known PLA_2 enzymes selected were Notexin from Tiger Snake, Pa 1G, Pa 3, Pa 5, Pa 9C, Pa 10A, Pa 11, Pa 12A, Pa 13, Pa 15, Pa 23, which were isolated from King Brown (Takasaki *et al*, 1990) and Acanthin I and Acanthin II from Death Adder. Amongst these venom PLA_2 enzymes, presynaptic neurotoxicity have been reported in Pa 13 and Pa 11 (Nishida *et al.*, 1985)

Figure 3.9 - The clustalW alignment of the putative proteins sequences

```
11              LGAASIPAQPLNILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
13              LGAASIPAQPLNIFQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
12              LGASSIPAQPLNILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
15              LGASSIPAQPLNILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
PA-15           -----------NILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 49
PA-13           -----------NILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 49
14              LGAASIPAQPLNLIQLSNMIKCAIPGSXPLFHYQDYGCYCGPGGHGKPVDKLDRCCKAHD 60
Notexin         -----------NLVQFSYLIQCANHGKRPTWHYMDYGCYCGAGGSGTPVDELDRCCKIHD 49
PA-1G           -----------NLIQFGNMIQCANKGSRPTRHYMDYGCYCGWGGSGTPVDELDRCCQTHD 49
PA-3            -----------NLIQFGNMIQCANKGSRPTRHYMDYGCYCGWGGSGTPVDELDRCCKVHD 49
PA-5            -----------NLIQFSNMIQCANKGSRPSLDYADYGCYCGWGGSGTPVDELDRCCKVHD 49
PA-10A          -----------NLIQFSNMIQCANKGSRPSLHYADYGCYCGWGGSGTPVDELDRCCKVHD 49
AcanthinII      -----------NLYQFGGMIQCANKGARSWLSYVNYGCYCGWGGSGTPVDELDRCCQIHD 49
AcanthinI       -----------DLFQFGGMIGCANKGARSWLSYVNYGCYCGWGGSGTPVDELDRCCQIHD 49
PA-9C           -----------NLIQFKSIIECANRGSRRWLDYADYGCYCGWGGSGTPVDELDRCCKVHD 49
PA-12A          -----------NLIQFGNMIQCANKGSRPSLNYADYGCYCGWGGSGTPVDELDRCCQVHD 49
PA-11           -----------NLIQFGNMIQCANKGSRPSLDYADYGCYCGWGGSGTPVDELDRCCQVHD 49
PA-12C          -----------NLIQFGNMIQCANKGSRPSLDYADYGCYCGWGGSGTPVDELDRCCQTHD 49
                :    :    ::: *:  :* **  *      * :****** ** *.***:*****: **
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Continued

```
11              DCYIEAGKDGCYPKLTWYSWDCTGDAPTC-NPKSKCKDFVCACDAAAAKCFAKAPYNKAN 119
13              DCYIEAGKDGCYPKLTWYSWDCTGDAPTC-NPKSKCKDFVCACDAAAAKCFAKAPYNKAN 119
12              DCYIEAGKDGCYPKLTWYSWDCTGDAPTC-NPKSKCKDFVCACDAAAAKCFAKAPYNKAN 119
15              DCYIEAGKDGCYPKLTWYSWQCTGDAPTC-NPKSKCKDSVCACDAAAAKCFAKAPYNKAN 119
PA-15           DCYIEAGKDGCYPKLTWYSWQCTGDAPTC-NPKSKCKDFVCACDAAAAKCFAKAAYNKAN 108
PA-13           DCYIEAGKDGCYPKLTWYSWDCTGDAPTC-NPKSKCKDFVCACDAAAAKCFAKAPYNKAN 108
14              DCYGEAGKKGCYPVLTLYSWECTEKVPIC-NSKTKCKRIVCDCDAAVAKCFAKAPYKKEN 119
Notexin         DCYDEAGKKGCFPKMSAYDYYCGENGPYCRNIKKKCLRFVCDCDVEAAFCFAKAPYNNAN 109
PA-1G           DCYGEAEKKGCYPKLTLYSWDCTGNVPIC-SPKAECKDFVCACDAEAAKCFAKATYNDAN 108
PA-3            DCYGEAEKKGCYPKLTLYSWDCTGNVPIC-SPKAECKDFVCACDAEAAKCFAKATYNDAN 108
PA-5            DCYAEAGKKGCYPKLTLYSWDCTGNVPIC-NPKTECKDFTCACDAEAAKCFAKAPYKKEN 108
PA-10A          DCYDQAGKKGCFPKLTLYSWDCTGNVPIC-NPKSKCKDFVCACDAAAAKCFAKAPYNKAN 108
AcanthinII      NCYGEAEKKRCGPKMTLYSWECANDVPVCN-SKSACEGFVCDCDAAAAKCFAKAPYNKNN 108
AcanthinI       NCYGEAEKKQCGPKMTSYSWKCANDVPVCNDSKSACKGFVCDCDAAAAKCFAKAPYNKNN 109
PA-9C           ECYGEAVKQGCFPKLTVYSWKCTENVPICD-SRSKCKDFVCACDAAAAKCFAKAPYNKDN 108
PA-12A          NCYEQAGKKGCFPKLTLYSWKCTGNVPTCN-SKTGCKSFVCACDAAAAKCFAKAPYKKEN 108
PA-11           NCYEQAGKKGCFPKLTLYSWKCTGNVPTCN-SKPGCKSFVCACDAAAAKCFAKAPYKKEN 108
PA-12C          NCYEQAGKKGCFPKLTLYSWKCTGNAPTCN-SKPGCKRFVCACDAAAAKCFAKAPYKKEN 108
                :** :* *. * * :: *.: *  . * *   :  *   .* **. .* *****.*:. *
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Continued

```
11              WNIDTETHC- 128
13              WNIDTETHC- 128
12              WNIDTETHC- 128
15              WNIDTETHC- 128
PA-15           WNIDTKTRCK 118
PA-13           WNIDTKTRCK 118
14              YNIDTETHC- 128
Notexin         WNIDTKKRCQ 119
PA-1G           WNIDTKTRC- 117
PA-3            WNIDTKTRCK 118
PA-5            WNIDTKTRCK 118
PA-10A          WNIDTKTRCK 118
AcanthinII      IGIGSKTRCQ 118
AcanthinI       IGIGSKTRCQ 119
PA-9C           YNIDTKTRCQ 118
PA-12A          YNIDTKKRCK 118
PA-11           YNIDTKKRCK 118
PA-12C          YNIDTKKRCK 118
                 .*.:::.:*
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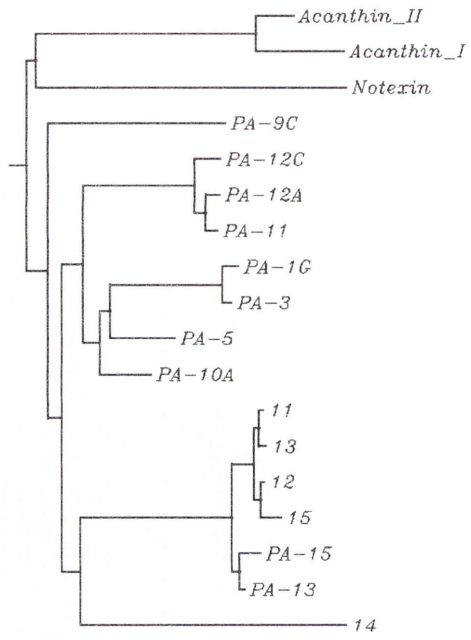

This is a clustal alignment of the group B amino acid sequences with some known PLA_2 amino acid sequences together with a Phylogram. The published sequences are PA-1G, PA-3, PA-5, PA-9, PA-10A, PA-11, PA-12A, PA-12C, PA-13 and PA-15 previously isolated from King Brown crude venom and Notexin and Acanthin I and II previously isolated from Copperhead and Death Adder respectively.

In all the sequences, the position of the 7 cysteine bridges are conserved, suggesting that all the novel sequences (including the highly polymorphic sequence 14) are PLA_2 enzymes.

Interestingly, unknown sequence 12 and PA-13 have only two residue differences at positions 125 and 127 which was an artifact introduced in primer design.

and although Acanthin I and II were reported to be potent inhibitors of platelet aggregation (Chow *et al.*, 1998), Acanthin is highly homologous to another venom PLA_2 enzyme from Death Adder, Acanthoxin which was reported to be a potent pre-synaptic neurotoxin (van der Weyden *et al.*, 2000). The phylogram was constructed based on the clustal alignment and is shown in figure 3.9.

3.20 Short Chain amino acid sequences

The phylogram indicated that the putative amino acid sequences of novel genes 11, 12, 13 and 15 were highly homologous to two amino acid sequences (Pa 13 and Pa 15) which were previously identified from King Brown venom glands. Pa 13 was isolated by Nishida *et al.*, in 1985 and is a neurotoxin and Pa 15 was isolated by Takasaki *et al.*, in 1990 but its toxicity was not reported. However, as the primary structure of these two amino acid sequences are highly homologous, it is suggested that Pa 15 may well be a venom gene and further testing of the enzyme for possible neurotoxicity is justified. When Pa 13 and Pa 15 were aligned to the two amino acid sequences which were most similar to them (novel sequences 12 and 15) for direct comparison, it showed a 95% sequence identity. The clustalW alignment is shown in figure 3.10. There were five sites of mutations at amino acid 14, 81, 114, 125 and 127. The variations in amino acid 125 and 127 were introduced by the reverse primer which was based on Notexin, thus these two variations could be accounted for easily. When these two variations were taken into account, the sequence data revealed that novel sequence 12 matches with Pa 13 completely and in all likelihood is the gene for the neurotoxin Pa 13. As for the other new sequences (11, 13 and 15), these are most likely to be homologues of Pa 13 and Pa 15 as they are highly similar in primary structure. The highly variable new sequence 14 only produced a truncated protein (residue 27 is a stop codon) when translated and it is probably a pseudogene.

While the identification of these novel gene sequences was encouraging, the absence of other venom genes isolated by Takasaki, e.g. Pa 5, Pa 12, etc was noted. A possible explanation for their absence is intra-species variation and a detailed discussion is offered in the last chapter.

Figure 3.10 - Clustal Alignment of Known Proteins from King Brown

```
Novel12         LGAASIPAQPLNILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
Novel15         LGAASIPAQPLNIFQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
PA13            -----------NILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 49
PA15            -----------NILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 49
                           **:**************************************************

Novel12         DCYIEAGKDGCYPKLTWYSWDCTGDAPTCNPKSKCKDFVCACDAAAAKCFAKAPYNKANW 120
Novel15         DCYIEAGKDGCYPKLTWYSWDCTGDAPTCNPKSKCKDFVCACDAAAAKCFAKAPYNKANW 120
PA13            DCYIEAGKDGCYPKLTWYSWDCTGDAPTCNPKSKCKDFVCACDAAAAKCFAKAPYNKANW 109
PA15            DCYIEAGKDGCYPKLTWYSWQCTGDAPTCNPKSKCKDFVCACDAAAAKCFAKAAYNKANW 109
                ********************:**********************************.******

Novel12         NIDTETHC- 128
Novel15         NIDTETHC- 128
PA13            NIDTKTRCK 118
PA15            NIDTKTRCK 118
                ****:*:*
```

This clustalW alignment of the putative unknown amino acid sequences 12 and 15 with two known amino acid sequences of PLA2 enzymes found in the King Brown snake, Pa 13 and Pa 15. The alignment confirms that novel sequence 12 and PA13 are the same amino acid sequence.

3.21 The Long Chain Amino Acid Sequences

While the group of short chain nucleotide sequences proved to be potential venom genes, the group of long chain sequences seemed to be something else. These nucleotide sequences were also translated into putative amino acid sequences and aligned with the shorter amino acid sequences of 11 to 15 for comparison. The alignment is shown in figure 3.11. The phylogram analysis and clustalW alignment suggested that the unknown sequences were significantly different from the shorter amino acid sequences. It is noted that there were some regions of similarity, in particular, the cysteine residues from the novel sequences which aligned with the cysteine residues of the other sequences. As mentioned in the introductory chapter, the monomeric PLA_2 enzymes have 14 cysteine residues which form seven disulfide bridges to give a stable tertiary structure to PLA_2 enzymes. However, the unknown sequences also contained an additional unpaired cysteine residue at position 81 and unpaired, highly reactive residues within an enzyme is rare. There were also two insertions of proline residues at positions 70 and 80, as well as several other insertions at positions 78 (glycine), 82 (tyrosine), 83 (asparagine) and 98 (glycine).

It is believed that the proline residues could introduce "kinks" to the overall tertiary structure and possibly disrupt the tertiary structure all together (Ballach and Schmid, 2000). On the other hand, a personal communication from Dr Brian Fry (2008) suggested that proline kinks could only alter the structure of a globular protein but not the structure of PLA_2 enzyme as PLA_2 enzymes are stabilized by seven cysteine bonds. However, PLA_2 enzymes are globular proteins and while the seven cysteine bonds might not be broken, the folding of the protein could still be altered to render the catalytic site and bonding sites inactive. In either case, further testing of these unique proteins must be done before any valid conclusions could be drawn.

It is interesting to note that there have been no reports of homologous amino acid sequences to this group of PLA_2 enzymes until recently when Pierre *et al.*, isolated possibly similar genes from the cDNA venom gland library of *Oxyuranus scutellatus* (St Pierre *et al.*, 2006). He reported three isoforms of putative PLA_2 enzymes based on nucleotide sequence translation which contain a six residue insert at positions 85 to 90. More importantly, these inserts also contained a proline (residue 86), cysteine (residue 88), asparagine (residue 89) and glycine (residue 90). Unfortunately, there was no report on the enzyme activity or toxicity of these novel putative

proteins. A brief discussion of the results presented by Pierre *et al.*, and the putative long PLA_2 sequences are covered in chapter 5.1. Taking these various factors into account, it is tentatively suggested that these may in fact represent a novel group of PLA_2 genes but the possibility that they may be pseudogenes are also not discounted. It is highly unlikely that these genes would have PLA_2 activity due to their disrupted PLA_2 fold, however, these genes reveal a facet of the gene duplication process which will be discussed in the last chapter.

Figure 3.11 – Clustal W alignment of Novel Long Sequences

```
1                LEAASIPPQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKAHD 60
9                LGAASIPPQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKAHD 60
4                LEASSIPAQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKAHD 60
6                LGASSIPAQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKAHD 60
2                LGAASIPAQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKTHD 60
3                LGAASIPAQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKTHD 60
7                LGAASIPAQPLNLYQLKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRSCKTHD 60
8                LGASSIPAQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKTHD 60
5                LEAASIPAQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKTHD 60
10               LEAASIPPQPLNLYQFKEMIECANKGTISGLAYAGYGCYCGNGGRGTPVDELDRCCKTHD 60
12               LGASSIPAQPLNILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
15               LGASSIPAQPLNILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
11               LGAASIPAQPLNILQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
13               LGAASIPAQPLNIFQFRKMIQCANKGSRAAWHYLDYGCYCGPGGRGTPVDELDRCCKIHD 60
14               LGAASIPAQPLNLIQLSNMIKCAIPGSXPLFHYQDYGCYCGPGGHGKPVDKLDRCCKAHD 60
                 * *:***.****: *: :**:**  *: .   * .****** **:*.***:***.** **
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Continued

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                    70       78   83              98
1                ECYGEAEKLPACNYAMS-GPCYNTYSYDCVGHQLTCKGDNDDCKAFICNCDRTAAICFAK 119
9                ECYGEAEKLPACNYAMS-GPCYNTYSYDCVGHQLTCKGDNDDCKAFICNCDRTAAICFAK 119
4                ECYGEAEKLPACNYAMS-GPCYNTYSYDCVGHQLTCKGDNDDCKAFICNCDRTAAICLAK 119
6                ECYGEAEKLPACNYAMS-GPCYNTYSYDCVGHQLTCKGDNDDCKAFICNCDRTAAICFAK 119
2                ECYGEAEKLPACNYVMTSGPCYNTYSYDCVEHQLTCKGDNDECKAFICNCDRTAAICFTK 120
3                ECYGEAEKLPACNYVMS-GPCYNTYSYDCVEHQLTCKGDNDECKAFICNCDRTAAICFAK 119
7                ECYGEAEKLPACNYVMS-GPCYNTYSYDCVEHQLTCKGDNDECKAFICNCDRTAAICFAK 119
8                ECYGEAEKLPACNYVMS-GPCYNTYSYDCVEHQLTCKGDNDECKAFICNCDRTAAICFAK 119
5                ECYGEAEKLPACNYVMS-GPCYNTYSYDCVEHQLTCKGDNDECKAFICNWDRTAAICFAK 119
10               ECYGEAEKLPACNYVMS-GPCYNTYSYDCVEHQLTCKGDNDECKAFICNCDRTAAICFAK 119
12               DCYIEAGKD-GCYPKLT------WYSWDCTGDAPTCN-PKSKCKDFVCACDAAAAKCFAK 112
15               DCYIEAGKD-GCYPKLT------WYSWQCTGDAPTCN-PKSKCKDSVCACDAAAAKCFAK 112
11               DCYIEAGKD-GCYPKLT------WYSWDCTGDAPTCN-PKSKCKDFVCACDAAAAKCFAK 112
13               DCYIEAGKD-GCYPKLT------WYSWDCTGDAPTCN-PKSKCKDFVCACDAAAAKCFAK 112
14               DCYGEAGKK-GCYPVLT------LYSWECTEKVPICN-SKTKCKRIVCDCDAAVAKCFAK 112
                 :** ** *  .*   ::       **::*. .   *:  : .**  :*  * :.* *::*
```

```
1          APFNKENWNIDTETHC 135
9          APFNKENWNIDTETHC 135
4          APFNKENWNIDTETHC 135
6          APFNKENWNIDTETHC 135
2          APFNKENWNIDTETHC 136
3          APFNKENWNIDTETHC 135
7          APFNKENWNIDTETHC 135
8          APFNKENWNIDTETHC 135
5          APFNKENWNIDTETHC 135
10         APFNKENWNIDTETHC 135
12         APYNKANWNIDTETHC 128
15         APYNKANWNIDTETHC 128
11         APYNKANWNIDTETHC 128
13         APYNKANWNIDTETHC 128
14         APYKKENYNIDTETHC 128
           **::* *:********
```

This is a clustalW alignment of ten putative amino acid sequences derived from the novel nucleotide sequences. The ten amino acid sequences are aligned with the five amino acid sequences from group B which were previously determined to be PLA_2 sequences.

Although the seven cysteine bridges motive is conserved amongst the 10 sequences, there are important additional residues at position 70, 78 to 83 and 98. Most importantly, at position 81, there is an additional unpaired cysteine and a proline at position 80.

Taking into consideration of the unpaired cysteine, the introduction of a proline in the middle of the chain and that these sequences are completely novel and do not show high homology with other venom PLA_2 enzymes previously isolated in King Brown snake, the corresponding genes for these putative proteins are most like to be pseudogenes which are still expressed as mRNA only in the venom glands.

3.11

3.22 Sequencing results for the α-neurotoxin genes

While the PCR based on the various primer sets (gene specific primers for group C α-neurotoxins; degenerate primers for long and short chain α-neurotoxins) yielded a rich number of bands. When these bands were ligated into a sequencing vector (pGEM-T) and sequenced, all the bands were false positive results due to mis-priming of the primers. The problem with mis-priming was particularly evident where degenerate primers were used. Thus no valid results nor conclusions could be drawn at this point in time. However, it is believed that the problems encountered here can be overcome by utilizing the correct primer sequences.

Chapter 4

Screening for PLA_2 and α-Neurotoxin genes in Death Adder snake venom gland

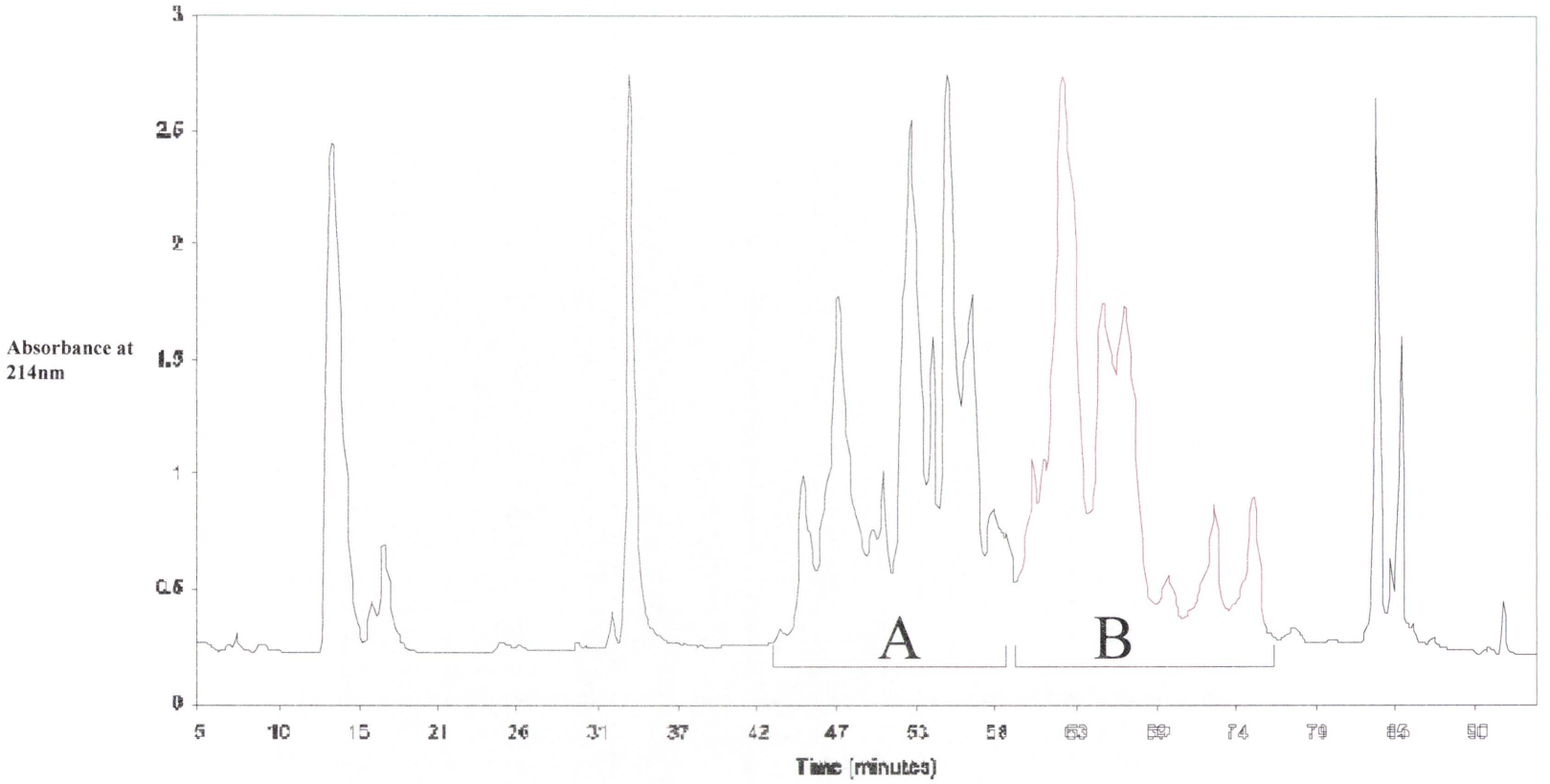
4.1 Introduction

It was mentioned in the introductory chapter that Death Adder envenomation was traditionally associated only with post-synaptic neurotoxicity with little or no signs of active α-neurotoxins at work. This has led to the assumption that Death Adder venom does not contain PLA_2 enzymes with pre-synaptic neurotoxicity. However, in 1997 a novel α-neurotoxin Acanthoxin was isolated from Death Adder crude venom by van der Weyden (van der Weyden *et al.*, 2000). In addition, another PLA_2 enzyme Acanthin I and its isoform Acanthin II were also isolated from Death Adder venom in the same year by Chow (Chow *et al.*, 2000). However, Acanthin was tested for its anti-platelet aggregation activity (which proved positive) rather than for neurotoxicity. The discovery of a novel α -neurotoxin in Death Adder raised the question of whether other isoforms of Acanthoxin existed.

4.2 PLA_2 enzymes in Death Adder Crude Venom

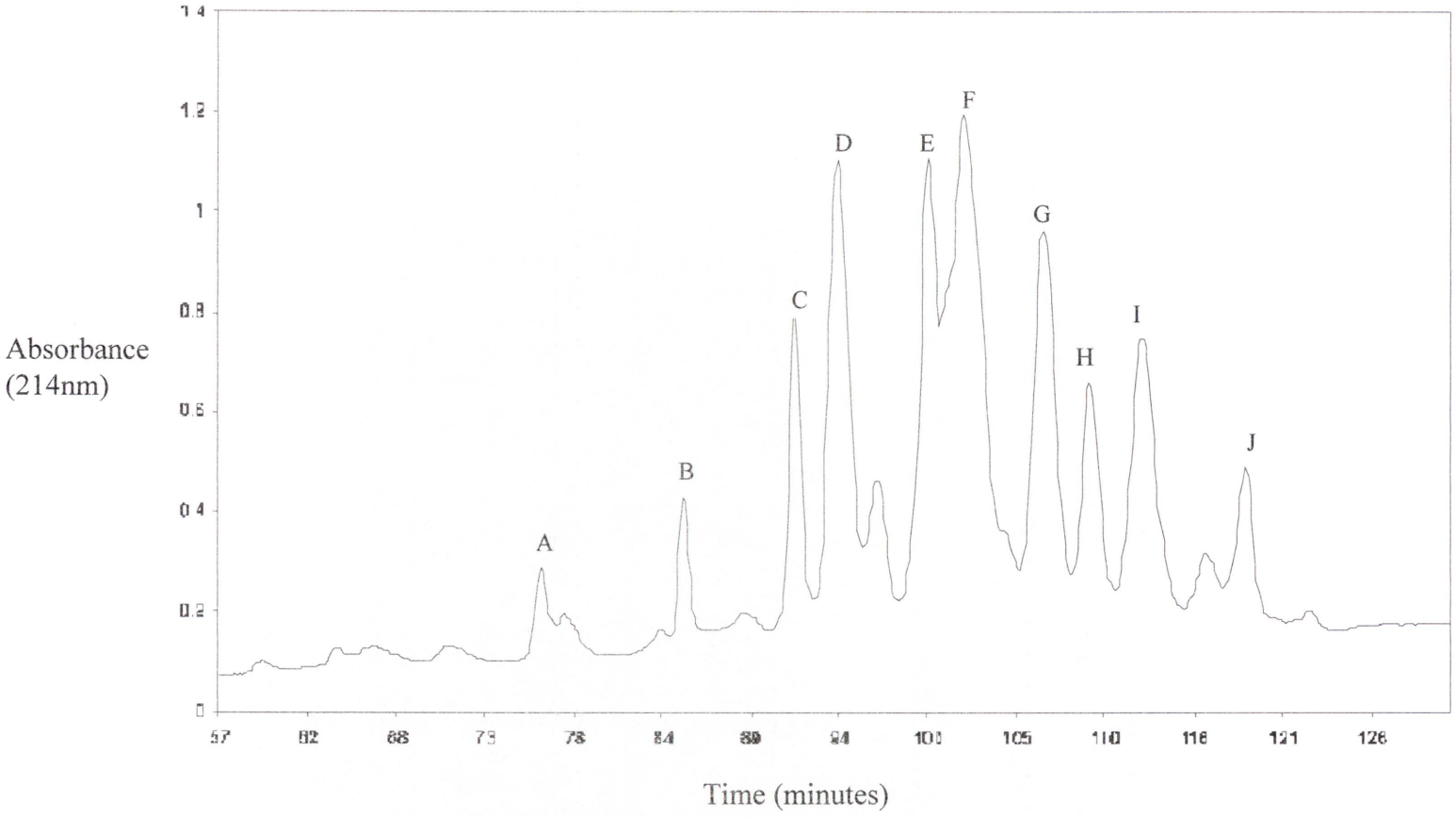
In 1998, Sung answered the above question and reported the isolation of seven novel PLA_2 enzymes isolated from Death Adder crude venom (Sung, 1998). These multiple isoforms were isolated using high performance liquid chromatography in combination with electrospray mass spectrometry. Initially, an aliquot of appropriately diluted crude venom was injected into a C2/C18 column for reverse-phase high performance liquid chromatography and individual components where then eluted from the column by an increasing concentration of acetonitrile. Thus, various components were separated based on hydrophobicity and a chromatogram could be generated by monitoring the time at which the components eluted from the column. A typical chromatogram of the separation is shown in figure 4.1, which shows the entire venom profile upon elution over a runtime of 95 minutes. The venom profile is a complex one, with three peaks detected at approximately 15 minutes into the run, another two peaks were observed at approximately 34-35 minutes, followed by a series of seventeen peaks beginning at 42 minutes to 79 minutes. Another three peaks were then eluted at 85 minutes followed by a last small peak at 92 minutes.

Figure 4.1 – Chromatogram of Crude Death Adder Venom



This is a chromatogram of crude death adder venom obtained from reverse-phase high performance liquid chromatography using a C2/C18 column. The region containing PLA_2 enzymes is marked as A. The region marked as B was also examined for presence of PLA_2 enzymes but no PLA_2 enzymes were isolated from it. Separation of the various venom components was achieved over a 3 hour gradient using 80% (V/V) acetonitrile.

Figure 4.2 – This is a chromatogram of Region A, showing further separation



In this chromatogram, the various fractions obtained from region A were pooled together, and re-injected into the C2/C18 column For RP-HPLC. While buffer A remained as water, better separation was achieved by substituting 80% (V/V) Acetonitrile with 100% Methanol. A series of 10 fractions (labelled A-J) were collected and fractions A to I were found to contain PLA_2 enzymes.

All the peaks were collected and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) which determined the respective sizes of the proteins contained in each peak. Only proteins around 12-14 kDa in size were deemed to be relevant as these were most likely to be monomeric PLA_2 enzymes, and the results of SDS-PAGE revealed that peaks found within 58 to 76 minutes contained proteins of that size. However, it could be observed that many of the peaks in this region contained "shoulders" which indicated that the peaks were not pure but contained two or more proteins in each peak. Thus, more stringent separation conditions were necessary to obtain peaks of single proteins.

In order to collect sufficient samples for the second separation, a total of ten runs of the first separation were performed and the peaks from region 58 to 76 minutes were collected, lyophilised to near dryness and pooled. The pooled sample was then re-injected into a freshly equilabrated C2/C18 column for analysis. Methanol (100% v/v) was used as the eluting buffer.

A typical result for this HPLC analysis is shown as a chromatogram in figure 4.2 which shows a series of thirteen peaks being eluted at 75 mins to 121 mins. These peaks were again collected and analysed by SDS-PAGE for size determination and the results revealed that eight of the thirteen peaks contained proteins of the correct size and are labelled 1 to 8 in the chromatogram. These peaks were then injected into an electrospray mass spectrometer for accurate size determination (to an accuracy of +/- 1 dalton) and purity evaluation. Finally, each of the peaks were also tested for PLA_2 activity using a spectrofluorimetry assay which is the demonstrative proof of the presence of the enzyme in question.

The results of electrospray mass spectrometry revealed that there were a number of different proteins in the eight peaks, ranging from 12-13 kDa in size, and the spectrofluorimetry assay found that peaks A to G contained PLA2 activity. The results of the mass spectrometry and spectrofluorimetry are summarised and set out as a table below (table 4.1) :-

Table 4.1 Size and PLA_2 activity of protein components from Crude Death Adder venom

Peak	Number and Size of proteins (in daltons)	PLA_2 activity
1	12890.28±1.13	Yes
2	13251.21±5.31 13471.88±4.29	Yes
3	12835.86±3.65 13060.31±2.89 13280.79±2.44	Yes
4	12796.00±2.53	Yes
5	12952.36±6.33	Yes
6	12105.05±6.27	Yes
7	13110.47±0.11	Yes
8	n/a	no

Finally, the partial amino acid sequence of the proteins from each of the eight peaks were determined by Edmond sequencing reaction and the results are listed below :-

```
Peak 1 :  DKFQFQFMIQCANLE
Peak 2 :  DKFQFGFMIQCANLG
Peak 3 :  DLFQFGGMIQCANLG
Peak 4 :  NLFQFQFMIQCANLG
Peak 5 :  NKFQFGGMIQCANLG
Peak 6 :  NLFQFGGMIQCANLG
Peak 7 :  DLFQFGFMIQCANLG
Peak 8 :  WIPQKAKNFEQFXNM
```

While these results clearly demonstrated the presence of multiple PLA_2 enzymes in crude venom, whether these PLA_2 enzymes were venom or not was not determined.

4.3 Aim : determining possible venom genes in Death Adder

The work of van der Weyden (2000), Chow (2000) and Sung (1997) clearly provided abundant evidence of the existence of PLA_2 genes in Death Adder. However, no nucleotide sequence data were determined at the time and thus, one of the aims of this project is to isolate and determine the nucleotide sequence of PLA_2 genes in Death Adder venom glands. While the mere determination of a nucleotide sequence cannot prove that the PLA_2 gene in question codes for a neurotoxins, it is believed that the provision of nucleotide sequence data is important for any subsequent projects for protein expression and neurotoxicity assays.

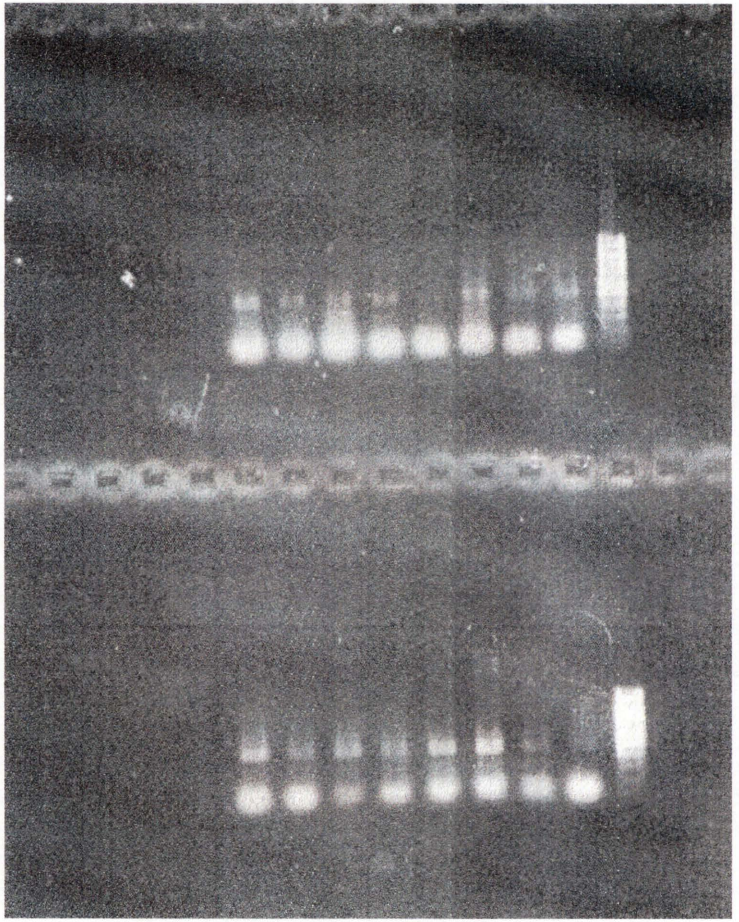
4.4 Methodology

The methods employed in this part of the project are the same as the methods in sections 3.2, 3.3, 3.6 to 3.11 for determination of neurotoxin genes (both PLA_2 and α-neurotoxins) in King Brown snake venom glands. In other words, the methods include screening the Death Adder cDNA library for genes of interest using Israel's method of PCR screening with the same primers used in sections 3.2 and 3.3; and screening for genes of interest using an optimised PCR protocol.

4.5 Results for Israel's PCR screening method

The PCR results from the pooled samples from the various rows and columns were analysed by gel electrophoresis on a 1.0% (w/v) agarose gel in TAE buffer; PCR products were stained by ethidium bromide and visualised by exposure to UV light. The results are shown in figure 4.3 below. A 100 bp ladder was also included in the sample wells as a standard in lane 1. Since monomeric venom PLA_2 enzymes isolated from Death Adder are similar in size to other monomeric venom PLA_2 enzymes from other Australian snakes, there was no reason to expect the genes to be significantly different either. Thus the expected product is 400 bp again since the same primers (NsPLA2 forward and reverse) were used and these primers should also be able to hydridise to 5' and 3' ends of the genes of interest, and effectively allowing entire genes to be sequenced.

Figure 4.3 – Results from Screening Death Adder venom gland cDNA library with Gene specific primers.



9 8 7 6 5 4 3 2 1

This is a 1.0% (W/V) agarose gel in TAE buffer visualized by ethidium bromide staining. The standards used were 100 bp ladder (Promega) and is found in lane 1 in both gels.

In the top gel, bands of the expected size were observed in lanes 2, 3, 4, 6, 8 and 9; and in the bottom gel, lanes 4, 5, 6, 7, 8 and 9 contained PCR products of the expected size (approximately 400 bp) as well.

PCR products of approximately 400 bp were observed in almost all the wells except in the fifth row of the top gel representing samples from Row 4. In the bottom gel, the bands from the second and third sample wells which corresponded to columns A and B were also significantly weaker when compared to other wells, and were also treated as negative results. The large amount of wells which tested positive for the presence of the PLA_2 gene indicated that the genes of interest were abundant in the cDNA library and could be readily sequenced from the library. However, as in section 3.4, the bacteriophage could not be further sub-cultured and this method of screening was abandoned.

4.6 Results for PCR approach for PLA_2 genes

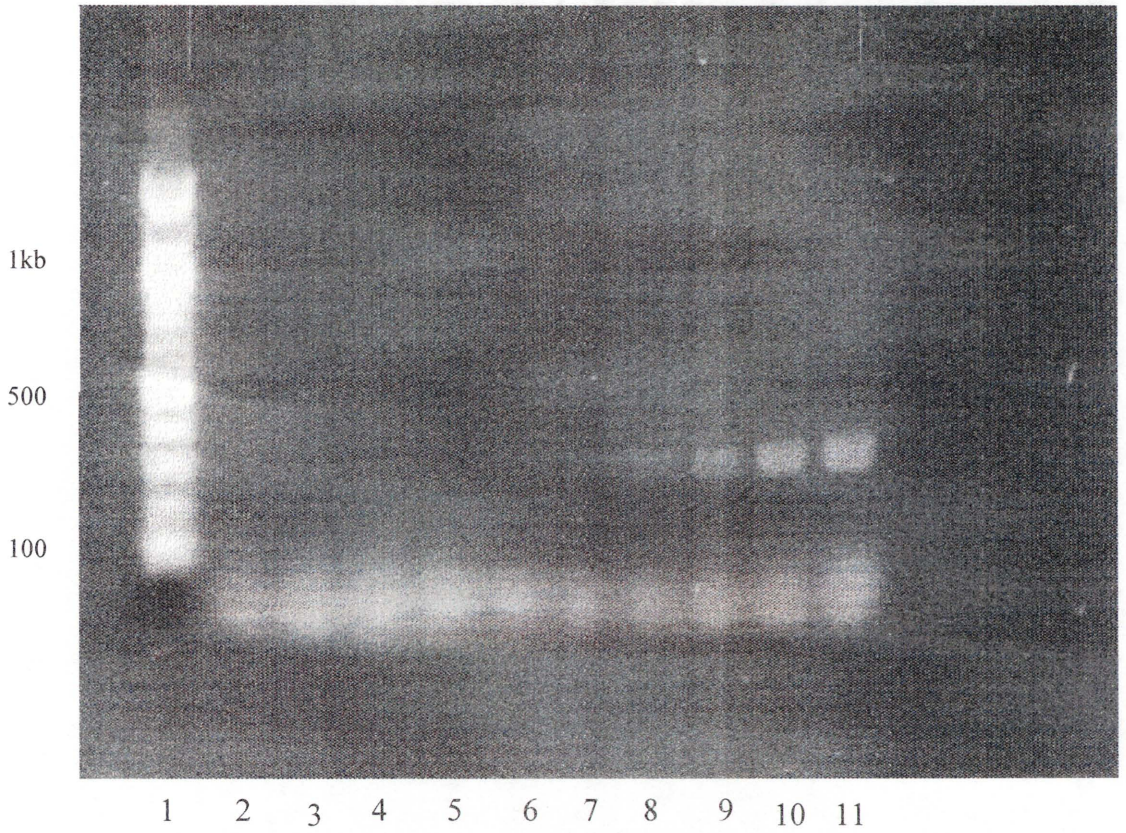
An alternative method to screen for genes of interest was to use PCR with optimised number of cycles to prevent any preferential amplification from occurring. As mentioned in section 3.5 and 3.6, this required an initial step of ascertaining the optimum number of cycles prior to PCR and the results for the optimisation experiments are shown in figure 4.4.

In figure 4.4, the products from PCR cycles 21 to 30 are shown in wells 2 to 11 and the first well contained a 100 bp ladder as standard. No PCR products were detected by visual inspection in wells 2 to 9 which indicated that the reaction was still possibly in the lag phase. However, it was observed that both cycles 29 (well 10) and 30 (well 11) produced high intensity bands at approximately 400 bp and this was the expected size of the PCR product. The bands in well 11 were slightly broader and more diffused, which could possibly indicate that at cycle 30, there were already some mis-priming of the primers occurring due to the increased concentration of template. For this reason, cycle 29 was chosen as the optimum number of cycles for PCR.

4.7 Results for Automated Sequencing

Following the same protocol as in section 3.14, after 29 cycles of PCR, the PCR product was excised and extracted from a 1.0% (w/v) agarose gel; then ligated into pGEM-T vector using the pGEM-T easy vector system (Qiagen). The vector was then precipitated and transformed into electro-competent DH5α *Escherichia coli* cells by electroporation.

Figure 4.4 – The Results for Optimizing the number of Cycles used for screening



This 1.0% (W/V) agarose gel in TAE buffer shows the PCR products obtained at different cycles of the PCR. Lane 1 contains the 100 bp ladder as a standard. lanes 2 to 11 contain pCR products from cycles 21-30 respectively.

Bands of the expected size were observed In lane 8 (cycle 27) and the strongest band was visible in lane 10 (cycle 30).

Thus, cycle 30 was the optimum number ff cycles for isolation of all PLA_2 genes in the cDNA library. Further amplification would result in more non-specific binding as shown in lane 11.

The transformed bacteria were then plated onto LB-ampicillin plates with Xgal and IPTG for blue/white colony selection and positive colonies (indicated by the white colour) were selected for automated sequencing.

Automated sequencing involved randomly selecting forty positive colonies (which was the same number used in 3.14) and inoculating the colonies into separate 10 ml aliquots of LB-ampicillin broth for amplification of the plasmid. The plasmid was then extracted from the cells by Fastprep plasmid purification system from Eppendorf (see section 3.10) and sent to AGRF for sequencing. Curiously, all forty colonies contained only a single nucleotide sequence which is set out in figure 4.5. In figure 4.5 the entire unknown nucleotide sequence (358 nucleotides) and its alignment with the neurotoxin gene - notexin is shown. It is noted that the entire unknown gene sequence could be aligned with notexin and the identity of the alignment was 83%, with 298 out of 358 nucleotides matching with notexin and thus indicating that this is indeed a PLA_2 gene.

4.8 Results of Protein Analysis

Since there are no nucleotide sequence data for PLA_2 genes in Death Adder, further analysis of the unknown sequence was based on the theoretical amino acid sequence translated from the nucleotide sequence. The translated nucleotide sequence yielded an amino acid sequence of 119 amino acid residues with fourteen cysteine residues. When the putative amino acid sequence was aligned with Acanthin I and II, two known PLA_2 sequences isolated from Death Adder (Chow *et al.*, 2000), it is observed that the cysteine residues align perfectly, indicating that there is a high probability that the fourteen cysteine residues in the unknown sequence also take part in the formation of the seven disulfide bridges which is a characteristic feature of the PLA_2 fold. The clustal alignment of the unknown sequence with Acanthin I and II is shown in figure 4.6. It is noted that 76 out of 119 amino acids (64% of the sequence) match perfectly with both sequences, but the unknown sequence is more similar to Acanthin II than Acanthin I, with an identity score of 66%.

Figure 4.5 - Novel PLA_2 gene for Death Adder

Notechis scutatus scutatus mRNA for phospholipase PLA2
Length = 621

Score = 234 bits (118), Expect = 1e-58
Identities = 298/358 (83%)
Strand = Plus / Plus

```
Query: 1   tctcaacctctatcagttcggcaaaatgattgaatgtgccaacaagggcagtcgaccttc 60
           ||||||||||||||||||||||||| |||||| |||||||||||| | |||||  ||||| |
PLA2 : 99  tctcaacctctatcagttcggcaacatgattcaatgtgccaaccatggcaggagacctac 158

Query: 61  tttggattatatgaactacggttgctactgcggcacagaagatcgcgggacaccggtgga 120
           ||||| ||||  | ||||||||||||||||||| ||| || | ||||||||||||||||
PLA2 : 159 tttggcttatgcggactacggttgctactgcggcgcaggaggtagcgggacaccggtgga 218

Query: 121 tgacttggataggtgctgcaaagcacatgacgactgctatgctgaagccgaaaaacatgg 180
           ||| |||||||||||||||||||||||| |||||||||| |||||||| |||| | ||
PLA2 : 219 tgagttggataggtgctgcaaagcacatgatgactgctatggtgaagccggaaaaaaagg 278

Query: 181 atgcttccccaagatgatgtcgtatagttggaaatgtgccaacaatgtgcccatctgcaa 240
           ||||| ||||| | ||| || |||||||||| |||||    | ||    |||| ||||||
PLA2 : 279 atgctaccccacgttgacgttgtatagttggcaatgtattgaaaaaacacccacctgcaa 338

Query: 241 ttcaaaaacgcagtgtcaacattttgtgtgtcattgtgacctctgggcaaccaagtgctt 300
           ||||||||||   ||| ||| || ||||||| |||||||   |   ||| ||||||||||
PLA2 : 339 ttcaaaaacgggatgtgaacgttctgtgtgtgattgtgatgccacagcagccaagtgctt 398

Query: 301 ttcccaagaaacttacaacaagaacaacttcggtatcgacaccgagaaacgttgccaa 358
           | || |||   |||||||||||||| |||| |  |||||||||||||||||||||||
PLA2 : 399 tgccaaagccccttacaacaagaagaactacaatatcgacaccgagaaacgttgccaa 456
```

This is the result from a database search of the unknown sequence (labelled Query) with other nucleotide sequences on the NCBI database.

The sequence which scored the highest with the unknown sequence was a PLA_2 gene from *Notechis scutatus scutatus*., scoring 83% in identity and proving that the unknown gene isolated was also a PLA_2 gene.

Figure 4.6 - Clustal alignment with Acanthins demonstrating unknown is Acanthin-like gene

```
                 1        10        20        30        40        50
Acanthin1        -DLFQFGGMIGCANKGARSWLSYVNYGCYCGWGGSGTPVDELDRCCQIHDNCYGEAEKKQ 59
AcanthinII       -NL QFGGMIQCANKGARSWLSYVNYGCYCGWGGSGTPVDELDRCCQIHDNCYGEAEKKR 59
Unknown          LNL QFGKMIECANKGSRPSLDYMNYGCYCGTEDRGTPVDDLDRCCKAHDDCYAEAEKHG 60
                  :*:*** ** *****:*. *.*:*******  . *****:*****: **:**.****:

                 60        70        80        90        100       110
Acanthin1        CGPKMT YSW CANDVPVCNDSKSACKGFVCDCDAAAAKCFAKAPYNKNNIGIGSKTRCQ 119
AcanthinII       CGPKMTLYSWECANDVPVCN SKSACEGFVCDCDAAAAKCFAKAPYNKNNIGIGSKTRCQ 118
Unknown          CFPKMT YSW CANNVPICN SKTQCQHFVCHCDLWATKCFSQETYNKNNFGIDTEKRCQ 119
                 * **** ***:***:**:** **: *: ***.**  *:***:: .*****:**.::.***
```

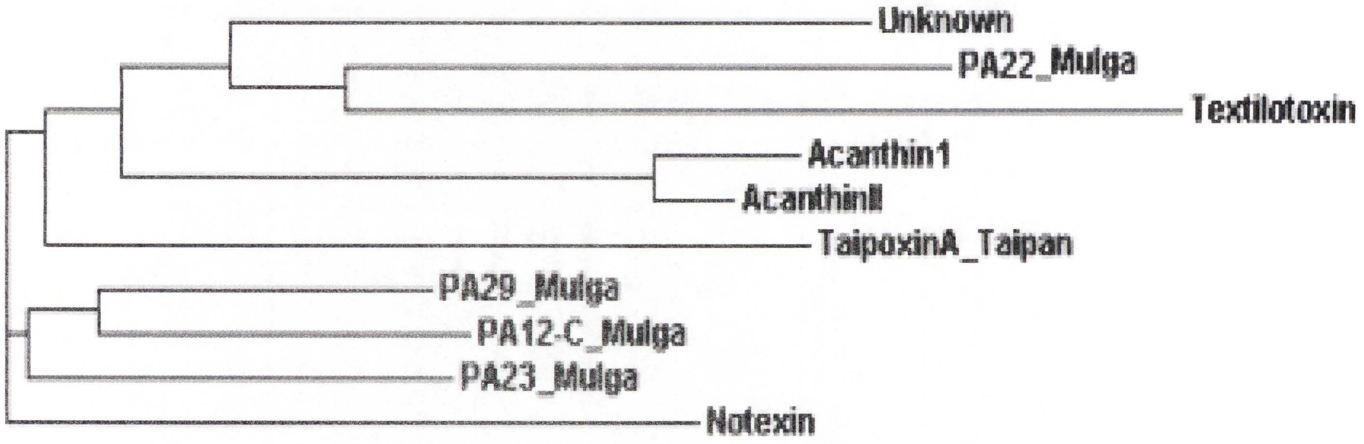
The above figure is a clustalW alignment of the unknown with the two highest scoring protein sequences – Acanthin I and II. A visual comparison of the novel putative protein to the two other protein sequences suggest the novel sequence is a hybrid protein of Acanthin I and II..

Comparisons with other amino acid sequences of known venom PLA_2 enzyme were also made with the unknown sequence. These were Textilotoxin (Brown snake), taipoxinA (taipan), Pa 29, Pa 22, Pa 12C and Pa 23 (King Brown) and Notexin (Tiger snake). A phylogram was constructed based on the clustal alignment of these sequences, but none of the amino acid sequences proved to be significantly more similar in primary structure than Acanthin II. In other words, amongst all the sequences, Acanthin II scored the highest at 66% identity. The phylogram is set out in figure 4.7 below.

Finally, there were also partial amino acid sequences isolated from Death Adder crude venom by Sung previously. Unfortunately the data is limited as sequencing by Edman degradation reaction could only determine 15 amino acid residues from the N–terminal from each sequence. Nonetheless, the alignment of those partial amino acid sequences with the first 15 amino acid residues from the N terminal of the unknown is shown below.

```
Sequence  1          DKFQFQFMIQCANLE
Sequence  2          DKFQFGFMIQCANLG
Sequence  7          DLFQFGFMIQCANLG
Sequence  4          NLFQFQFMIQCANLG
Sequence  5          NKFQFGGMIQCANLG
Sequence  6          NLFQFGGMIQCANLG
Sequence  3          DLFQFGGMIQCANLG
Acanthin_I           DLFQFGGMIGCANKG
Novel Sequence       NL QFGKMIECANKG
Acanthin_II          NLYQFGGMIQCANKG
                      : *** **:***:*
```

Figure 4.7 - Phylogram of Alignment with other proteins



Known protein sequences of the pre-synaptic neurotoxins were aligned with the novel sequence obtained and the differences are represented as a phylogram above. The degree of similarity is illustrated by the relative proximity between each protein. The phylogram suggests that the novel putative protein sequence is similar to PA22, Textilotoxin and Acanthin.

Interestingly, when the identity scores were examined, Acanthin II scored the highes identity at 66%, with a similarity score of 78%. The range of scores were 66% to 55%.

As can be seen from the alignment above, the unknown sequence has regions which match perfectly with the previously isolated sequences as well as a few residues which are not homologous. For example, in position 3, a deletion seems to have occurred in the primary structure, but interestingly, this deletion was also present in Acanthin II. In position 14, the unknown sequence contains the residue lysine which again is present in Acanthin but not the other sequences. Unique amino acid residues lysine and glutamic acid are observed in position 7 and 10. These two amino acids do not occur in any of the other sequences.

4.9 Screening Results for α-neurotoxin genes

The results here were similar to the results in section 3.22. In other words, the primers used were mis-primed to other irrelevant cDNA strands and generated a large number of false positive bands. Variation in PCR conditions did not yield any improvement to the results. Thus no valid results can be reported at this stage and no conclusions regarding α-neurotoxins could be drawn.

4.10 Conclusion

After sequencing forty colonies and yielding only a single nucleotide sequence, it was reluctantly concluded that no more sequences could be found. However, the novel sequence was significantly homologous to other venom PLA_2 genes and the putative amino acid sequence was also highly homologous to known PLA_2 enzymes. Thus, a novel PLA_2 gene was found in Death Adder venom gland, but whether this gene is a α-neurotoxin or not could not be determined given that no further tests were performed. Interestingly, there is a discrepancy between the large number of amino acid sequences previously found in Death Adder crude venom and the single gene found in this part of the project. While it is possible to suggest that this is due to the experimental procedure not being sensitive enough to isolate other PLA_2 genes, this suggestion can be refuted by the results from King Brown snake venom gland in which the same experimental procedure not only produced several novel sequences of PLA_2 genes, a novel group of genes were also isolated. Another explanation is that there may be large variation in the venom composition between individual snakes and further discussion is offered in the next chapter.

CHAPTER 5

Conclusions and Caveats

5.1 The screening results from King Brown snake venom gland cDNA library

As stated in sections 3.15 to 3.16, a total of ten novel long chain putative PLA_2 genes and five novel short chain PLA_2 genes were isolated from the King Brown cDNA venom gland library. The library was screened and tested to confirm that it contained a representative sample of the all messenger RNA in the venom gland.

With respect to the short chain PLA_2 genes, it is believed that these genes are translated into functional PLA_2 enzymes and are active components of King Brown snake venom. Although no neurotoxicity tests were performed to confirm their function, the perfect matching of codons from sequence 13 to the amino acid sequence of Pa 13 (a PLA_2 enzyme which has been confirmed to be a neurotoxin by Nishida *et al.*, 1985) strongly indicates that these genes are functional venom genes.

Furthermore, the presence of multiple genes is strong evidence that gene duplication has occurred in the King Brown snake during the course of its evolution. Although there is no direct evidence of accelerated evolution, the results of the screening tests support the proposition that accelerated evolution of the neurotoxin genes has occurred within this group of short chain PLA_2 genes as nucleotide substitution has occurred at specific sites rather than at random intervals in the exons.

However, it could be argued that the substitutions were not site directed but occurred as random mutations. The reason that they appeared site directed is that with random mutations, genes which are not functional were eliminated in the course of evolution or are no longer expressed (and thus no RNA were produced in either case). On the other hand, the presence of the large number of novel long chain PLA_2 venom genes from the results would not support this argument as these genes seemed to be expressed at the mRNA level but are not translated into functional proteins. This dilemma could be resolved if the long chain PLA_2 genes were pseudogenes but again there is insufficient evidence to demonstratively prove the long chain PLA_2 genes are, in fact, pseudogenes. Since these genes are expressed at the mRNA level and no definitive evidence that there are pseudogenes was found, it is concluded that they are genes with unknown functions.

Thus, in all likelihood, the substitutions noted in the results are products of the process of accelerated evolution in a site directed manner. The other interesting result was the novel nucleotide sequence 14, which contained a large number of substitutions. While it was tempting to propose that accelerated evolution in the form of nucleotide substitution is a highly controlled process involving changes only at a few regions (and thus conserving the overall structure and function of the original gene), the presence of the highly mutated sequence 14 further complicates matters. Perhaps sequence 14 was derived from a different PLA_2 gene originally during gene duplication. However, this is purely conjectural and no valid inference could be drawn unless a proper rooted tree analysis is performed.

Another interesting observation from the results is that the number of homologous genes found in the cDNA library was lower than the previously reported number of proteins isolated from crude King Brown venom i.e. while five novel PLA_2 genes were found in this project, ten different PLA_2 enzymes have been previously reported by various authors (Nishida *et al.*, 1985a, Nishida *et al.*, 1985ab, Takasaki and Tamiya, 1985, Rowan 1989, Takasaki *et al.*, 1990). The difference in the number of isomers can be reconciled if we take into account species variation within King Brown snake. It was most likely that the PLA_2 enzymes reported by the various authors relied on pooled venom samples, quite possibly from a number of snakes and were obtained from different regions and variation in venom composition from snakes from different region have been reported. However, a more detailed discussion of this aspect is covered in the following section as the same reasoning is applied to the results from Death Adder cDNA library.

With respect to the long chain PLA_2 genes. Their presence was unexpected as it was previously believed that genes which were non-functional due to accelerated evolution would be eliminated by some unknown mechanism. The existence of these genes suggest that the process of accelerated evolution can occur not only as point substitutions but also (possibly) as large additions and deletions resulting in drastic changes to a protein molecule. Furthermore, since these genes are produced at the RNA level, it seems at the DNA level, there has been no significant changes to the inducer region and signal peptide region of the genes, thus further demonstrating that accelerated evolution is a site directed process and not a random process.

The validity of the experimental results presented in this project is further supported by St Pierre *et al.*, (2006). Through the use of microarray hybridisation techniques, they were able to screen 621 transcripts from a cDNA library constructed from the venom gland of the Taipan snake *Oxyurans scutellatus*. While they reported a large number of novel sequences, including a large number of potential Pseudectoxin-like toxins. They were only able to isolate three isoforms of PLA_2 enzymes from the Taipan venom gland cDNA library. Further screening of the cDNA library with gene specific primers yielded eight isoforms. Most importantly, while five of the eight isoforms shared high homology with other known PLA_2 sequences, the remaining three had unusual characteristics, they were longer than the other PLA_2 amino acid sequences, and contained an addition of six amino acid residues at position 85 to 90. The residues of interest are lysine 85, proline 86, alanine 87, cysteine 88, asparagine 89 and tyrosine 90. While the inserts for the pseudogenes that were isolated in this project were glycine 79, proline 80, cysteine 81, tyrosine 82 and asparagine 83. In other words, it could be argued that the three novel sequences reported by Pierre *et al.*, were homologues of the ten pseudogenes reported in this project. Furthermore, it is noted that the number of novel PLA_2 gene sequences reported by Pierre *et al.*, were comparable to the number of sequences isolated in this project despite the differences in the number of clones sequenced.

5.1.1 The validity of the putative proteins

Throughout this project, all novel putative amino acid sequences were produced by translation from the nucleotide sequences. However, due to the binding site of the primers used, the signal peptide region was only partially obtained. In a personal communication by Professor Kini (2008), he warned that this may be important in the determination of some mature protein sequences, such as γ-taipoxin which have an 8-residue propeptide region. Fortunately, the lack of the entire signal peptide region does not impact on the process of translation from the nucleotide sequence into putative amino acid sequence, and does not affect the validity of the sequence homologies. It is, however, important to obtain the entire signal peptide region if further work is considered, especially if a functional mature protein is to be produced.

5.2 Definitive proof of accelerated evolution by sequencing the introns

Definitive proof that accelerated evolution has occurred in King Brown snake could be found by sequencing the intron segments and comparing the number of changes in the nucleotide sequence within the introns to the number of changes in the exons. In other words, by ascertaining the rate of mutation of the introns, the base rate of mutation of the relevant gene could be derived (the ds value). Without the added effects of accelerated evolution, the number of changes in the introns should exceed the number of changes in the exons significantly. This is because any mutation which occurs in the exon region may lead to undesirable changes in the protein structure and function, eventually leading to the elimination of the mutated gene from the gene pool. Thus only changes which conserve present function or those rare changes which actually have an added benefit would remain within the exon region. However, introns are not subjected to the same selective pressure and all random mutations should remain within the region. Therefore, by sequencing introns, it is possible to determine the base rate of mutation for the gene of interest. Then the rate of mutation (dn value) in the exons are compared to the base rate and if the changes exceed this base rate of random mutation (i.e. dn/ds is greater than 1), then accelerated evolution has occurred.

5.3 The Death Adder conundrum

While the screening procedure for King Brown cDNA library produced a large number of genes and pseudogenes, only a single PLA_2 gene was isolated using the same procedure on the Death Adder cDNA library. Furthermore, previous experiments on the protein content of the crude venom using HPLC suggested that Death Adder venom contained a number of PLA_2 genes (van der Weyden *et al.*, 2000, Chow *et al.*, 2000 and Sung 2001). Since the cDNA library was tested and screened to ensure that it was representative of the genes expressed by the venom gland at the time of harvesting, could the differences in the number of PLA_2 enzymes observed between the gene level and the protein level be reconciled? The conundrum could be resolved if intra-species variation were taken into account.

5.4 Intra-Species Variation

The venom composition between venomous snakes of the same species varied from one individual to another was observed by a number of authors (Furtado *et al.*, 2006, Mirtschin, *et al.*

2002, Sasa, 1999, Chippaux *et al.*, 1991). More recently, Daltry *et al* (1996) also documented venom variation (at a protein level) in the Pit viper, *Calloselasma rhodostoma*, when he compared pit vipers from 36 different sites in Southeast Asia; and Menezes *et al.*, (2006) reported variation amongst eighteen siblings in the pit viper based on sexual dimorphism. A number of hypotheses were offered to explain the forces driving the variation :- firstly, it was a function of geographical distance; secondly, it could be due to phylogenetic relationship amongst the population and lastly, it could be due to diet/prey availability (Sasa, 1999). Interestingly, Li *et al*, (2005) reported that the Marbled Sea snake - *Aipysurus eydouxii* which feed exclusively on fish eggs did not produce any venom. In fact, the α-neurotoxin that was expressed was truncated and resulted in a loss of biological activity. Thus illustrating that diet plays an important role in venom evolution. While it would be interesting to speculate which of these could be the primary reason for causing variation in venom composition, it is beyond the scope of this project. Thus, for the purposes of this project, it is suggested that all three factors could potentially contribute to the variation of venom within the same species. More importantly, when pooled crude venom is used in analysis of venom composition, in all likelihood, the pooled samples were not derived from a single individual milked over a long period of time, rather the venom from several individuals were milked and pooled to form the sample. Thus, this could account for an increase in the number of isoforms observed at the protein level.

Intraspecies variation in venom composition has also been observed in Death Adder. Fry *et al* (2002) obtained venom samples from New South Wales (Eden), Queensland (Gold Coast), South Australia (Eyre Peninsula) and Western Australia (Darling Range); and the various samples were fractionated and analysed by liquid chromatography-mass spectrometry (LC-MS). A large number of components were isolated using this sensitive method of detection and the results relating to potential PLA_2 enzymes are worth more detailed scrutiny. In Table 5.1 below, the results for putative PLA_2 enzymes found in the snakes from the four regions are listed, for the purposes of clarity, the results for other components have been edited.

Table 5.1 - Various PLA_2 enzymes from *A. antarcticus* from different regions of Australia

	NSW	QLD	SA	WA
Number of isomers	4	3	4	3
Molecular Size	1.2897128e+19	1.2841128e+14	1.2896129e+19	1.2898128e+14

A number of interesting points could be made about these results. Firstly, these results show clearly that there is intra-species variation in venom composition within Death Adder. For example, when comparing the results between NSW and QLD (and assuming the accuracy of LC-MS is approximately +/- 1 dalton), it is observed that snakes from NSW share 2 common PLA_2 enzymes to snakes from QLD, and have 2 unique PLA_2 enzymes. Secondly, and surprisingly, only the 13.8 kD PLA_2 enzyme seem to be common to snakes from all four regions. A few of the 12.8 kD PLA_2 enzymes could be found in 3 regions but are not common to all 4 regions. Thirdly, Fry *et al* (2002) confirmed the identity of these enzymes by N–terminal sequencing but did not report any homology to Acanthin, nor Acanthoxin (unfortunately, the actual sequences were not published and no comparisons could be made to the various N–terminal sequences isolated by myself in 2001; and lastly, Fry *et al.*, used pooled venom samples from different snakes from within the same region. Thus, these results support that geographical isolation could be a factor in causing venom variation, however, there was no evidence as to whether phylogenetic differences could also be a contributing factor. If phylogenetic difference is also a factor i.e. the genetic difference between each individual snake is a source of variation, then by pooling the venom, these differences cannot be observed. This leads to the related question of whether there are multiple PLA_2 genes in Death Adder.

5.5 Multiple venom genes or single copy?

Quite clearly, the results from chapter 4 indicate that there is only a single copy of PLA_2 gene in Death Adder as only a single nucleotide sequence was isolated from 40 clones, the validity of this result is supported by other research. Chuman *et al.*, (2000) constructed a cDNA library from the venom glands of a single mature *Naja naja kaouthia* snake and screened the library for group I PLA_2 enzymes using southern blot analysis. They found only two cDNA (NnkPLA-I and NnKPLA-II) were expressed. Similarly, Bharati *et al.*, (2003) constructed cDNA libraries from the venom glands of three different carpet vipers (*Echis pyramidum leakeyi*, *E. sochureki* and E. *ocellatus*). However, rather than using a single individual, each cDNA library was constructed

using the pooled total RNA from three individuals. When the cDNA libraries were screened for PLA_2 enzymes, they reported that only a single cDNA sequence was isolated from each library. These results support the proposition that not all PLA_2 venom genes undergo gene duplication and in some cases, PLA_2 genes can and does exist in low copy numbers.

5.6 Further work

While we have offered some experimental results and research material demonstrating that the PLA_2 gene in Death Adder may exist in low or even as a single copy (which may vary from individual to individual depending on the location), there is no definitive proof of whether this is necessarily true. Ideally, the crude venom from a single Death Adder could be collected over time and analysed by LC/MS and the PLA_2 components within the venom is then compared to the PLA_2 gene sequences obtained from a cDNA library of the same individual.

Furthermore, it is possible that Death Adders have not relied on PLA_2 enzymes as the main agent for incapacitating their prey as they have distinctly different hunting habits to King Brown snakes. Thus, the PLA_2 gene(s) in Death Adder have not been subjected to the same evolutionary forces as those found in King Brown snakes. Rather, Death Adders may rely on the neurotoxicity of the α-neurotoxins in their venom and these toxins might be undergoing accelerated evolution and gene duplication. Thus, further work on studying α-neurotoxin genes would be justified.

5.7 Proper Phylogenetic Study

The screening results from chapter 3 produced fifteen novel sequences which are homologous to each other and are clearly related to each other in terms of evolution and function. However, the analysis provided was strictly a study in sequence similarities. While the graphs indicated the similarities of the sequences to each other, they do not suggest any proximity in evolutionary terms. In order to demonstrate the evolutionary relationship to each other and to other known PLA_2 venom genes, a proper rooted tree analysis must be performed. Dr Fry, in a personal communication (2008) suggested that to accurately reconstruct the molecular evolution of these venom genes, the rooted tree analysis would have to be based on a large number of replicates. He proposed that the number of replicate should be 1000, 10000 or 1 million based on the technique of choice (maximum parsimony, neighbour joining or Bayesian respectively). Unfortunately, this

is far beyond the limited resources available for this masters project.

5.8 Concluding remarks - a cautionary tale

In the introductory chapter, it was stated that snake venom is a complex mixture of toxins and accelerated evolution and gene duplication have contributed directly to this complexity. Since multiple isomers of PLA_2 enzymes have been found in both King Brown snakes and Death Adders, it was tacitly accepted that PLA_2 genes in Death Adders and King Brown were undergoing accelerated evolution and thus, the aim of this project was to isolate all the venom genes in Death Adder and King Brown snake in order to demonstrate the processes of accelerated evolution and gene duplication do occur amongst Death adders and King Brown snakes.

While the results from King Brown snake cDNA library has offered strong evidence of gene duplication and accelerated evolution in King Brown snake, a contrary result was found in the Death Adder cDNA library. Whether accelerated evolution does or does not occur in Death Adders, it seems the use of pooled venom samples in venom composition research projects coupled with intra-species variation of venom composition have led to the misconception that isolation of multiple isomers of a PLA_2 or α-neurotoxin protein is evidence for accelerated evolution and gene duplication.

The End

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