Chapter 1

Introduction and literature review

1.1 INTRODUCTION

Animal venoms are an evolutionary adaptation to immobilise and digest prey and are used secondarily as a defence mechanism (Tu and Dekker, 1991). Intriguingly, evolutionary adaptations have produced a variety of venom proteins with specific actions and targets. A cocktail of protein and peptide toxins have varying molecular compositions, and these unique components have evolved for differing species to quickly and specifically target their prey. The compositions of venoms differ, with components varying within the toxins of spiders, stinging fish, jellyfish, octopi, cone shells, ticks, ants and snakes.

Toxins have evolved for the varying mode of actions within different organisms, yet many enzymes are common to different venoms including L-amino oxidases, esterases, aminopeptidases, hyaluronidases, triphosphatases, alkaline phosphomonoesterases, phospholipases, phosphodiesterases, serine-metalloproteases and Ca^{2+}/Mg^{2+}-activated proteases. The enzymes found in venoms fall into one or more pharmacological groups including those which possess *neurotoxic* (causing paralysis or interfering with nervous system function), *myotoxic* (damaging muscle), *haemotoxic* (affecting the blood, especially those that affect blood clotting), *haemorrhagic* (causing damage to blood vessels and so cause bleeding), *haemolytic* (causing damage to red blood cells), *nephrotoxic* (damaging the kidney), *cardiotoxic* (affecting the heart) or *necrotoxic* (causing death of tissue) activities. Since these toxins block specific physiological processes, through understanding of the mechanism of actions, may lead to the development of useful biochemical and pharmaceutical tools or give an insight into molecular mechanisms (Narahashi, *et al.*, 1964; Wang, *et al.*, 2001b; Wang, *et al.*, 2001c).

Given the diversity of venom structure and function it should not be surprising that natural toxins may prove to be useful drugs or may be used as a template for drug development (Lewis and Garcia, 2003). Research of animal venoms is often undertaken

to isolate and identify components with potential use as diagnostic agents, for medical therapies, or to use as aids to understand normal physiological function such as coagulation or membrane receptor sites (Narahashi, *et al.*, 1964). The approaches taken to develop drug leads from bio-active peptides and proteins are being applied to many hundreds of venomous compounds from species including spiders, scorpions, marine snails, bees, ants, and jellyfish and specifically within this research project, snakes.

There is an increasing number of examples of the identification and development of venom proteins into pharmaceuticals. The toxins isolated from cone shell venom contain a large variety of small peptides. These peptides are highly specific for targets in the neuronal and muscular systems of their prey: these include highly specific sodium, potassium and calcium channel blockers (Lewis, *et al.*, 1996). An example of a toxin's action and possible applications of this is seen in the cone shell toxin MVIIA, which targets voltage-gated calcium channels found largely in sensory organs (Bowersox, *et al.*, 1996). A synthetic derivative of this small peptide has now been approved by the American FDA for the treatment of intractable pain. Other toxins that block n-methyl-d-aspartate (NMDA) receptors (Zhou, *et al.*, 1989) may enter medical practice as anticonvulsants or as therapy for stroke.

Other experimental applications of venoms are being explored with venom from the Australian snakes *Notechis scutatus* (tiger snake: notexin) and *O. s. scutellatus* (taipan: taipoxin) to study muscle regeneration (Harris, 1975; Harris, *et al.*, 1977). Furthermore a cardiac calcium channel blocker from taipan venom, taicatoxin (Possani, *et al.*, 1992; Fantini, *et al.*, 1996), has become recognised as a specific Ca^{2+} -activated potassium blocker on chromaffin cells (Doorty, *et al.*, 1997). A plasmin inhibitor isolated from *N. scutatus* venom (Willmott, *et al.*, 1995) is being trialed as a possible treatment for the prevention of post-surgery haemorrhage. An example of the successful development of an antihypertensive drug now used in humans comes from the South American lance-headed viper (*Bothrops jararaca*). This snake's venom contains inhibitors of the angiotensin-converting enzyme (ACE) (Camargo, *et al.*, 2000).

The identification, isolation and a subsequent understanding of the structure-function properties of proteins within the venom were essential for development of the non-

peptide ACE inhibitors now in widespread use e.g., 'captopril', 'enalapril' and 'lisinopril' (Ernst and Harrison, 1999).

Snake venoms have evolved to be specialised for either mammals, birds, frogs or other reptiles. Australia harbours about 157 snake species of which 96 are venomous (Wilson and Swan, 2003). In Australia there are about 3,000 human snake bites per year, of which 200 to 500 receive antivenom. On average only one or two human snake bites per annum are fatal (Sutherland and Tibballs, 2001). Australian snakes, with few exceptions, belong to the family Elapidae and are listed in the top ten most venomous snakes of the world (Broad, *et al.,* 1979; Fry, 1999) (for phylogeny see Figures 1.1,1.7 and 1.8).

The genus *Oxyuranus* consists of three of the largest and most feared Australasian snakes, and are listed within the top three of the world's most venomous snakes, one bite producing enough venom to kill 50,000 mice. European settlers came to fear the taipan (*Oxyuranus scutellatus scutellatus*); one early report stating that while four out of seven settlers bitten by *Acanthophis antarcticus* (death adder) recovered, all 12 settlers bitten by 'brown snakes' (*O. s. scutellatus*) had perished (Garde, 1890). The Papuan taipan (*Oxyuranus scutellatus canni*) has also long been feared by Papua New Guinean natives as a snake whose bite was fatal (O'Shea, 1996). On the other hand, the inland taipan (*Oxyuranus microlepidotus*)[1] was virtually 'lost' to science and did not receive wide recognition until live animals were 'rediscovered' in 1974; the venom appearing to be more lethal than any other described snake venom (Covacevich and Wombey, 1976).

The *Oxyuranus* species have been reported to be closer to those of each other than to those of any other large Australian elapid (Sutherland and Tibballs, 2001). Despite the similarities between the species comparative differences in the proportions of venom components have been reported. A distinction in the clinical presentation of envenomed patients and antivenom efficacy have also been described between species (Broad *et al.*, 1979). The effects of the venoms from the three species are primarily neurotoxic.

[1] (Note for future annotation: *O. s. scutellatus* may be taken to mean the Australian species, the coastal taipan or *Oxyuranus scutellatus scutellatus* (OS in figures), *O. microlepidotus* to mean the inland taipan or *Oxyuranus microlepidotus* (OM in figures), and *O. s. canni* to mean the Papuan subspecies *Oxyuranus scutellatus canni* (OSC in figures)).

These venoms also cause coagulant and myotoxic effects, common to many Australian snakes. The symptoms of most bites by *Oxyuranus* species tend to follow a common pattern. The classic early non-specific symptoms of elapid envenomation include headache, vomiting and/or abdominal and/or lymph gland pain. Laboratory investigations may also confirm symptoms such as coagulopathy, haemolysis, rhabdomyolysis or renal failure. Specific signs and symptoms of systemic envenomation include facial, bulbar and /or peripheral neurotoxicity, clinical evidence of bleeding, convulsions or difficultly in breathing and/or swallowing (Sutherland and Tibballs, 2001). There have been few case reports of bites by *O. microlepidotus*; to date all envenomations from this species have involved either amateur or professional herpetologists (Pearn *et al.*, 1994). The techniques used to provide evidence of envenomation varies dependant upon the resources available. Limited resources in Papua New Guinea have resulted in a dependence upon observable symptoms, whereas clinical medicine within Australia places more reliance upon laboratory evidence. Although the standards of proof are different, the results enable a solid comparative summary of the envenomation of the three *Oxyuranus* species (Table 1.1).

Comparative electrophoretic studies have been conducted with the venoms from *O. s. scutellatus* and *O. microlepidotus*, and indicated significant differences exist between the components of these venoms (Broad *et al.*, 1979a). Further studies have shown that *O. microlepidotus* venom has a higher hyaluronidase activity compared to *O. s. scutellatus* venom. *In vitro, O. microlepidotus* possesses post-synaptic neuromuscular activity blocking smooth muscle contraction and vasorelaxation activities, and *in vivo*, produces transient respiratory and cardiovascular collapse (Bell *et al.*, 1998 and 1999). Another study demonstrated the venom was slightly less potent than that of *O. s. scutellatus* in reducing the nerve-mediated twitch of chick biventer cervicis muscle preparations (Crachi, *et al.*, 1999a). Although CSL antivenom neutralised the effects of their respective presynaptic neurotoxic (paradoxin and taipoxin) and the post-synaptic activity of *O. s. scutellatus* venom, the post-synaptic activity of *O. microlepidotus* venom was not neutralised.

Significantly, the experimental design of this study bore no resemblance to the physiology of envenomation. Firstly, muscle preparations were avian not mammalian. *Oxyuranus* species prey on mammals and subtle prey-specific evolution may cause

differences in toxin binding specificity between avian and mammalian receptor sites (Harris and Maltin, 1982). Secondly, few patients receive antivenom before they are bitten; chick biventer cervicis muscle preparations were saturated and incubated with antivenom to which venom was added. The design failed to take into account the latency period that toxins, (eg taipoxin) undergo before irreversible neurotoxicity occurs. A study based on rescue experiments with envenomed mammals (ie: > 4 hours) may have produced vastly different results that more closely reflected clinical reality.

Electrophoretic comparisons between the venoms of *O. s. scutellatus* and *O. s. canni* also indicated some variations between venom components (Sutherland, 1979). In one series of *O. s. canni* envenomations 77% of cases resulted in incoaguable blood after a 20WBCT test (if whole blood in a glass tube has not coagulated within 20 minutes the blood is considered incoaguable) with systemic bleeding from gingival sulci, nose, bite and venipuncture sites (Lalloo *et al.*, 1995). Defibrinogenation with depletion of coagulation factors has also been reported following *O. s. canni* envenomation (Trevett *et al.*, 1994; Lalloo *et al.*, 1995b; Lalloo *et al.*, 1997; Sutherland and Tibballs, 2001). These symptoms have not commonly been reported after *O. s. scutellatus* envenomation.

It has also been shown that the antivenom, produced using *O. s. scutellatus* venom may not be as effective in bite victims of *O. microlepidotus* or *O. s. canni* (Lalloo *et al.*, 1995a; Trevett *et al.*, 1995; Southern *et al.*, 1996; Crachi *et al.*, 1999a; Currie, 2000). Experiments have shown that although *O. s. scutellatus* antivenom neutralised *O. s. canni* venom, it did not neutralise it as rapidly or efficiently as for *O. s. scutellatus* envenomations (Currie *et al.*, 1991). In response to this finding it is advised that more antivenom is needed to treat for *O. s. canni* envenomations (Currie *et al.*, 1991).

Research has also shown that no significant neutralisation of *O. s. scutellatus* venom could be effected using antivenoms raised against venom from the two largest Australian elapidae groups, the tiger snakes (*Notechis*) and brown snakes (*Pseudonaja*) (Morgan, 1956; Coulter *et al.*, 1978; Broad, *et al.* 1979). The apparent distinction of venom components from other Australasian elapid species and the substantial differences between venom of Australian snakes and those from overseas venomous

viperid and crotalid snake families, also make *Oxyuranus* a good candidate and source from which to find novel compounds.

To understand the physiological basis underlying the functional activities of these venoms, and to isolate and identify potential peptides of interest, a consistent venom source and a basic understanding of these proteins is required.

To date, the majority of components in the venom of the taipan have not been characterised and little molecular research has been undertaken on *Oxyuranus* species. The amino acid sequences of only ten proteins from *O. s. scutellatus*, seven from *O. microlepidotus* and three from *O. s. canni* have been submitted to SWISS-PROT databases (ExPASy http://au.expasy.org/cgi-bin/sprot-search-de?*Oxyuranus*). This number represents a small proportion of possibly hundreds of proteins within each of these venoms not yet described. Due to the number of peptides as yet undiscovered, there is much exploratory work that can be conducted.

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This project was a comparative study designed to substantially build upon previous research into venom of *Oxyuranus* species. This study was primarily conducted utilising fundamental proteomic tools including chromatography, one and two dimensional gel electrophoresis (2DE) mass spectrometry (O'Farrell, 1975; Wilkins, *et al.*, 1999) and N-terminal sequence determination. In addition, a cDNA expression

library was constructed using mRNA from a venom gland of a coastal taipan. This library was screened with taipan antivenom.

The steps following were considered necessary as part of the quality control process to ensure scientifically valid and consistent results.

1. the snakes used in this study were professionally identified,
2. the snakes used were maintained in captivity and therefore their history, environment and feeding habits were regulated and known and
3. most significantly, fresh venom samples from a consistent source were stored and used under standard laboratory conditions. Previous research has been conducted using lyophilised commercial venoms. Potentially, commercial venoms are isolated from varying sources and labels often report to mix species venoms within a sample.

1.2 LITERATURE REVIEW

1.2.1 Biology, ecology and distribution of *Oxyuranus*

The name *Oxyuranus* stems from the Greek words Oxus; sharp pointed, Oura; the tail and Anus (Latin); belonging to, meaning it has a long pointed tail. *Scutellatus* is derived from Scutellata (Latin); a chequered garment (Sutherland and Tibballs, 2001), *Microlepidota*; small eyed and *Canni* named in honour of Cann, the then curator of reptiles at the Taronga Park Zoo (Slater, 1956)(Figure 1.1) Appendix I lists *Oxyuranus* within current accepted phylogeny, and groups snake families and genera for quick reference of phylogenetic relationships. Appendix II lists the common and scientific names of Australian snakes.

All taipan species are large; females of each species reach similar maximum sizes with mean snout-vent length of about 145 cm, but the male *O. s. scutellatus* and *O. s. canni* can grow much larger than male *O. microlepidotus* (mean snout-vent length of *O. s. scutellatus* is 156 cm, and average length of *O. s. canni* of 175-250 cm and mean

snout-vent length of *O. microlepidotus* 132 cm). Mid-body scalation is: *O. s. scutellatus* 21 or 23, and *O. s. canni* 21-23, *O. microlepidotus* 23, rarely 25; anal: single, single, single, ventrals: 220-248, 220-250, 211-224, subcaudals: 48-76 paired, 48-80 paired, 54-66 paired. *O. scutellatus* colouring ranges from unmarked light olive to dark russet brown dorsally; specimens from the Tully area, North East Queensland are almost black, and the head is usually lighter coloured (Cogger, 2000, Shine and Covacevich, 1983)(Figure 1.2). *O. canni* differs in colour from *O. s. scutellatus* from brown to black with an orange streak down the back (Figure 1.3), and some differences in skull characteristics. *O. microlepidotus* colours vary from pale to very dark brown dorsally, often with dark flecks (Figure 1.4). The head can be glossy black in wild specimens and lighter in captive specimens (Queensland Museum and (O'Shea, 1996). This species shows some morphological resemblance to the *Pseudonaja* species (Figure 1.5).

Figure 1.1 Classification of Genus *Oxyuranus*.

CLASS	Reptilia
ORDER	Squamata (Snakes and Lizards)
SUB-ORDER	Serpentes (Snakes) Suborder Ophidia (Serpents)
SUPERFAMILY	Xenophidia (Colubroidea = Caenophidia)
FAMILY	Elapidae/Hydrophiidae (Elapid: front fanged [proteroglyphous] venomous snakes)
GENUS	*Oxyuranus*
SPECIES	scutellatus (Peters 1867): Australian coastal taipan microlepidotus (McCoy, 1879):(Australian inland taipan)
SUBSPECIES	scutellatus canni (Slater, 1956); (Papuan taipan)
AETIOLOGY	*Oxyuranus* - Oxus(Greek) sharp pointed; Oura (Greek) the tail; Anus (Latin) belonging to, it has a long pointed tail *scutellatus* - scutellata (Latin) a chequered garment *microlepidotus* - "small scaled" *canni* - Named after Australian herpetologist George Cann Sr

Figure 1.2: Coastal taipan (*O. s. scutellatus*) photograph courtesy of Mr D. Williams.

Figure 1.3 Papuan taipan (*O. s. canni*), photograph courtesy of Mr D. Williams.

Figure 1.4: Inland taipan (*O. microlepidotus*) photograph courtesy of Mr D. Williams.

Figure 1.5: Brown snake (*Pseudonaja*), photograph courtesy of Mr D. Williams.

Populations of *O. s. scutellatus* and *O. microlepidotus* are biogeographically separated by distances of more than 750 kilometres. *O. microlepidotus* is found in dry, arid, ashy downs areas of Australia from the Channel Country, south-western Queensland and north-eastern South Australia and live in burrows or cracks in the ground. *O. scutellatus* occurs in and around open forests, dry closed forests, coastal heaths, fore-dunes and cultivated areas of northern and eastern Australia. *O. canni* occurs in lowland areas of southern coastal West Papua (Irian Jaya) and eastwards across southern Papua New Guinea through areas of savannah woodland, grassland and

open forests. This species often frequents urban and rural vegetable gardens in conditions that are often more humid, wet and forested (see Figure 1.6).

Figure 1.6: Geographical locations of *Oxyuranus*.

Map courtesy of Mr D. Williams. Map data sources: Data for 52 *Oxyuranus microlepidotus* (—) and 198 *Oxyuranus scutellatus* (—) came from the records of: West Australian Museum, Northern Territory Museum, Queensland Museum, Peter Mirtschin (Venom Supplies). Data for 137 *Oxyuranus scutellatus canni* (—) compiled from the National Museum & Art Gallery of PNG and David Williams field records from 2001-2004.

1.2.2 Clinical presentation after envenomation by *Oxyuranus*

Responses to envenomation may vary from case to case, although most *Oxyuranus* species snakebites tend to follow a common pattern. The classic early non-specific symptoms of elapid envenomation include headache, vomiting and/or abdominal and/or lymph gland pain. Specific signs and symptoms of systemic envenomation include facial, bulbar and /or peripheral neurotoxicity, convulsions, or difficultly in breathing and/or swallowing. Laboratory investigations often reveal clinical evidence of bleeding, confirming symptoms such as coagulopathy, haemolysis, rhabdomyolysis or renal failure (Sutherland and Tibballs, 2001).

The following case reports are a general reflection of taipan envenomation but are by no means a finite description of envenomation of these species. Case studies are presented for *O. s. scutellatus* and *O. canni*. There have been few case reports of bites by *O. microlepidotus*; to date all envenomations from this species have involved either amateur or professional herpetologists. In a survey conducted of 28 Queensland herpetologists four reported they had been bitten although no clinical information was provided (Pearn, *et al.*, 1994). Cases of *O. microlepidotus* envenomation report convulsions and at times up to four weeks to recover from a bite. A summary of clinical symptoms and values is shown for *Oxyuranus* envenomation in Table 1.1.

Case Reports:

O. s. scutellatus

'On 10 April 1980 a 39-year-old tobacco farmer presented to Mareeba Hospital, Queensland, with a one hour history of nausea and vomiting. The morning before admission he had been out on his farm, working barefoot. Six hours after admission he developed fixed dilated pupils and bilateral ptosis. Progressive muscle paralysis developed rapidly, involving the respiratory muscles, and necessitated intubation and artificial ventilation. He was transferred to Cairns Base Hospital. On arrival at Cairns Base Hospital he was totally paralysed. Pupils were fixed and dilated, tone was flaccid and tendon reflexes were absent. He was febrile; his pulse was 90 bpm, and bp 170/115 mm Hg. Investigations of admission demonstrated a marked leucocytosis, and bleeding studies showed a severe coagulopathy. On the evidence it was felt that snake bite was the most likely diagnosis, occurring between 6 am and 9 am that day, unnoticed by the

patient. There were no obvious puncture marks. He was given four units of polyvalent antivenom, after blood and urine samples had been taken for specific venom assay. Over the next twelve hours he became hypertensive and oliguric. Further investigation the next day showed a markedly elevated creatine phosphokinase (19,600 IU/L) and reversal of his coagulopathy. He was maintained for 19 days on mechanical ventilatory support, hyper-alimentation and peritoneal dialysis, being discharged with no residual problems 27 days after admission.' taken from Sutherland and Brigden (1981) in Sutherland and Tibballs, 2001.

O. canni

'A woman (52) presented at a rural Health Sub-Centre at 12.45 pm. She had seen a bandicoot running through grass in front of her, and a " long, pale black snake" chasing it had bitten her on the leg. Two bleeding puncture marks were found. A standard 20 minute whole blood clotting time (20WBCT) test at 1.25 pm clotted within 15 minutes. An intravenous line was established and the patient placed under observation. At 9.30 pm bilateral ptosis, dysarthria and diplopia were noted and she was premedicated with 25 mg IV Phenergan and 0.25 ml SC adrenaline. Tachycardia (128 bpm) and bulbar paralysis developed, RR dropped to 13 per minute. Although drowsy, she spat out bloodstained saliva when roused. Infusion of one ampoule of CSL polyvalent antivenom was commenced at 9.45 pm and she was referred by road to a larger health centre. On arrival at 2.30 am, pronounced bilateral ptosis, diplopia, bulbar paralysis and conspicuous bleeding from the gums were observed. Tachycardia (116 bpm persisted, with BP 130/90, and RR of 24 per minute. She was unresponsive to verbal commands and became comatose. Further premedication was followed by two ampoules of CSL polyvalent antivenom, commencing at 3.00 am. Suction was used to clear airways. At 4.50 am she stopped breathing with no discernible peripheral pulse or heart beat, fixed and dilated pupils, and cold extremities. Death was pronounced at this time.' (Williams and Bal, 2003).

The techniques used to provide evidence of envenomation varies dependant upon the resources available. Limited resources in Papua New Guinea have resulted in a dependence upon observable symptoms, whereas clinical medicine within Australia places more reliance upon laboratory evidence. Nevertheless, although the standards of proof are different, the results enable a solid comparative summary of the envenomation of the three *Oxyuranus* species. The effects of the venoms from the three species are

primarily neurotoxic. These venoms also cause coagulant and myotoxic effects, and can be found in many Australian snakes.

Neurotoxins, common to most venoms, are often PLA_2s and may differ from venom to venom or within a venom, in which several forms may be present or they may show differing substrate preferences but all can disrupt neuronal conduction, neuromuscular junction transmission or the contractile responses of muscles. As paralysis increases, vital centres in the brain fail due to lack of ventilation and oxygen (Strong, *et al.,* 1976; Lee, 1982; Balass, *et al.,* 1997).

Severe taipan envenomation may often result in disseminated intravascular coagulopathy (DIC). DIC is a serious clinical syndrome associated with the potent prothrombin activator in *Oxyuranus* species venoms. DIC occurs when the blood clotting mechanisms are activated throughout the body instead of being localised to an area of injury. Small blood clots form throughout the body, and eventually the blood clotting factors are used up and not available to form clots at sites of tissue injury. Clot dissolving mechanisms are also increased. This disorder is variable in its clinical effects, and can result in either clotting symptoms or, more often, bleeding (Lalloo, *et al.,* 1995b). Bleeding can be severe. Only mild coagulopathy has been reported for *O. microlepidotus* envenomation (Sutherland and Tibballs, 2001).

Myolytic activity is present in varying degrees in many terrestrial and marine Australian snake venoms. The myotoxic breakdown of muscle fibres results in rhabdomyolysis; the release of muscle fibre contents into the circulatory system. When the skeletal muscle is damaged, myoglobin, an oxygen-binding protein pigment found in the skeletal muscle, is released into the bloodstream and is filtered out of the bloodstream by the kidneys. Myoglobin may occlude nephrons within kidney, causing damage such as acute tubular necrosis or kidney failure.

As well as releasing myoglobin the damaged muscle loses enzymes, including creatine (phospho) kinase (CPK, CK) (Mebs, 1986). Necrotic (dead tissue) skeletal muscle may cause massive fluid shifts from the bloodstream into the muscle, reducing the relative fluid volume of the body and leading to shock and reduced blood flow to the kidneys. Some of these are toxic to the kidney and frequently result in kidney damage

leading to myoglobinuria. Nephrotoxicity usually follows myoglobinuria due to severe rhabdomyolysis.

Table 1.1: Summary of clinical signs and symptoms of *O. s. scutellatus*, *O. microlepidotus* and *O. s. canni.*

Clinical signs and symptoms of envenomation of *O. s. scutellatus* (OS), *O. microlepidotus* (OM) and *O. s. canni* (OSC) taken from: (Lester, 1957; Trinca, 1969; Sutherland, 1975; Mirtschin, *et al.* 1984; Lalloo, *et al.* 1995; Southern, *et al.* 1996; Cobcroft, 1997; Barrett and Little, 2003; Williams and Bal, 2003)

Symptoms	**OS**	**OM**	**OSC**
Headache	√	√	√
Vomiting	√	√	√
Lymph node pain	√		√
Abdominal pain.	√	√	√
General weakness	√	√	√
Collapse	√	√	√
Bleeding from the mouth			√
Vomiting blood and/or blood in stool	√	√	√
Tachycardia		√	√
Hypotension	√	√	√
Hypertension	√		√
Neurotoxic symptoms			
Ptosis	√		√
Dysarthria	√	√	√
Dysphagia			√
Diplopia	√		√
Dyspnoea	√	√	√
Opthalmoplegia	√		√
Signs			
Swelling at bite site			
Facial, bulbar and /or peripheral neurotoxicity	√	√	√
Difficultly in breathing and/or swallowing	√	√	√
Convulsions/ altered consciousness	√	√	√
Clinical evidence of bleeding; 20WBCT >20min, PT>25sec	√	√	√
APPT > 35sec	√	√	√
Rhabdomyolysis	√		√
CK > 270U/L	√	√	√
Creatine > 53 umol	√		√
Platelets < 150 x 10^9/L	√	√	√
Fibrinogen depletion	√		√
Antivenom (MV; monovalent, PV; polyvalent)	3-4 amp PV 4 amp MV	7 amp MV 1 amp PV	1-4 amp PV
Required intubation &/or ventilation	√	√	√

Normal ranges: Prothrombin time (PT): 11-12 sec, Activated partial prothrombin time (APTT): 37-42 sec, Platelet count (Plt): 150x10^9-500x10^9/L, Creatine kinase (CK) 22-269 U/L Or 0.8-1.2 IU, Creatine 8-53umol

Coagulopathy of *O. s. scutellatus* and *O. s. canni* are shown to be comparable to the coagulopathy effects of *Pseudonaja*. There is currently much research being undertaken regarding the medical importance of the *P. textilis* prothrombin activator (Masci, *et al.*, 1988, Masci, *et al.,* 1990, United States Patent Application 20040208205). The prothrombin time for *O. s. scutellatus* and *Pseudonaja* has been reported up to 1 to 6 hrs (normal PT 11-12 sec) although bleeding from gingival sulci, bite sites and venipuncture wounds have not been described within *O. s. scutellatus* envenomation. The activated partial prothrombin times were comparable between the species (normal APPT: 27-42 sec vs *Pseudonaja and scutellatus* 127-160 sec), as were the platelet counts (normal Plt: 150-500x10^9/L vs 89-92x 10^9/L) and creatine (normal creat: l8-53umol vs 210-460 umol). Creatine kinase levels were measured for *O. s. scutellatus* only in literature cited (normal CK: 22-269 U/L vs up to 7000U/L) supporting the finding of myotoxicity by Harris (Harris, *et al.*, 1976) (Harris and Cullen, 1990). As yet there have been no reports available comparing and identifying specific protein differences between *Oxyuranus* venoms that may cause the varying toxicity or systemic coagulopathies described.

1.2.3 Present state of knowledge of *Oxyuranus* venoms-summary

The study of Australian snakes dates back to the 1890s when initial morphological data was published (Thomson, 1933). In 1916 the Commonwealth Serum Laboratories (CSL) was established in Melbourne because World War I threatened the shipment of supplies of vaccines and antiserum from the United Kingdom. Studies of snake venoms were initiated due to innumerable deaths caused by local snakes in the agricultural society of the early 1900s and the public outcry for an antidote. The first Australian snake antivenom was produced against the venom of the tiger snake (*Notechis scutatus*) in 1931, and an antivenom raised against coastal taipan (*O. s. scutellatus*) was produced in 1953. Despite several early important publications, it was not until the 1960s and 70s that venom research emerged as an important field of scientific investigation.

After antivenom therapy was established for the major snake species within Australia (the venom of *Oxyuranus scutellatus* was used to produce antivenom against taipan bite), investigations were undertaken to determine the composition of the lethal

components observed in snakebite victims. Typical methods used included column chromatography of whole venom and investigation into the most toxic fractions, invariably neurotoxins, via injection into mice (Kamenskaya and Thesleff, 1974). Research conducted on *O. s. scutellatus* venom in the 1970s isolated a highly lethal component, a neurotoxin, taipoxin which was found to block presynaptic nerve transmission and produce myolytic effects (Fohlman, *et al.,* 1976;; Chang, *et al.,* 1977; Harris, *et al.,* 1977; Fohlman, *et al.,* 1979; Jeng, 1978b; Lind, 1982; Lind and Eaker, 1982; Harris and Maltin, 1982; Lee and Ho, 1982; Dodds, *et al.*, 1995; Crachi, *et al.,* 1999; Crachi, *et al.,* 1999; Harris, *et al.*, 2000). Current research has revealed taipoxin accumulates in the plasma membrane, independently of calcium, causing fragmentation of the F-actin cytoskeleton of cells (Neco, 2003). *O. microlepidotus* was described to contain an analogue of this toxin, and was named paradoxin (Fohlman, *et al.*, 1979; Hamilton, *et al.,* 1980; Brown, *et al.,* 1987).

This research was followed by a brief report regarding the isolation of another neurotoxin from *O. s. scutellatus*, taicatoxin. This toxin contains not only subunits of neurotoxic Ca^{2+} blockers but a serine protease inhibitor (Mebs, 1979; Brown, *et al.,* 1987; Possani, *et al.,* 1992a). The full peptide sequence has not been established.

During the 1980s, methods of chromatography were utilised and research was focused on other properties of *O. s. scutellatus* venom, using lethal fractions. Snake venom proteins containing procoagulant or anticoagulant activity received much attention, and from these studies scutelarin was characterised (Walker, 1980; Walker, *et al.,* 1982; Pirkle and Marsh, 1992; Stocker, 1994). Later work examined this component by gel electrophoresis and anion exchange chromatography, using chromogenic substrates to observe amidolytic activity (Speijer, *et al.*, 1986; Tan and Ponnudurai, 1990). Despite these studies the structure and sequence of scutelarin remains unknown. Fohlman (1979) reported that *O. microlepidotus* venom also contains a prothrombin activator.

Further research later published the pharmacological studies of four new neurotoxins from *O. s. scutellatus* venom. These were named OS1, OS2, OS3 and OS4 and were isolated using chromatography. Sequence from OS1 and OS2 was obtained and further characterisation was continued with OS2, observed to be toxic (Lambeau, *et al.,* 1989;

Lambeau, *et al.,* 1990; Gandolfo, *et al.,* 1996). Further toxic neurotoxins were characterized and named toxins 1 and 2 (Zamudio, *et al.*, 1996). Finally, protein sequences from liver tissue have been identified to sequences matching PLA_2 precursors (Hains and Broady, 2000), whilst the identification of protein sequences through LC/MS/MS from the three *Oxyuranus* venoms have matched portions of natriuretic peptides from other species (Fry, *et al.,* 2003). These sequences have been placed onto public databases.

The majority of *Oxyuranus* venom characterisation has centred on lethal fractions and physiological assays. Due to this, *Oxyuranus* venom components are more easily categorised by their pharmacological action. The methods used in the 1970's and 80 have used cutting edge technology of the time, and have been a limiting step in efforts to characterise and study venoms. The new technologies are opening new doors to re-examine current knowledge and to make new discoveries. A summary of toxins within *Oxyuranus* species are found in Appendix 3 and further expansion of specific venom proteins are undertaken within later Chapters.

1.2.4 Evolution of Australian snakes and their venoms

It has been postulated that a common ancestor of Australian snakes moved from Asia into Australia when the continents were joined, moving to the east coast later. Until the early Palaeocene (approximately 64 million years ago) Australia was effectively still part of Gondwana, with land connections to South America via Antarctica (Audley-Charles, 1987; Woodburne and Case, 1996). A period of isolation followed as the Australian plate moved north from the Antarctic; the isolation ended as the tectonic collision with southeast Asia produced a bridge allowing the dispersal of terrestrial animals from the north (Hall, 2001).

Superimposed onto this trend are several intervals of reduced sea level (Hoffstetter, 1939; Stoor, 1964). Australian snakes have now evolved in relative isolation for some 30 - 35 million years (Cogger, 1981). It has been suggested that the early Australian elapid fauna, due to limited diversity, were *Pseudechis-like* ("black" snake) and *Pseudonaja-like* ("brown" snake) (Scanlon, 2003). Polymorphism of genes is suggested for the speciation seen (Hoffstetter, 1939; Hoffstetter, 1955) while climatic

deterioration, fragmentation of forests and specialisation produced both the intra- and inter-species variation. Inferred phylogeny trees from Keogh can be seen in Figures 1.7 and 1.8 (Keogh, *et al.*, 1998).

Based on the current understanding of the phylogenetic relationships of Australo-papuan venomous snakes it has been suggested that the morphological diversity and innovation seen in *Oxyuranus* species might be traceable to a *Pseudonaja*-like ancestor (Scanlon, *et al.*, 2003). *O. microlepidotus* has retained many physiological and behavioural traits resembling present-day *Pseudonaja* species, such as small head size, short fangs (3.4-6.2 mm), lower venom yields (average 44 mg), high toxicity (0.01 mg/kg)[2] and distinctive threat posture.

In contrast *O. s. scutellatus* and *O. s. canni* have developed several recently derived, innovative features: larger head, larger eyes and jaw gape, large fangs (7.9-12.1 mm), large venom yields (average; 120 mg), high toxicity (0.064 mg/kg), larger body size and a unique "snap and release" biting strategy.

These innovations have been attributed to diet and foraging behaviour (Shine, 1983). All *Oxyuranus* species are active predatory hunters that feed almost solely on mammals; mainly rats, mice, bandicoots and quolls. In contrast, *Pseudonaja* species are opportunistic feeders and pursue a range of small prey animals whereas *O. microlepidotus* has diverged to hunt moderately sized mammals such as the plague rat *Rattus villosissimus* in the confined spaces of narrow earth cracks and animal burrows. *Oxyuranus scutellatus* and *O. s. canni* hunt larger mammals such as bandicoots both in burrows and above ground (Dr W Wüster personal communication, D.J. Williams personal communication comm.).

Some of the morphological differences between the species are attributable to environmental selection pressures.

[2] Broad et al (1979); 0.1% bovine serum albumin in saline was used as a diluent and injected subcutaneously.

Figure 1.7: Phylogeny of the Family of Elapidae.

Phylogeny ascertained using cytochrome *b* and 16S rRNA data. *Pseudechis* is noted within this tree and can be treated as synonymous with *Oxyuranus*. Taken from Keogh, *et al.*, (1998) and http://www. embl-heidelberg. de /~uetz/LivingReptiles.html

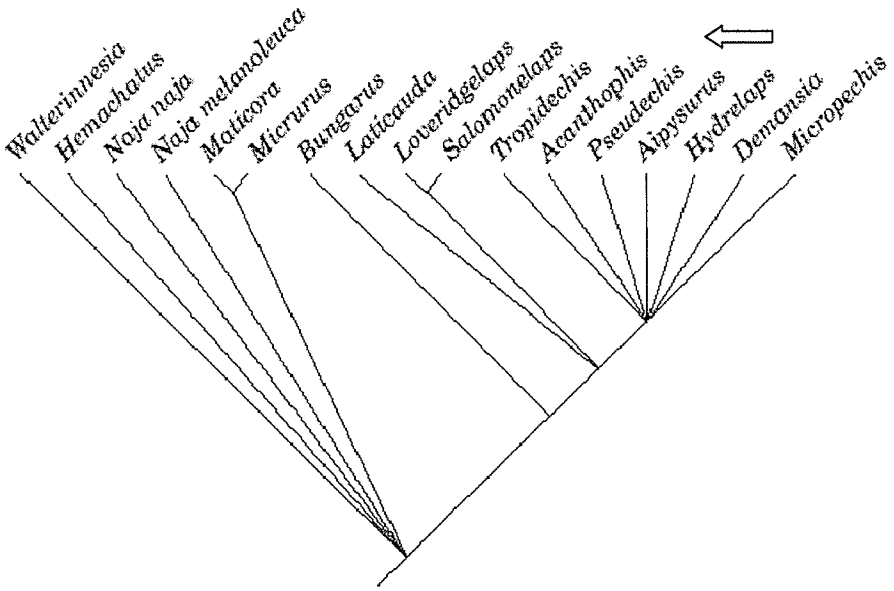
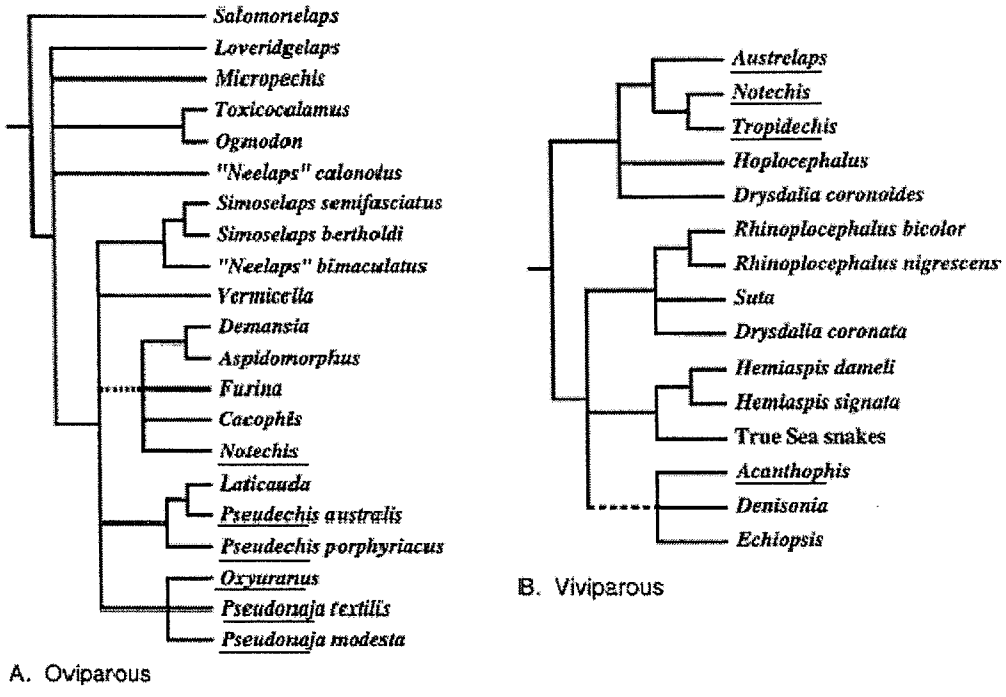


Figure 1.8: Phylogeny of Hydrophiines (sea snake).

There is much overlap between hydrophiines (sea snake) and Elapidae. Australian snakes are underlined. This conservative summary of phylogenetic relationships was obtained from the combined analyses of the cytochrome b and 16S rRNA data for (A) the oviparous hydrophiines plus *Notechis ater* and (B) the viviparous *hydrophiines* (Keogh, *et al.*, 1998).
Taken from http://www.embl-heidelberg.de/~uetz/LivingReptiles.html



Cephalic melanism in *O. microlepidotus* is a thermoregulatory adaptation to life on the searing hot, bare "Ashy Downs" of inland Australia. As black absorbs heat rapidly these snakes are able to limit exposure to potential predators by only revealing their black heads and necks outside burrows while basking. Similarly in the dense tropical grasslands of southern Papua New Guinea, the darker body colour of *O. s. canni* may be a selective adaptation to enable rapid thermoregulation in obstructed light conditions.

The phylogeny of Elapidae, indeed serpents in general is constantly revised and up-dated as new information comes to light or differing methods of comparison are used. Currently, most phylogenetic comparisons are based on mitochondrial DNA (mtDNA); sequence from cytochrome C, ND4 (Wüster and Thorpe, 1994; Keogh, *et al.,* 1998; Slowinski and Lawson, 2002), cytochrome b, 16S rRNA genes (Keogh, *et al.,* 1998) and c-mos genes (Slowinski and Keogh, 2000; Slowinski and Lawson, 2002). Together with phylogeny trees based upon morphological characteristics such as scalation and hemipenial structure (Keogh, 1999), this data is contributing to a better understanding of the evolution and phylogenetics of snakes, and in small part, of the genus *Oxyuranus*. Alignments of the mitochondrial ND4 genes from *O. microlepidotus* and *O. s. scutellatus* found variations of approximately 13%. This indicates an evolutionary divergence from a common ancestor around 9-10 million years ago (Dr W Wüster per com; unpublished data). Compared to other taxa, these two *Oxyuranus* species are their closest relatives. Significantly, alignments of around 1,300 base pairs of the mitochondrial ND4 and cytochrome C genes from *O. s. scutellatus* (Cairns and Airlie Beach, Australia) and *O. s. canni* (Moreguina, Papua New Guinea and Merauke, Irian Jaya) were identical down to the last base pair (Dr W. Wüster per com; unpublished data). This has important implications for the continued recognition of *O. s. canni* as a subspecies, and may eventually lend support to synonymy with Australian *O. s. scutellatus*.

1.2.5 Variance of Venoms

The evolution of venomous snakes leads to discussion regarding the evolution of the snakes' venom. The diversification of the toxins within venoms has generated

tremendous research and considerable debate. As venoms of more species are investigated, broader generalisations about venom compositions, functions, structures and biological roles are made.

One of the essential questions for this thesis is an exploration of the extent of variation (and similarity) between the venoms of these three species. Based on the phylogenetic information, the venoms of *O. s. scutellatus* and *O. s. canni* should be very similar, despite the reports of reduced antivenom efficacy in cases of envenomation by the latter (Lalloo, *et al.,* 1995a; Trevett, *et al.,* 1995). *O. microlepidotus* venom is expected to exhibit the greatest variation among the three species, given the 13% variation in the mitochondrial ND4 gene.

Many suggestions have been made about the pressures that may drive the evolution and development of proteins and enzymes. The presence, or absence, of particular proteins in individual venoms has been attributed to varied activities that stem from differences in genomic organisation, toxin evolution and selective mechanisms, although the reasons for the differences in the production of toxins and their specificity remains obscure (Williams, *et al.,* 1988; Magro, *et al.*, 2001). The combination of multigene-families in the venom genome (Duda and Palumbi, 2000; Conticello, *et al.*, 2001; Barbour, *et al.*, 2002), point mutations, gene duplications, recombination's and post-translational modifications of the gene product, has lead to a wide array of peptide and protein toxins (Moura da Silva, *et al.,* 1995; Chang, *et al.,* 1997; Slowinski, *et al.,* 1997; Afifiyan, *et al.,* 1999; Kordis and Gubensek, 2000; Takacs, *et al.,* 2001). For example, multigene families have been reported within *Conus* species, relating the likeness of functional diversity to major histocompatibility loci (Duda and Palumbi, 1999; Duda and Palumbi, 2000). Whereas the development of a zinc-binding toxin in vipers effecting haemostasis has been suggested to have evolved from a common ancestor with mammals, suggesting a conservation of motifs within gene families (Metalloproteinase disintegrin cysteine-rich (MDC) toxins) (Moura da Silva, *et al.,* 1996). Natural selection is attributed with sorting out inappropriate and inefficient genes.

For evolutionary success through natural selection, it would be assumed several constraints would be imposed upon proteins. The molecular structure of a particular

target site, for example a neurotoxin target site, should be conserved phylogenetically across a wide spectrum of lineages. In addition, the same target site must be associated with fundamental physiological mechanisms, such as release, binding or degrading of a neurotransmitter at the neuromuscular junction, in order to provide a basis for the immediate and potentially lethal pharmacological effect of toxins (such as neurotoxins in this example).

Therefore it may be possible that the potential for producing a large diversity of toxin functions may be limited to a number of structural frameworks. For example, the venom from the predatory cone snail (*Conus* species) can contain a great diversity of up to 50 peptides with varying functions. These peptides appear to meet particular requirements for affecting receptors or ion-channels of a particular type of prey (Rockel, *et al.*, 1995; Mebs, 2001). These toxins exhibit a conserved disulfide framework consisting of two, three, or four loops, reinforcing a possible structural limitation for toxins (Oliveira and Gomez, 1990; Favreau, *et al.*, 1999).

Another example of conserved protein structures may be seen in the snake venom three-finger-shape toxins (3FTx). Three finger toxins typically contain an alpha-neurotoxic folding motif, with four invariant disulfides in a central globular core from which three β-sheet-rich loops stem (Endo, *et al.,* 1987). These toxins have widespread activity not only in elapid venoms but also in the venoms of the various 'colubrid' families. This also suggests that a conservation of structure does exist, though not automatically translating to a conservation of function. The cDNA sequences show 96-98% identity although the pharmacological activities vary (Endo and Tamiya, 1987a; Ménez, 1998; Tsetlin, 1999), indicating that a change in as few as one amino acid within the coding region of a gene can have large repercussions upon the function of a toxin (Fry, *et al.,* 2003). Although these snake venom toxins differ in the type of pharmacological effects they induce, the toxicity may not be directly related to enzymatic activity. One explanation has been put forward within PLA_2 toxins, suggesting the presence of a specific 'toxic' site, which interacts with a cellular target site (Kini and Evans, 1989a). It is thought the interaction with a membrane receptor confers toxicity whereas enzymes lacking this interactive site are non-toxic (Lambeau, *et al.*, 1989; Lambeau, *et al.,* 1991; Ishizake, *et al.*, 1995; Lambeau and Lazdunski, 1999).

Species must also be resistant to their own venom action. Takacs (2001) noted that α-neurotoxins from the Egyptian cobra (*Naja haje*), which targets the nicotinic acetylcholine receptor (nAChR) of the neuromuscular junction of prey, was not functional against itself. This immunity was assumed to be due to amino acid substitutions within the receptor, although this was incompatible with the evolutionary theory that "1) the molecular motifs forming the α-neurotoxin target site on the nAChR are fundamental for receptor structure and /or function, and 2) the α-neurotoxin target site is conserved among Chordata lineages" (Takacs, *et al.,* 2001). An N-glycosylation signal in the ligand binding domain that was unique to *N. haje* was found. This glycosylation caused a modification of the receptor to which the neurotoxin could not bind. These theories also support the hypothesis that toxins are targeted against evolutionary conserved molecular motifs (Alape-Girón, *et al.*, 1999, Takacs, *et al.*, 2001).

Interestingly, a study describing the complete inhibition of PLA_2 toxicity *in vivo* by purified snake PLA_2 inhibitors (PLI) (eg Ohkura, *et al.*, 1993) has been described, and provides another example of proteins evolving to share common binding sites (Ohkura, *et al.*, 1997). These PLIs are large multimeric glycoproteins found in snake serum, which form soluble complexes with PLA_2s, thus inhibiting their enzymic action. That these PLIs are expressed in the snake's liver, and not in the venom, suggests these inhibitors are secreted into the snake's circulation as a protective mechanism against accidental self-envenomation. This does not, however, explain the presence of PLIs in the serum of non-venomous snakes. It has been suggested they are present as protection against predation by venomous snakes or that these peptides are evolutionary remnants from a common ancestor (Kogaki, *et al.,* 1989; Inoue, *et al.,* 1991; Ohkura, *et al.,* 1993; Lizano, *et al.,* 1997; Dunn and Broady, 2001; Okumura, *et al.,* 2002; Thwin, *et al.,* 2002). Research of both PLA_2s and their inhibitors is ongoing within Elapidae, although not specifically with the Australian Elapid, *Oxyuranus.* Perhaps further characterisation of these may aid in antivenom research (Harrison, *et al.* 2003).

Varying venom composition and toxin versatility has also been attributed to selection-pressures. It is assumed that the genes encoding the proteins present in venom

have been subjected to an accelerated evolution accompanied by functional diversity of their products (Ohno, *et al.,* 1998). These pressures include factors such as adaptation to a specific type of prey (Daltry, *et al.,* 1996; Wüster, 1999), the season of venom collection (Gubensek, *et al.,* 1974) (Williams and White, 1992), the age of the snake (Marsh and Glatston, 1974; Meier and Freyvogel, 1980; Meier, 1986), and differences in geographic location (Barrio and Miranda, 1966; Irwin, *et al.*, 1970; Williams, *et al.,* 1988; Chippaux, *et al.,* 1991; Yang, *et al.,* 1991; Assakura, *et al.*, 1992; Francischetti, *et al.,* 2000).

Hypotheses have been proposed to explain these geographic differences. Firstly, the variation in venom could be a function of the geographical distance between groups. The opportunity to exchange venom-coding genes is expected to be higher between spatially close populations, causing them to produce more similar venom than remote populations. Secondly, variation in venom may be associated with the patristic phylogenetic relationships among groups (a patristic distance analysis is based on constructing trees from distance matrices). This hypothesis predicts that populations of recent common ancestry produce more similar venoms than populations separated by greater patristic distances. Thirdly, variation in venom might be associated with a geographical variation in diet.

Venom variation within species between geographic locations has been described for the Australian tiger snake (*Notechis scutatus*), and Malaysian pit viper (*Calloselasma rhodostoma*). Populations of *N. scutatus* from Melbourne and Mt Gambier, 377 kilometres apart, differed in venom compositions and toxicity (Yang, *et al.,* 1991), whereas a population of *N. scutatus* in South Australia at Lake Alexandrina (582 kilometres) varies considerably from both of these groups in the neurotoxic components of its venom (Williams, *et al.,* 1988) see also (Williams and White, 1990, Williams and White, 1992). Williams, *et al.,* (1988) suggested that differences found in *Notechis* venoms are a reflection of the duration spent in separate habitats, due to time spent at the differing geographical locations. *Notechis* species are opportunistic feeders, eating reptiles, birds, frogs, eggs and mammals: it is possible differences may be related to the major prey found within the varying habitats. Venom variation within *C. rhodostoma* species from differing geographical areas has also been reported, these variations were suggested to be due to prey differences (Daltry, *et al.,* 1996).

Variations have also been described within the venoms of Australian death adders (*Acanthophis* species) (van der Weyden, *et al.,* 2000), yellow-faced whip snakes (*Demansia psammophis*) (Williams & White, 1990) and common brown snakes (*Pseudonaja textilis*) (Williams and White, 1990). Significant variability in geographically distinct populations of *Acanthophis* species venoms may provide evidence for the elevation of some populations to new species (Fry, *et al.,* 2001; Fry, *et al.,* 2002; Wickramaratna, *et al.,* 2003b; Wüster unpublished).

The toxin amino acid sequences have been used to infer toxin evolution (Slowinski, *et al.,* 1997; Fry, *et al.,* 2003). Several groups of proteins such as phospholipases (PLAs) (Alape-Giron, 1999; Betzel, *et al.,* 1999; Valentin and Lambeau, 2000; Jeyaseelan, *et al.,* 2000; Fujimi, *et al.,* 2002; Fujimi, *et al.,* 2002), and haemolytic and serine proteases (Magalhaes, *et al.,* 1997; Joseph, *et al.,* 1999; Nirthanan, *et al.,* 2003) have been characterised with evolutionary implications associated across genera and families (Slowinski, *et al.,* 1997, Fry and Wüster, 2003). The analysis of peptide sequences may facilitate a better understanding of the evolutionary position of toxins and, under strict analysis, may provide an evolutionary insight into these phyla (Page and Charleston 1996; Page, 2000; Fry *et. al.* 2003). Unfortunately, samples of Australian elapid toxin sequences are underrepresented, thus putative species trees may be misleading at this time.

1.2.5.1 Examples and biochemistry of venom toxins with a focus upon Oxyuranus

"As in all areas of biology, if there is more than one way to carry out a particular function, life has evolved a variety of equally viable strategies for confronting a challenge" (Poran, *et al.,* 1987; Daltry, *et al.,* 1996; Heatwole and Powell, 1998). The diversity of venoms may be due to survival mechanisms; through surpassing prey defences and preventing the evolution of venom immunity within prey. Yet many groups of enzymes are common to venoms. The variation of the biochemical compositions of venoms amongst snake species has been described between closely related species or even within species (Jimenez, 1975). Due to the significant role of neurotoxicity after envenomation by Australian Elapids, the role and activity of neurotoxins (PLA_2 and post-synaptic) will be expanded.

Activities described within elapidae include;

Acetylcholinesterases (AChE) (EC 3.1.1.7). AChE catalyses the hydrolysis of acylcholinesters with a relative specificity for acetylcholine. This enzyme binds to cellular membranes of excitable tissue (synaptic junction, endoplasmic reticulum, etc.) (Nachmansohn, 1970; Friedenberg, 1972; Politoff, 1975).

Phosphodiesterase (EC 3.1.15.1) and Phosphatase (EC 3.1.31. and EC 3.1.3.2). Activity successively hydrolyses 5'-mononucleotides from 3'-hydroxy-terminated ribo- and deoxyribo-oligonucleotides. Phosphodiesterase from rattlesnake venom (*Crotalus adamanteus,* I.U.B.: 3.1.4.1) and the Japanese mamushi venom (*Agkistrodan halys blomhoffi*) have been well characterised.

L-amino oxidase, (E.C.1.5.3.2). N-methyl-L-amino-acid oxidase catalyses the oxidative deamination of a number of basic amino acids and L-amino acids (L-arginine and L-lysine). The enzyme is absolutely specific for L-isomers (An N-methyl-L-amino acid + $H_2O + O_2$ = an L-amino acid + formaldehyde + H_2O_2). These enzymes have been well characterised in *Crotalus adamanteus venom* (I.U.B. 1.4.3.2) and Malaysian Pit viper venom *(Calloselasma rhodostoma)* (Pawelek, *et al.,* 2000).

Hyaluronidases: This is any of a group of enzymes that catalyse the hydrolysis of complex carbohydrates such as hyaluronic acid and chondroitin sulphates. These enzymes have also been found in insects, leeches, mammalian tissues (testis being the richest mammalian source) and in bacteria. This is an important spreading factor that degrades hyaluronate, one of the major connective tissue constituents in animals.

1.2.5.1.1 Phospholipase A_2's

Phospholipase A_2 (PLA_2, EC 3.1.1.4) catalyses the Ca^{2+} dependent hydrolysis of the 2-acyl ester bond of 3-sn-phosphoglycerides (De Haas, 1961). PLA_2s form a diverse class of enzymes with regard to function and localisation. PLA_2s also play a pivotal role in cellular processes, including phospholipid digestion and metabolism, host defence and signal transduction (Dennis, 1994). The intracellular PLA_2s, which are thought to play an important role in the inflammation processes, are found in low concentrations in nearly every mammalian cell. The secretary enzymes are found abundantly in mammalian pancreas and in snake or bee venoms serving a digestive function (Dennis,

1997). Snake venom peptides may contain two regions; an enzymic and a PLA_2 region. High enzymic activity does not automatically translate into high PLA_2 activity and vice versa. Extensive studies have been carried out on the toxic effects of snake PLA_2s with regard to their structure, function and mechanism of action (Kini, 1997; Bailey, 1998). The enzymic areas of these proteins possess varying pharmacological actions including, oedema forming (Lloret and Moreno, 1993), hypotensive (Huang, 1984), cardiotoxic (Fletcher, *et al.*, 1981), platelet aggregating (Gerrard, *et al.*, 1993; Yuan, *et al.* 1993), hemorrhagic (Condrea, *et al.*, 1981; Gutierrez, *et al.*, 1980b), myotoxic (Mebs, 1986; Gutierrez, *et al.*, 1995; Ponraj and Gopalakrishnakone 1995 and 1996), convulsant (Fletcher, *et al.*, 1980) and neurotoxic activities (Hseu, *et al.*, 1999; Sundell, *et al.*, 2003) (Lambeau, *et al.*, 1999) (Figure 5.1).

PLA_2s have been classified from type I to IX based on their molecular size, substrate specificity, amino acid sequence and disulphide bond pattern. The first three types include venom-derived proteins. *Type I* are secreted in the pancreas and are found in elapid venom. This group has been divided into two groups, the IA (toxic) and IB (pancreatic). These proteins have seven disulphide bridges, and average in size from 13 to 15 kDa (Six and Dennis, 2000).

Type I snake PLA_2s

Within Elapids, Davidson and Dennis (1990) and Ohno, *et al.*, (1998) postulated a gene duplication event gave rise to two type 1 PLA_2s; types IA (toxic) and IB (non-toxic, pancreatic) PLA_2s. The non-lethal pancreatic (1B) groups are characterised by the presence of an extra loop which is characteristic of the pancreatic PLA_2s. This loop, which is absent in group IA PLA_2s, is referred to as the "pancreatic loop", and is thought to be an ancestral property present in venom PLA_2s of the elapids that appear at the lower end of the evolutionary tree such as the king cobra (*Ophiophagus hannah*) (Huang, *et al.*, 1999), Brazilian coral snakes (*Micrurus*)(Francis, *et al.*, 1997) and Australian snakes (*Notechis, Pseudechis, Pseudonaja, Oxyuranus, Austrelaps*) (Jeyaseelan, *et al.*, 1998).

The type IB PLA_2s, are been considered to have evolved under neutrality, possibly retaining the pancreatic loop for the maintenance of properties such as procoagulant activity. This activity remaining possible by keeping the structural scaffolds of the

proteins conserved and minimising the rate of mutation of the gene (Ogawa, *et al.,* 1996; Armugam, *et al.,* 2004).

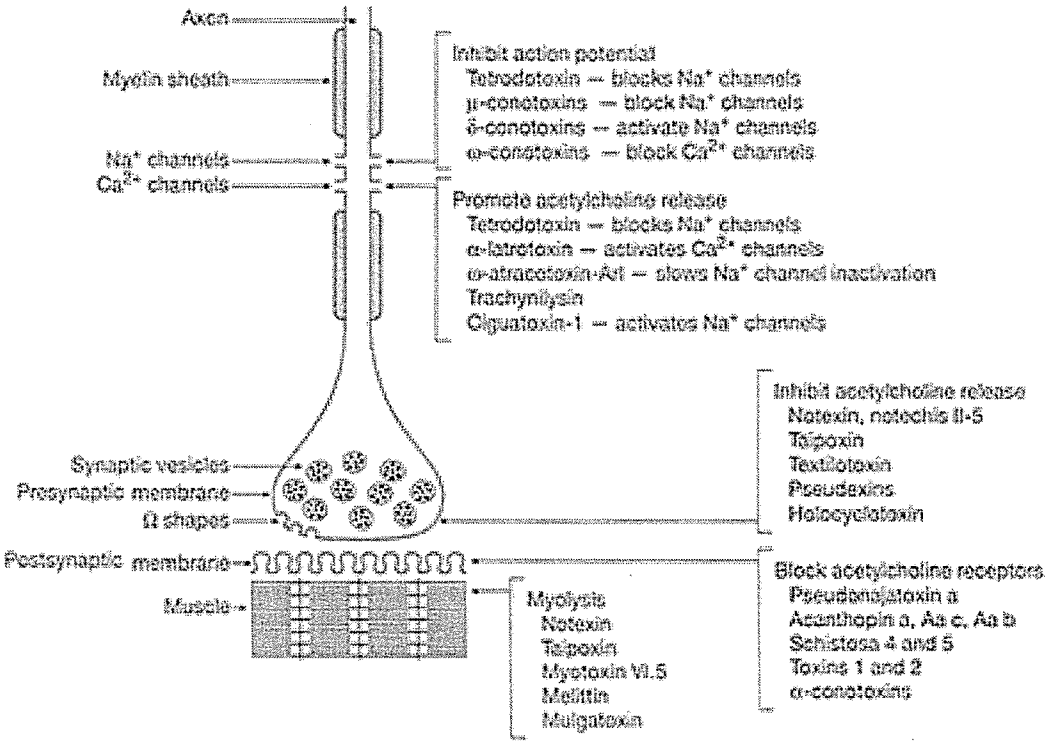
Alternatively, the loss of the pancreatic loop seen in type IA was suggested as an added adaptive advantage to promote pathophysiological properties and lethality among the PLA_2s (Davidson and Dennis, 1990).

Between both group 1A and 1B, the most important active sites are the PLA_2 catalytic site (His^{48}, Asp^{49}, Tyr^{52}, Tyr^{64}), and the Ca^{2+} binding site (Tyr^{24}-gly^{25}-Cys^{26}-Tyr^{27}-Cys^{28}-gly^{29}-Arg^{30}-gly^{31}-gly^{32}-Ser^{33}-gly^{34}) (Kramer, *et al.,* 1989), which are conserved in most PLA_2s. Yet there is much discussion between researchers regarding differing structures and areas causing the lethality of these toxins (Ogawa, *et al.,* 1996).

Example: Neurotoxins

Neuromuscular paralysis occurs through PLA_2 activity by several enzymic methods (Yang and Chang, 1993). The *blockade of nerve conduction* occurs through stabilising nerve membranes by blocking sodium channels. This stops action potentials and may cause death by paralysis (eg: tetrodotoxin (TTX) from Tetraodontiformes fish (pufferfish and toadfish). Some conotoxins (from *Conus* shells) activate sodium channels while other conotoxins block potassium channels effectively depolarising neurons and preventing any conduction. S*pontaneous action potentials* slow sodium channel inactivation during depolarisation. The prolonged action potential increases transmitter release from nervous tissue, causing the production of nerve impulses leading to gross muscle twitching. Robotuxin from *Atrax robustus* (Sydney funnel web spider venom) shows these effects (Sutherland, *et al.,* 2001). Other toxins affect the acetylcholine processing and release from the nerve ending (*Pre-synaptic*, Hodgson and Wickramaratna, 2002), whereas others block acetylcholine receptors on the muscle fibre (*post-synaptic*, Figure 1.9) (Lee and Tsai, 1976; Rosenberg, *et al.,* 1989; Mebs and Ownby, 1990; Ramirez, *et al.,* 1990; Mackessy, 1993; Tu, 1996; Currie, 2000). Cardiotoxic effects have also been described (Barrington, *et al.,* 1986; Gong, *et al.,* 1989; Kumar, *et al.,* 1997; Bell, *et al.,* 1998).

Figure 1.9: Highly schematic drawing of nerve fibre and motor end plate region.
Highly schematic drawing of nerve fibre and motor end plate region showing sites and mode of actions of various toxins (Taken from Sutherland and Tibballs, 2000).



Pre-synaptic (β) neurotoxins

Several physiological mechanisms of venom-induced pre-synaptic neurotoxicity have been discovered. *Loatodetus mactans* (North American Black Widow spider) venom contains a protein, latrotoxin (MW 130 kDa), and this activates calcium influx in neural tissue. This causes the vesicles filled with acetylcholine to empty and remain empty leading to patchy paralysis of muscle and release of transmitters, including catecholamines, in other tissues (Holz and Habener, 1998).

The Australian snake neurotoxins infiltrate the plasma membranes independently of the presence of calcium, the ensuing physical changes causing the prevention of vesicle recycling (taipoxin, notexin). This action leads to an inability to release transmitter and hence paralysis (Cull Candy, *et al.,* 1976; Fohlman, 1979; Hamilton, *et al.,* 1980; Harris, *et al.*, 2000; Neco, *et al.,* 2003).

Pre-synaptic neurotoxins appear to be a variable group of toxins in size and structure. Examples of varying structures from snake venoms include the three subunit toxins of *O. scutellatus* (taipoxin), the two subunit toxins of *Bungarus multicinctus* (B-bungarotoxin), and single subunit toxins from *Notechis scutatus* (notexin) and *O. scutellatus* (OS1 and 2). These multi-chain neurotoxins are made of two, three or four polypeptide subunits that are not linked by disulphide bridges and have a sequence homology with PLA_2s from mammalian pancreas and snake venoms. It has been shown that at least one of the subunits possesses phospholipase A_2 activity and is responsible for the enzymic activity of the neurotoxin. Whereas the other subunits may or may not contain phospholipase activity.

Taipoxin is a 45.6 kDa complex composed of three similar subunits that can be dissociated at acidic pH or in the presence of 6M guanidine (Fohlman, 1976). This toxin causes flaccid paralysis by blocking neuromuscular transmission without affecting muscle sensitivity to acetylcholine (Fohlman, 1976). Taipoxin has also been shown to cause the appearance of omega figures in the pre-synaptic membrane and a loss of synaptic vesicles, suggesting it may act after binding to or gaining entry to the pre-synaptic terminal (Cull Candy, *et al.,* 1976). Suggestions for the mediation of movement of this toxin into the pre-synaptic sites have included binding to secreted neuronal proteins aiding the uptake of taipoxin into neurons (Dodds, *et al.,* 1995; Tzeng, *et al.*, 1995; Kirkpatrick, *et al.*, 2000) although it has also been shown taipoxin readily enters cultured cells suggesting this toxin did not require mediators or specific ion channels (Fathi, *et al.*, 2001; Neco, *et al.,* 2003). Other research has suggested the cause of neuromuscular paralysis observed in envenomed subjects was due to the myolytic action of venom causing the degeneration of motor nerve terminals and axonal cytoskeleton (Harris, *et al.*, 2000). Of the three subunits α and β are classified type IA phospholipases. α-Taipoxin is a basic 14.6 kDa peptide consisting of 119 or 120 amino acid residues. This subunit is the only one of the three that has both neuronal and enzymic properties on its own (Fohlman, *et al.,* 1976; Lipps, 2000).

The isolated α-subunit is-500 fold less toxic than the full toxin and, as yet, it is unknown why this occurs or why this subunit is toxic. The β-subunit is a neutral protein that has mitogenic activity (Lipps, 2000) though devoid of toxic and enzymic

activity (Cull Candy, *et al.,* 1976; Lind and Eaker, 1980; Lind and Eaker, 1982; Lambeau, *et al.,* 1989). This subunit has been separated into at least two slightly different isocomponents by ion-exchange chromatography of 14.3 kDa (beta-1 and beta-2) with each containing 119 or 120 amino acid residues.

The third 26.9 kDa subunit, γ-taipoxin, is homologous in sequence to the proenzyme form of pancreatic phospholipase, and is classified as a type IB peptide. The acidic γ chain of taipoxin is devoid of direct toxic activity but still retains a non-lethal enzymic activity. This subunit displays only weak phospholipase activity and is also unique in having eight disulphide bonds (Fohlman, *et al.,* 1977; Ramlau, *et al.,* 1979) and a carbohydrate moiety within this complex (Coulter, *et al.,* 1980). It has been shown that only the basic ∝-chain and acidic γ-chain evoke antibody production.

Basic peptide monomers OS1, 2, 3 and 4 (Lambeau, *et al.,* 1989) from *O. scutellatus* consist of a polypeptide chain of 14 to 16 kDa and display high phospholipase activity. OS1, a non-toxic peptide, binds to M-type (muscle type) neuronal PLA_2 receptors but not the N-type (neurone type) receptors. Whereas the toxic OS2 is capable of binding to both M- and N-type PLA_2 receptors (Lambeau, *et al.,* 1990; Lambeau, *et al.,* 1991; Gandolfo, *et al.,* 1996).

Enzyme binding

Phospholipase binding, activity and toxicity appear to involve a fine balance of differing factors. In the absence of crystal structures, varying models of pre-synaptic neurotoxins have been suggested. These models include specific residues involved with binding, enzyme orientation and toxicity (Chang *et al.,* 1997). With no biochemical data available to support the following assumptions, comparisons of the sequences of secretary neurotoxic PLA_2s from differing origins have shown several trends.

The PLA_2s must first bind to their substrate. Electrostatic interactions between positive charges from the PLA_2 recognition site, and a negatively charged anionic head group of phospholipids optimise catalysis and are important for the adsorption and orientation of the enzyme at the lipid water interface (Yu and Dennis, 1993; Betzel, *et al.,* 1999). The hydrophobicity of the enzyme recognition site is also likely to be important, as increased hydrophobicity will improve enzyme binding to the respective target sites on the membrane substrate. Biochemical studies of PLA_2s from various

sources have also indicated that Leu^2 (Volwork and De Haas, 1982; Liu, 1995), Trp^3 (Volwork and De Haas, 1982; Baker, *et al.*, 1998; Liu, *et al.*, 1995), Arg^6 (Van-Scharrenburg, *et al.*, 1983; Baker, *et al.*, 1998), Lys^{10} (Dua, *et al.*, 1995; Van Der Wiele, *et al.*, 1988), Met^{20} (Volwork and De Haas, 1982; Lee, *et al.*, 1996), Leu^{31} (Volwork and De Haas, 1982; Kuipers, *et al.*, 1990), Lys^{56} (Dua, *et al.*, 1995), Noel, *et al.*, 1991; Beiboer, *et al.*, 1995), Leu^{64} (Kuipers, *et al.*, 1990), Val^{65}(Kuipers, *et al.*, 1990), Asn^{67} (Kuipers, *et al.*, 1990) and Lys^{116} (Van der Wiele, *et al.*, 1988; Dua, *et al.*, 1995) may be involved in PLA_2 binding to aggregated phospholipids. Based on crystal structure, Asn^{117} and Asp^{119} have been proposed to be part of the interfacial binding face within porcine PLA_2 (Dijkstra, *et al.*, 1981; Dijkstra, *et al.*, 1983).

Within venoms, varying substrate binding sites from Lys^7 and Lys^{10} (Han, *et al.*, 1997) and Leu^2-Phe^5, Ile^9 (Wang, *et al.*, 1992; Scott, 1997) have also been suggested. Kini and Iwanaga (1986) suggested that a hydrophobic region around residues 73 and 100 was important for the pre-synaptic neurotoxicity from the comparison studies of hydropathy profiles between pre-synaptically acting PLA_2s and non-neurotoxic PLA_2s (Kini, *et al.*, 1986). Yet Takasaki 1990 (Takasaki, *et al.*, 1990a, b, c) demonstrated that toxins from *P. australis*, which also shows pre-synaptic neurotoxicity, did not contain marked differences of hydrophobicity in this region.

Enzyme penetrability of the phospholipid membrane is also reported to be improved by positively charged residues flanking hydrophobic segments of the recognition site, this reported to facilitate hydrolysis of phospholipids. In addition, hydrophobic side chains, such as tryptophyl residues, may penetrate into membranes enhancing PLA_2 binding (Kini, 1997). Other research has also suggested aromatic residues at the C-terminus are used for binding (Janssen, *et al.*, 1999). These sites are not comparative with the non-venom pancreatic sequences.

"Toxic sites"

Specific residues inferring toxicity of PLA_2s also reportedly vary. In snake venom toxins, β-bungarotoxin, notexin and taipoxin, the alkylation of His^{48} in the active site with p-bromophenacyl bromide irreversibly abolished the phospholipase A_2 activity and destroy toxicity in a parallel manner (Fohlman, *et al.*, 1976; Jeng and Fraenkel-Conrat, 1978a; Marlas, 1982), indicating specific residues may be responsible for toxicity. It

was suggested substitution of an invariant residue, from Gly^{30} to Ser^{30} also resulted in a decrease of toxicity (Dufton and Hider, 1983) after sequence comparisons were undertaken between the toxic PLA_2s from notexin (*Notechis scutatus*), II5 (*Enhydrina schistose*) a myotoxin from a true sea snake and non toxic PLA_2s PLA I, III, IV (*Laticauda semifasciata*) and from a sea krait (*N. scutatus*).

Comparisons of Australian *Pseudechis* and *Notechis* species, a true sea snake (*Enhydrina*, sp); sea kraits (*Laticauda*) and cobras (*Naja* and *Hemachatus*) revealed that Asp^{50}, Lys^{58} and Asp^{90} may also be important for toxicity. Another three residues; Lys^{46}, Asn^{74} and Glu^{94} were also found to be predominant within toxic PLA_2s. A proposed domain for the pre-synaptic toxicity consisting of seven hydrophilic residues, Arg^{43}, Lys^{46}, Asp^{50}, Glu^{54}, Lys^{58}, Asp^{90} and Glu^{94} has also been reported (Takasaki and Tamiya, 1985; Takasaki, *et al.*, 1990). It was suggested that enzymes which possess the complete set of the seven residues were strong neurotoxins while PLA_2s which lack a few of them, especially Asp^{50}, Lys^{58} or Asp^{90}, were weak neurotoxins.

Alternatively there are suggestions that differing regions cause lethality. Residues such as Phe^{100}-Tyr^{105} (Gubensek, *et al.*, 1994) and 'pre-synaptic clusters', residues 55-65 and 80-89 (Arriagada and Cid, 1989) have also been reported to cause differences in toxicity. A train of basic residues 63, 86 and 58/81 were also suggested to lower toxicity in the Bs-bungarotoxin A chain and *E. schistosea* myotoxins (Kondo, *et al.*, 1989). Some of these residues were also identified by sequence comparisons of some *P. australis* peptides. Comparisons have also indicated sequences containing Lys^{63} and Lys^{81} are classified as weak neurotoxins (Takasaki, 1989).

Further to this study, it has also been that suggested proteins containing a phenylalanine at position 63 were toxic whereas a basic residue would cause this protein to be non toxic (Takasaki, *et al.*, 1990a; Takasaki, *et al.*, 1990c). Alternatively, Tsai, (1987) suggested that the charge scores of residues 57, 58, 63, 70 and 86 of elapid PLA_2 correlated with toxicity, that is, pre-synaptically toxic PLA_2 enzymes have higher scores of charge (+4 or +3) than the non toxic PLA_2 enzymes (0 to +1) (Tsai, *et al.*, 1987).

From the literature studied, it appears that there are specific residues required for toxicity, though these residues differ between species.

1.2.5.1.2 Post-synaptic (α) neurotoxins

Although many studies have investigated pre-synaptic neurotoxins, relatively few studies have been carried out on the post-synaptic neurotoxins in the venom of Australian elapids. The nicotinic acetylcholine receptor (AchR), a pentamer composed of four subunits (α2,β,γ,δ), plays a central role in post-synaptic neuromuscular transmission by mediating ion flux across the cell membrane in response to binding of acetylcholine (Tsetlin, *et al.*, 1982; Changeux, *et al.*, 1984; Conti-Tronconi and Raftery, 1982; Hucho, 1986; Servent, *et al.*, 1997; Karlin, 1980, McCarthy, *et al.*, 1986; Gong, *et al.*, 1999). This regulatory activity is inhibited by binding to an α-neurotoxin (Lee, 1979) or to some anti-AchR antibodies.

Functional studies have focused mostly on the α-subunit because it is responsible for binding acetylcholine (Changeux, 1981; Moore and Raftery, 1979; Sobel, *et al.*, 1977; Tzartox and Changeux, 1983) and α-neurotoxins (Bon, *et al.*, 1979; Dufton and Hider, 1983; Endo, *et al.*, 1987; Joubert and Viljoen, 1979; Lee, 1979; Noda, *et al.*, 1982; Noda, *et al.*, 1983a). Toxin binding to post-synaptic membrane receptors causes flaccid paralysis of striated muscles (Pillet, *et al.*, 1993). This is usually more easily reversed by antivenom than are the effects of the pre-synaptic neurotoxins.

Over 50 of these single chain polypeptide snake neurotoxins have been described. All the main Australian snake venoms have at least one of type, usually with a molecular weight around 6 kDa. To date only two post-synaptic neurotoxins have been isolated from *Oxyuranus*.

Two classes of elapid α-neurotoxins or 'three finger toxins' are distinguished; the 60 to 62-residue, four disulphide bond "short-chain or type I neurotoxins" and the 66 to 79 residue, five disulphide bridge "long-chain or type II neurotoxins" that include membrane toxins (Karlsson, *et al.*, 1984; Endo and Tamiya, 1991). These toxins, which form a three-finger-shape, have a folding motif with four invariant disulphides in a central globular core from which three β-sheet-rich loops emerge (Endo, 1991). Two potential short chain neurotoxins were isolated from the *O. s. scutellatus* cDNA library.

Short chain neurotoxins

Ten short chain neurotoxins have been isolated from Australian elapids. The venom proteins from *Acanthophis antarcticus* (Aa c) (Kim and Tamiya. 1981) *Pseudechis australis* (Pa a) (Nishida, *et al.,* 1985; Rowan, *et al.,* 1989), *O. scutellatus* (toxin 1 and toxin 2) (Zamudio, *et al.,* 1996) and *Pseudonaja textilis* (Pt sntx 1, 2, 3, 5, 6, 7) (Gong, *et al.,* 1999) are homologous. These peptides are suggested to produce peripheral paralysis. Other well characterised short chain neurotoxins include those from *Bungarus multicinctus* (kappa bungarotoxin), *Naja naja* (cobratoxin) and *Laticauda semifasciata* (erabutoxin).

Erabutoxin is a type I post-synaptic neurotoxin from the venom of *L. semifasciata* of which the crystal structure has been solved. Three forms of erabutoxin have been characterised: erabutoxin a, a lecithinase, erabutoxin b an anticoagulase and erabutoxin c a hyaluronidase. Erabutoxin b is the most toxic and extensively studied. Upon envenomation the toxin binds to the muscular nicotinic acetylcholine receptor on the motor end plate blocking it irreversibly. This produces peripheral paralysis by blocking neuromuscular transmission at the post-synaptic site (Endo, *et al.,* 1971; Sato and Tamiya, 1971). This is a basic peptide composed of 62 amino acids with four disulphide bonds. The resolved structure of erabutoxin found the Tyr^{25} was buried in the protein and nitration of this residue inactivates the toxin. Trp^{29} was exposed to the surface of erabutoxin b and, when modified, the lethal activity of the venom also disappeared (Gaucher, *et al.,* 2000). The site on erabutoxin b where it binds to the acetylcholine receptor was proposed to be at Pro^{44} to Gly^{49} (Obara, *et al.,* 1989). This toxin is very similar to another elapid post-synaptic neurotoxins such as α-cobratoxin (*N. atra*).

O. scutellatus toxins 1 and 2, which differ by only one amino acid residue, inhibit the binding of α-bungarotoxin to nicotinic acetylcholine receptors in skeletal muscles but not to central neuronal nicotinic receptors (Zamudio *et al.*, 1996). These toxins are 5-fold less potent than α-bungarotoxin or the two short-chain α-neurotoxins erabutoxin a and erabutoxin b. It was suggested that nonconservative substitutions at position 32 in both taipan toxin 1 and 2 may be responsible for the observed decreases in affinities of the toxins of 5-fold for muscle receptors (compared to alpha-bungarotoxin) and over 10-

fold for alpha-bungarotoxin-sensitive nicotinic receptors in brain (compared to the structurally similar short-chain alpha-neurotoxins erabutoxin a and erabutoxin b) (Zamudio, *et al.*, 1996).

Like pre-synaptic neurotoxins, specific residues have been implicated in the toxicity of post-synaptic peptides. The global head (core) formed by four disulphide bridges of the molecule, is thought to localise the invariant residues in the immediate vicinity of the disulphide bridge or, they are found toward the distal ends of the three major loops. In contrast, the least conserved residues tend to be grouped across the loop of the globular head (Atassi, 1995). Potent neurotoxins in cobra and sea snake venoms have a Tryptophan residue at position 29 and it has been found that Trp^{29}, Lys^{27}, Asp^{31} and Arg^{33} are essential for toxicity (Pillet, *et al.*, 1993). Homologues of this group of toxins show sequence similarities, in particular, the characteristic Cys^{41}, Gly^{42}, Cys^{43} sequence but lack their toxicity.

This lack of toxicity can usually be attributed to the absence of certain key residues, most typically Trp^{29}. The secondary structures of these homologues are not believed to be substantially different from the group of short neurotoxins (Tu and Dekker, 1991). Differences in the core residue sequences may by interpreted as representing evolutionary distance, if the assumption that the core residues play mostly structural roles is correct. As an example, *Pseudonaja* Pa toxins form a special cluster different to other snakes including Australian snakes. These toxins may have diverged from an ancestral gene at a very early stage in evolution and may have evolved into taipan toxins and the short chain neurotoxins found in sea snakes and land snakes of Asia and Africa (Housset ,1996).

1.2.6 Proteomic and genomic characterisation of *Oxyuranus* venom

The utility of proteomics has been demonstrated for many organisms (Patton, 1999; Belghazi, *et al.*, 2001; Pennington and Dunn, 2001; Wang and Huang, 2002; Nawarak, *et al.,* 2003) and has been used as an approach to characterise elapid venoms (Fry, *et al.,* 2003). Proteins are functional entities within the cell and, like mRNA, there is a direct relationship between proteins and genes, reflecting phylogeny. However, this relationship is not of the one to one kind. A single gene may have multiple protein

products due to differential splicing of its primary transcript, proteolytic cleavages, or the covalent modification of the mature protein product (Butt, *et al.*, 2001). Therefore, the amino acid sequence of proteins has been used to analyse not only sequence similarity of venom proteins but structural motifs inferred from the sequence (Slowinski, *et al.*, 1997; Alape Giron, *et al.*, 1999; Betzel, *et al.*, 1999).

Full length cDNA sequences are also extremely useful for determining the genomic structure of genes, especially when analysed within the context of genomic sequence (Strausberg, 2002). The coding sequence of cDNA has also contributed to this information, allowing identity and function to be predicted for some peptides (Serrano, *et al.*, 1998; Yamazaki, *et al.*, 2002). This interpretation has been predicted with PLA_2s (Valentin and Lambeau, 2000a), three finger toxins (Alape-Giron, *et al.*, 1999; Fry, *et al.*, 2003; Nirthanan, *et al.*, 2003) and conotoxins (Duda and Palumbi, 2000; Conticello, 2001). In many cases functions have been described through sequence alone (Kini and Evans, 1989; Joseph and Kini, 2002; Pennacchio and Rubin, 2003; Assakura, *et al.*, 2003; Torres, *et al.*, 2003).

It is using these methods of analysis that a greater picture of *Oxyuranus* venoms will be achieved.

1.3 OBJECTIVES

The objectives of this study were:

Firstly, to conduct a comparative study of the venom composition from the three *Oxyuranus* species. The results obtained may present additional information by which to assess the similarities of these species and to aid in identifying specific proteins causing clinical variations after envenomation.

The venom from specific individuals were collected and recorded for the three species over a period of two years. These species were chosen for study due to the ready access to snake venom and the paucity of knowledge regarding components of venoms from these species. To date, most research has been conducted using *O. s. scutellatus* venom. This is the first characterisation and comparison of whole venom from all three species of *Oxyuranus*.

Secondly, the comparative analysis of these species may aid the identification of proteins for specific assays, functions, targets or structure analysis.

Thirdly, data produced from a cDNA library representing the open reading frame from genes would be used to identify sequences representing venom proteins. It was predicted that due to similar roles of venoms from snakes, *Oxyuranus* venoms would have some components in common with other elapidae snake venoms, but because of differences in geographical location, prey bases and in phylogeny, novel components would also be present. Furthermore, identification of some of the many unknown venom components and sequencing of proteins with activities similar to those found in characterised snake venoms will be essential to future understanding of both functional and evolutionary relationships between venoms components and the snakes which produce them. Although a strict phylogenetic analysis was not within the bounds of this project, the information obtained within this project will allow supplementary phylogenetic assignment in assistance to morphological and genetic data within venomous snakes.

Chapter 2

Materials and Methods

This chapter describes the general methods and materials used throughout the project. Recipes for buffers and solutions appear in Appendix VIII as do the primer sequences used in chapter 5.

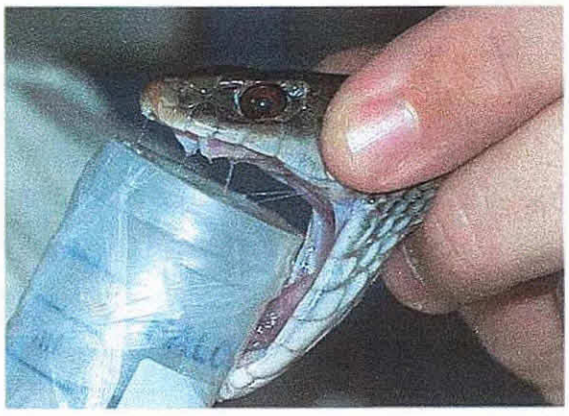
2,1 MATERIALS

2.1.2 Venom

Whole venom was obtained from captive *O. s. scutellatus* and *O. microlepidotus* housed at Billabong Sanctuary, Townsville (refer Table 3.1). The venom from *O. s. canni* were obtained from Papua New Guinea and supplied by Wilsha Holdings (PNG Wildlife Export no. 020255, AQIS no. 2001056617). The venom samples from each species were pooled and stored in the same buffer. Snake venom milking was performed by Wilsha Holdings. Snakes were physically restrained before being held behind the jaws and allowed to bite the top of the venom collection container, a 50 ml polyethylene tube with the open top covered with parafilm (see Figure 2.1). The expressed venom was transferred from the 50 ml polyethylene tube into a 1.5 ml microcentrifuge tubes and placed on ice. All snake venoms were stored at –80°C in 25 mM HEPES (pH7.6) + 10% glycerol.

Figure 2.1 Venom milking

Venom milking performed by Mr D. Williams with a Papuan taipan



2.1.2.1 Venom gland from O. s. scutellatus

A venom gland (lab no. 02-1002871) stored in 1ml of TRIzol (Gibco BRL) was kindly donated by Peter Mirtchin from Venom Supplies (ABN number 39 458 465 843 - PO Box 547, Tanunda, South Australia 5352 Web: www.venomsupplies.com).

2.1.3 Reagents

All general laboratory reagents were supplied by Sigma, InVitrogen, ICN, Amersham or BioRad laboratories.

2.2 METHODS

2.2.1 Protein protocols

2.2.1.1 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Protein purity and molecular masses were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5, 12 or 15% acrylamide in a TRIS-glycine buffering system as described by Laemmli (Laemmli, 1970). SDS-polyacrylamide running and stacking gels (buffers and solutions, appendix VIII) were cast in a Mini-PROTEAN II electrophoresis cell apparatus (BioRad).

One to 10 μg of sample were pre-treated in 2 x or 4 x cracking buffer (see solutions, appendix VIII) and heated at 100°C for 2 min to denature the proteins. Samples were vortexed vigorously, then centrifuged for 5 min at 13,000 rpm prior to loading onto the gel. Electrophoresis was carried out in 1 x SDS-PAGE running buffer (see solutions, appendix VIII) at 150 V for 50 min or until the bromophenol blue dye front had migrated off the bottom of the gel. For visualisation of all proteins, gels were removed from the apparatus and stained with Coomassie blue or silver stain. The molecular weights were estimated by interpolation from a linear semi-logarithmic plot of relative molecular mass versus distance of migration, using Broad Range Markers (BioRad).

Tricine gel electrophoresis

Tricine gel electrophoresis was useful for the resolution of small (15-35 kDa) proteins of similar size (Schagger and von Jagow, 1987). This technique utilised different buffers and stacking and resolving gels compared to the SDS-PAGE described above. Both the stacking and separating gels were polymerised at the same time; the stacking gel was poured directly onto the separating gel. Samples (5 to10 µl) were loaded onto the gels and were electrophoresed at 25 to 35 mA (constant current) per gel in a minigel set-up for 12 to 16 hours.

Staining of SDS-polyacrylamide gels

Coomassie

Two staining methods were used. The first Coomassie stain (1) (0.25 g Coomassie R-250, 100 ml methanol, 20 ml acetic acid in a total volume of 200 ml) was used routinely for staining SDS polyacrylamide gels. Following electrophoresis, gels were placed directly into the stain for 20 min with gentle rocking. Stain was poured off and the gel destained (1:1:8 methanol/acetic acid/water) for 12 hrs, changing the destain solution several times. An alternate Coomassie Stain (2) (34% methanol, 17% ammonium sulphate, 3% orthophosphoric acid and 0.1% Coomassie blue G250 in a total volume of 200 ml, Herbert, personal communication) was used for resolution of two dimensional gel electrophoresis spots. This method stained 2D gels well and gave better resolution of proteins compared to the previous method. The mixture was stirred overnight and then used once only. Gels were placed directly into the stain and placed in fresh stain solution after one hour. The gel was then stained overnight and destained in 1% acetic acid.

Silver staining

This technique was derived from an Amersham protocol written for the Amersham 2D flatbed electrophoresis system. All buffers are summarised in Appendix VIII. Immediately after electrophoresis gels were immersed in fixing solution for 30 minutes. This solution was poured off and gels then placed in incubation solution for 30 min or overnight. After incubation gels were washed 3 times for 5 min in distilled water. The water was removed and the gels immersed in silver solution for 40 minutes. The stain was developed after removal of the silver solution by the addition of developing

solution. When the protein bands reached the desired intensity (30 sec to 10 min) the reaction was quenched with stop buffer. The gels were placed in preserving solution overnight before being dried.

Drying SDS-Polyacrylamide gels

Stained SDS-polyacrylamide gels could be stored in cellophane. SDS polyacrylamide gels were soaked in 20% methanol for 30 min and then in water for 10 minutes. Two cellophane gel drying films (25.5 × 28 cm gel drying film, Promega) were immersed in distilled water. The gels were sandwiched between the cellophane and secured onto a frame. After 24 to 48 hrs the gels were cut out and stored.

2.2.1.2 Venom stability studies

The degradation of crude and fractionated venom was analysed by SDS-PAGE after being subjected to variations of pH, temperature and time exposed to room temperature. Fractionated venom samples (0.1 - 10 mg/ml) eluted at 4°C were removed and subjected to the same parameters as whole venom. The concentrations varied, dependent upon the initial concentration and will be stated.

Effect of Time

Frozen whole venom was thawed on ice and a 40 µl aliquot (100 mg/ml) was removed and placed at room temperature (25°C). At time points 0, 1 hr, 6 hr, 12 hr, 24 hr, 48 hr and 72 hrs, 5 µl of each venom sample was removed. Venom was diluted 1 in 10 with buffer and 8 µl (80 µg) of this sample was added to 8 µl of 2 x cracking buffer, and stored for SDS-PAGE analysis. Control samples were taken from the thawed stock sample, diluted 1 in 10 with buffer then treated with cracking buffer and subjected to SDS-PAGE.

Effect of Temperature

Whole venom (100 mg/ml) was thawed on ice and aliquots of 40 µl of whole venom (100 mg/ml) from *O. s. scutellatus*, *O. microlepidotus* and *O. s. canni* were removed and placed on ice. Samples (5 µl) were divided into separate microcentrifuge tubes for each species and placed at temperatures from 0, 40, 50, 60, 70, 80, 90 and 100°C for 20 minutes. Eight microlitres (80 µg) of this sample was added to 8 µl of 2 x cracking buffer, after a dilution of 1 in 10 with buffer, and stored for analysis on SDS-PAGE.

Effect of pH

Crude whole venom was thawed, and aliquots placed in varying pH buffers from pH 3, to 11 (buffers summarised below) for 20 min at room temperature. Thirty micro litre samples were examined by SDS-PAGE. Ten microlitres of buffer solution (800 mM, different pHs) was added to 40 µl of venom sample buffer. After incubation for 20 min the pH was altered to 7.6. Fifty microlitres of the resultant solution was added to 20 µl of 2 M HEPES (pH 8.8). Previous buffer pH checks showed the pH returned to between pH 7.5 and 7.6. This step was necessary to prevent any artifactual changes of samples due to different running conditions during SDS-PAGE. The effect of the varying pH buffers were tested on SDS-PAGE prior to analysing samples. Buffers used for stability assay were; pH 3.5-5, 0.8 M sodium acetate; pH 5.4-8.4, 0.8 M /TRIS/maleate/NaOH; 0.2 M maleate (24.2 g TRIS + 23.2 g maleic acid/L); pH 9-11, 0.8 M glycine/NaOH.

Stability of isolated 100 kDa protein (B2)

The effect of pH, temperature and time upon the activity of B2 was determined. The protein was incubated for 20 min at temperatures of 0, 20, 30, 40, 50, 60, 70 and 80°C and allowed to reach room temperature for 5 min before assaying. The stability of the enzyme activity of the protein was determined by incubating the enzyme for 1, 4, 24, 48 and 71 hrs at room temperature. Enzyme activity was measured following the addition of substrate as described above. The effect of pH was determined using 40 µl of 400 mM sodium acetate (pH 3-5), 400 mM TRIS/maleate/NaOH (pH 6-8) and 400 mM glycine/NaOH (pH 9-11) at a final concentration of 100 mM NaCl for 20 minutes. Sample was returned to pH 7.6 by the addition of 50 µl of the resultant solution to 20 µl of 2M HEPES (pH 8.8) and run in a total assay volume of 100 µl after 30 min recovery at 22°C. Previous buffer pH checks showed the pH returned to between pH 7.5 and 7.6. Enzyme activity was measured with the addition of chromogenic substrate. Controls were run at each pH.

2.2.1.3 Liquid chromatography

All buffers used for chromatography were prepared within the laboratory using analytical grade reagents. The pH of buffers was adjusted at 4°C and were filtered

(Millipore Durapore Membrane Filters 0.22 μM) and degassed (Poretics system under suction) prior to each chromatographic run. In preparation for use, columns were rinsed in 10 column volumes (cv) of water followed by 10 cv of the equilibrating buffer (buffer A). All cleaning and storage protocols were followed as recommended by the manufacturer. All chromatography was undertaken on a BioRad Biologic DuoFlow HPLC system and run at 4°C. Protein samples were loaded onto the column via a static loop (0.25 or 1 ml) and the column eluate monitored (280 nm). Data was analysed via BioRad BioLogic Duo-Flow software.

Gel filtration

Whole venom (100 mg/ml) was thawed on ice from storage at –70 °C. Ten milligrams of whole venom diluted to 1 ml with equilibration buffer was loaded onto a column of Phenomenex BioSep SEC S-2000 (300 x 21.2 mm) previously equilibrated with 50 mM HEPES, pH7.2, 200 mM NaCl at 4°C and run at 5 ml/min and 1 ml fractions collected. This was compared with gel filtration standards (BioRad) run under the same conditions. The MW standards consisted of thyroglobulin (bovine) 670 kDa, gamma globulin (bovine) 158 kDa, ovalbumin (chicken) 44 kDa, myoglobin (horse) 17 kDa and vitamin B-12 1.35 kDa.

Ion exchange chromatography

Anion exchange column chromatography

Anion exchange chromatography was undertaken using a Uno Q continuous matrix column (6 ml, 12x53 mm, BioRad). The equilibration buffer, or buffer A was run at two to 5 ml/min and consisted of 50 mM HEPES, pH 8.0, 20 mM NaCl while the elution buffer or Buffer B was 50 mM HEPES, pH 8.0, 1 M NaCl. Protein loading and column flow rate and backpressure are described in Chapter 3 and were within manufacturer's guidelines. One millilitre fractions were collected.

Cation exchange column chromatography

Cation exchange chromatography was undertaken using a Uno S continuous matrix column (1 ml, 7 x 35 mm, BioRad). The equilibration buffer, or buffer A was run at 0.5 to 2 ml/min and consisted of 50 mM HEPES, pH 7.6, 20 mM NaCl or 50 mM MES, pH 6.0, 20 mM NaCl dependant upon the experiment stated in Chapter 3. The elution

buffer or Buffer B was the appropriate Buffer A plus 1 M NaCl. Protein loading and column flow rate and backpressure are described in Chapter 3 and were within manufacturer's guidelines. One millilitre fractions were collected.

Affinity chromatography

Whole and fractionated venom was loaded onto a 1 ml High Trap benzamidine column (Amersham) equilibrated with 50 mM HEPES, pH 7.6, 500 mM NaCl and run at 1 ml/min. A 1 M NaCl wash of 5 to 10 cv was used to elute any non-specific binding and the bound serine proteases eluted with 1 cv of 0.5 M glycine, pH 3.0. Sixty microlitres of 1M HEPES, pH 9.0, was added to the elution tubes. The eluted samples utilising a low pH were visualised by SDS-PAGE and silver staining.

2.2.1.4 Quantification of proteins

Protein concentration was determined as described by Bradford (Bradford 1976), using BSA as standard or determined via UV absorbance at A_{280} in a Beckman DU 650 spectrophotometer assuming an E = 1.00.

2.2.1.5 Enzyme activity assays

The activity of protein fractions were performed in assays at in 50 mM HEPES, pH 7.6. Buffers for chromogenic substrates (Chromogenix, Helena Laboratories), p-nitroaniline (pNA) derivatives, were 50 mM HEPES + 20 mM NaCl for H-D-Ile-Pro-Arg-pNA (*S-2288*) and Bz-Ile-Glu (γ-OR) Gly-Arg-pNA (*S-2222*) and 50 mM HEPES + 16 mM NaCl for H.D-Val-Leu-Arg-pNA (*S-2266*) in a total volume of 100 µl. These p-NA derivatives were substrates for broad serine protease activity, factor Xa activity and glandular kallikreins and factor XIa respectively.

The reactions were initiated by the addition of enzyme (*Oxyuranus* protein/s) at a final concentration of $8x10^{-6}$ g (or 2.6×10^{-5} M assuming a molecular weight of 300, 000 Da) with substrate concentrations of 0.01 M used for initial specific activity and 0.0048 M (2 x Km) used for Michaelis-Menten kinetics. The linearity of enzyme activity was established for SEC peak one, this could not be established for B2. The substrate concentration was determined at 342 nm using an extinction coefficient of 8.27×10^3 M^{-1} cm^{-1} (Chromogenix), depending on the substrate used). The proteolytic activity of *Oxyuranus* proteins was measured using a Beckman DU 650

Spectrophotometer with a 1 cm path. The formation of p-nitroaniline was monitored continuously (405 nm). The amount of substrate hydrolysed was calculated from the absorbance at 405 nm using a molar extinction coefficient of 9600 M^{-1} cm^{-1} for free p-nitroaniline. The kinetic parameters, K_m and K_{cat} (or $K_{0.5}$ for allosteric enzymes), were determined by analysis of double reciprocal plots for the initial velocity as a function of substrate concentration utilising Sigma Plot. Enzyme units were defined as the activity that converts one mole of substrate per minute under the conditions stated above. Specific activity is described as a rate in Units (U) per milligram of enzyme (protein). All assays were performed in triplicate. Initial enzyme rates were linear with saturating levels of substrate (10 mM), with the substrate concentration required for maximal activity of the enzyme found to be 4.6 mM.

Preparation of membrane vesicles

The procedure used in the preparation of the membrane vesicles was based on a method described by John & Whatley (John, 1970, personal communication, Prof. J. N. Burnell). "The vesicles were used in activity assays when searching for protein activators. All the solutions and apparatus used were maintained between 1 to 4°C and all operations, except the lysozyme treatment, were carried out at these temperatures. Stock buffer solutions at a concentration of 0.4 M were adjusted to pH 7.3 at 4°C, and then diluted to 10 or 100 mM as required.

One colony of *E. coli* XL1 Blue cells grown on Luria-Bertani media plus carbenicillin (50 µl) (LBC) plates was inoculated into 100 millilitres of LBC. After 7 hrs incubation at 37°C with agitation, 5 ml of this culture was inoculated into 2 L of LBC and shaken at 200 rpm maintained at 35°C. The 2 L of mid-exponential phase culture were sedimented by centrifugation at 5000 g for 30 min and were washed in 800 ml of 150 mM NaCl containing 10 mM TRIS-HCl buffer. The cells were then suspended in 400 ml of 0.5 M sucrose containing 10 mM TRIS-HCl (pH 7.3) buffer, so that a sample of the suspension had an absorbance at 550 nm of 0.7. Lysozyme was added to the cell suspension at a concentration of 250 µg/ml, which was then left in a water bath at 30°C for 20 to 30 min until the A_{550nm} was below 0.2. After treatment with lysozyme the cells were sedimented by centrifugation at 40 000 g for 10 min and resuspended in 4 ml of 100 mM TRIS-acetate buffer. The suspension was then diluted

with 360 ml of water and shaken immediately to disrupt the cells. The suspension was left for 20 min then a trace of deoxyribonuclease (EC 3.1.4.5) and 2 mM magnesium acetate were added. The suspension was shaken gently to distribute the deoxyribonuclease and magnesium acetate and then centrifuged at 40,000 g for 40 min in a TLA 25.50 to yield a pellet and a clear supernatant; the supernatant was discarded. Pellets were resuspended in 4 ml of 100 mM TRIS-acetate buffer and the suspension of membrane vesicles was stored at 1 to 4°C and could be used for experiments over a period of three days."

2.2.1.6 Inhibition studies

Various inhibitors were incubated with the venom protein for 20 min after which chromogenic substrate was added and the activity measured immediately for 1 hr at room temperature. The differing responses of the enzyme to inhibitors assisted in characterizing the activity of various venom proteins.

2.2.1.7 Two dimensional gel electrophoresis

Two dimensional gel electrophoresis (2DE) was undertaken following protocols of (Galvani, et al., 2001a; Galvani, et al., 2001b; Herbert, et al., 2001). Samples of whole or fractionated venom were desalted using lipoextraction or tricholoroacetic acid (TCA) precipitation methods (see precipitation methods).

1st Dimension

The first dimension of isoelectric focusing was performed using 7 or 11 cm immobilized pH gradient (IPG) dry strips (Amersham) rehydrated with 10 to 40 µg of desalted sample (respectively in rehydration buffer (8 M urea, 2 M thiourea, 40 mM TRIS (pH 8.8-9), 2% CHAPS, 10 mM DTT and 5% ampholytes). Acrylamide (final concentration 2 mM) was added to these samples and left at room temperature for 1 hr before focusing on a BioRad Protean IEF cell. The focusing protocols are summarised below.

2nd Dimension

The focused IPG strips were equilibrated in SDS equilibration buffer (0.375 M TRIS-Cl, 6 M urea, 20% glycerol, 2% SDS, trace Coomassie) for 25 min immediately after focusing. These strips were placed onto SDS polyacrylamide gels with size markers

(BioRad Broad Range Protein marker) and run at 10 mA per gel for 15 min then 20 mA per gel for 1 hr or until the bromophenol blue band reached the bottom of the gel. Proteins were visualised after SDS-PAGE by silver staining or Coomassie blue staining (2).

Linear voltage / 7 cm strip / max 50 uA per strip / 20°C

Phase	Voltage (V)	Current (A)	Watts (W)	Time (hh:mm)	Volt hours
1	250	50 uA/strip		00.01	2.5
2	250	50 uA/strip		00.30	125
3	500	50 uA/strip		01.00	500
4	3500	50 uA/strip		02.00	7000
5	3500	50 uA/strip		05.00	17500
					25kVh total

Run using Volt hours as the strips do not usually reach max Voltage. (25kVh≈11hrs)

Linear voltage / 11cm strip / max 50uA per strip / 20°C

Phase	Voltage (V)	Current (A)	Watts (W)	Time (hh:mm)	Volt hours
1	250	50 uA/strip		00.01	0.01
2	250	50 uA/strip		01.00	250
3	500	50 uA/strip		01.00	500
4	3500	50uA/strip		02.00	7000
5	3500	50 uA/strip		05.50	19250
6	8000	50 uA/strip		04.50	36000
					63 kVh total

Run using Volt hours as the strips do not usually reach max Voltage. (63kVh≈18hrs)

2.2.1.8 ELISA

A checkerboard enzyme linked immuno-sorbent assay (ELISA) was developed to determine the optimal ratio of taipan monovalent antivenom (CSL) to add to whole taipan venom. All incubations were carried out at room temperature. Venom was bound to the wells of a 96 well plate (Sarstedt round well base) up to a 1 in 1,000 dilution in carbonate coating buffer (CCB) (0.1 M $NaCO_3$, 0.1 M $NaHCO_3$, 0.02% NaN_3, pH 9.6) by overnight incubation. Unbound venom was removed by washing the wells three times with phosphate buffered saline plus Tween 20 (phosphate buffered saline,0.9% NaCl, 0.3% Triton X-100, pH 7.4). Free sites on the plate were blocked

with 2% (w/v) bovine serum albumin (BSA) in PBST for 1 hour. Antivenom was diluted where appropriate in 2% BSA in PBST- before being added to the wells and the plates incubated for 1 hour. Plates were washed three times with PBST to remove unbound antivenom. Secondary antibody (goat anti-horse-HRP conjugate, Jackson Laboratories) was incubated for one hour. Plates were washed for 1 hr and washed a final 3 times in PBS only. Fifty millilitres of ABTS/H_2O_2 (1%, v/v) in citrate buffer (50 mM citric acid, 100 mM Na_2HPO_4, pH 5) was added to each well and incubated for 30 minutes. The presence of bound horseradish peroxidase was detected at A_{405} using a multiscan Ascent microtest plate reader (Pathtech).

2.2.1.9 Western blotting

Western blotting was carried out on gels to be analysed by antibody probing (Towbin *et al.,* 1979; Burnette, 1981). SDS-PAGE was undertaken in duplicate, one for antibody probing, the other to be silver stained to visualise proteins for a comparison. After SDS-PAGE, the gel was placed against nitrocellulose membrane (BioRad), fibre pads and filter paper pre-soaked in transfer buffer (Sambrook *et al.*, 1989) and the orientation marked. The electrophoretic transfer of proteins was performed using a Mini Trans-Blot Electrophoretic Transfer Cell from BioRad and was run at 80 V for 1 hr (for large proteins up to 2 hrs). All steps were undertaken at room temperature. After transfer, membranes were removed and prepared for Immuno screening.

2.2.1.10 Immuno screening

After the completion of electro-transfer of proteins, membranes were blocked in 10 ml of TBST (10 µl Tween 20/0.5 g skim milk powder) for 1 hour. Membranes were then washed once for 15 min then twice for 5 min in TBST. The primary antibody, taipan monovalent antivenom (CSL) was bound for 1 hour or overnight at the dilution of 1 in 10,000. Ten ml of TBST was added per membrane (80mm x 100mm). After primary antibody incubation the membrane was again washed once for 15 min then twice for 5 min in TBST. The secondary antibody, goat anti-horse HRP conjugated antibodies (H+L, Jackson laboratories) was added to 10 ml TBST per membrane at the dilution of 1 in 5,000. Bound antibodies were detected by DAB (Sigma) or ECL

(Amersham) by manufacturers' instructions and visualised on Kodak Hyperfilm. All steps were undertaken at room temperature.

Controls to check non-specific binding of the secondary antibody revealed these antibodies bound only to the primary antibody and not to venom proteins.

2.2.1.11 Glycoprotein detection

Whole *O. s. scutellatus* venom was fractionated by 7.5 or 15% SDS-PAGE. Proteins were electro-blotted onto a nitrocellulose membrane (BioRad) and probed with Concanavalin A-HRP conjugate with 1 mg/ml of Ca^{++} and Mn^{++} (Con A, ICN) (1 in 1000 dilution of TBST).

2.2.1.12 Antibody binding

Isolation of specific antibodies

Samples of *O. s. scutellatus* whole venom were blotted onto PVDF membrane and bound with taipan monovalent antivenom (CSL). The protein bands with bound antibodies were visualised with Ponceau S and the band of interest excised using a sterile scalpel blade. The antibodies were stripped from the protein band isolated by placing these into a microcentrifuge tube with 100 mM glycine (pH 3.0). Using the smallest volume possible this was shaken vigorously on a rotary shaker at room temperature for 1 hr after which the supernatant was removed and one tenth of the volume of 1M TRIS (pH 8.0) was added. These antibodies were used to probe an expressed cDNA library and were also bound to *O. s. scutellatus* whole venom separated on reducing 15% SDS-PAGE as a control. Antibody binding was visualised using DAB. Controls to check non-specific binding of the secondary antibody revealed these antibodies bound only to the primary antibody and not to venom proteins.

2.2.1.13 Precipitation of proteins

Precipitation methods were used to desalt venom samples for two-dimensional gel electrophoresis.

Lipoextraction

Following the procedure described by Mastro (Mastro and Hall, 1999), ice cold tributyl phosphate: acetone: methanol (1:12:1: v/v/v) was added to each sample. Following incubation at 4°C for 90 min the solution was centrifuged at 2800 g for 15 min, the pellet washed sequentially in 1 ml of tributyl phosphate, acetone, methanol and allowed to air dry.

Trichloroacetic acid (TCA) precipitation

A one tenth volume of 90% (w/v) TCA was added to each sample and left on ice for 30 minutes (or 4°C overnight). Samples were centrifuged at 15 000 g for 15 min and the supernatant poured off. The remaining pellets were washed in ice cold acid ethanol (40 mM acetic acid in EtOH). Samples were sonicated to resuspend the pellet then re-centrifuged at 15,000 g for 15 minutes. Again the supernatant was poured off and the pellet washed in ice cold acetone. The pellet was resuspended by sonication and centrifuged at 15,000 g for 15 minutes. The supernatant was removed and the sample dried in a Speedivac.

2.2.1.14 Determination of amino acid sequences of proteins

N-terminal Edman degradation sequencing

O. s. scutellatus venom samples (80 µg) were run on an SDS-PAGE gel (7.5 or 12% reducing conditions) and blotted onto an MSI PVDF membrane (2 hours at 70 V). The PVDF membrane was stained for 10 min in Ponceau S (0.1% (w/v) in 5% acetic acid and destained with 3 washes of distilled water. The visualised bands were cut out from the air-dried PVDF membrane and the N-terminal sequences determined commercially at the Intistute of Biomelecular Sciences, University of Queensland, Australia.

Mass spectrometry

Eighty micrograms of *O. s. scutellatus* whole venom was separated on a 7.5% reducing SDS-PAGE, as per N-terminal sequencing and sent to the Australian Proteome Analysis Facility, Macquarie University, North Ryde, Australia. From this a 100 kDa protein band was isolated and sent to APAF (Appendix 4). The sample (RW300S) underwent a 16 hr tryptic digest at 37°C. The resulting peptides were purified using a GELoader POROS R2 column to concentrate and desalt the sample. The sample was then analysed by ESI-TOF MS/MS using a Micromass Q-TOF MS equipped with a

nanospray source and data manually acquired using borosilicate capillaries. Data was acquired over the m/z range 40-1800 to select peptides for MS/MS analysis. After five peptides were selected, the MS was switched to MS/MS mode and data collected over the m/z range 50-2000 with variable collision energy settings. APAF was not able to distinguish between leucine and isoleucine and this is reported as [L/I]. Also, phenylalanine and methionine sulphoxide could not be distinguished, as together with lysine and glutamine, they differ in mass by only 0.04 Da.

2.2.2 Molecular Biology

2.2.2.1 cDNA Library

Construction

Total RNA was isolated from a venom gland excised from *O. s. scutellatus* and stored in TRIzol (Gibco BRL). Poly (A)+ mRNA was purified using a MicroPolyA Pure kit (Ambion) utilising an oligo-dT column. A cDNA library was constructed using a ZAP-cDNA® Gigapack® III Gold Cloning Kit (Stratagene). Packaging of this library resulted in 1.875 x 10^3 pfu/ml.

Screening

Excision

Both mass and single clone excision of the pBluescript® Phagemid from the Uni-ZAP® XR vectors and the use of ExAssist® Helper Phage with SOLR™ Strain were carried out according to the manufacturer's instructions (Stratagene).

Preparation of host bacteria

Host bacteria were prepared according to the ZAP-cDNA® manufacturers instructions (Stratagene).

Library screening

Library screening using protein expression

The cDNA library was screened by incubating 2 µl of *O. s. scutellatus* amplified venom gland library with 600 µl of host cells and incubated at 37°C for 20 minutes.

Following the addition of 8 ml of soft top agar (precooled to about 47°C) the contents of the tube were poured onto prewarmed, (37°C), pre-dried NZY + tetracycline (0.02 mg/L) plates (LB^{tet}). Once phage and agar had set plates were incubated for 4 to 5 hrs or when plaques were seen the size of small pinholes. Plates were overlaid with Hybond C-extra (Amersham) filters impregnated with 1mM IPTG and incubated at 37° C for a further 3 hrs (using the protocol from picoBlue Immunoscreening kit, Stratagene). After incubation, plates and filters were placed at 4°C for half an hour to firm the agar and the orientation of the filter was marked with needle pricks and these places noted on the plate using a pen. Filters were carefully removed and probed using the immunoscreening protocol utilising taipan antivenom (section 2.2.1.10).

Secondary screen

Plaques aligning with detected spots via phosphorimaging (DNA) or CSL taipan monovalent antivenom (protein) were removed using a cut yellow pipette tip, and re-plated for a secondary screen or excised following the Stratagene protocol. Tertiary screens were completed as necessary.

Radiolabelling

The cDNA library was screened by incubating 2 µl of *O. s. scutellatus* amplified library with 600 µl of host cells and incubated at 37°C for 20 minutes. Following the addition of 8 ml of soft top agar (precooled to about 47°C) the contents of the tube were poured onto prewarmed, (37°C), pre-dried NZY + tetracycline (0.02 mg/L) plates. After incubation at 37°C for 6 to 8 hrs the plates were placed at 4°C for half an hour to firm the agar and the plates overlaid with nitrocellulose filters (Hybond N^+ filters - Amersham). The orientations of the filters were marked with needle pricks, these places noted on the plate using a pen. Plate lifts were made according to the manufacturer's instructions, with the DNA fixed onto filters by exposure to UV light (210 nm) for 2 minutes. Filters were rinsed with 2 x sodium buffered sodium citrate (SSC) and placed in a sealable plastic sleeve with pre-hybridisation buffer at 42°C for 1 hour. Prehybridisation buffer (400 µl 100 x Denhardt's solution, 2 ml 20 x SSC, 0.8 ml 0.5 M Pi, 40 µl 20% (w/v) SDS, 4 ml formamide, 2 ml water and 200 µl of Herring sperm DNA (10 mg/ml) that had been boiled then placed immediately on ice for 5 min) was added to the mixture when cool. This mixture was left overnight at 42°C with

shaking. The filters were removed from the hybridisation solution and washed twice for 10 min with two times SSC/0.1% SDS followed by two washes of 10 min in 0.1 x SSC/0.1% SDS to remove unbound DNA. All washes were performed at 42°C. DNA probes were radiolabelled according to Ambion DECA Prime II random priming DNA labelling kit (Ambion) and added to the prehybridization mix after boiling for at least 2 minutes. Overnight incubation and washes were performed as above. Filters were then mounted between two sheets of plastic wrap supported by a cardboard backing. This was placed against a blanked Phosphor Imager cassette for 2 hrs and then scanned using a Molecular Dynamics Phosphor Imager and visualised by ImageQuant software.

2.2.2.2 Agarose gel electrophoresis

DNA fragments were analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989). Depending on DNA fragment sizes, agarose gels were prepared from 0.7 to 2.0% (w/v) agarose and dissolved with heat in 1X TBE buffer. DNA was visualised using ethidium bromide (final concentration 0.15 μg/ml) which was added to agarose before casting the gel. The agarose gels were run in 1X TBE buffer (buffers and solutions, Appendix 8) at 70 to 100 V for 20 to 40 minutes. A marker (a 100 bp or 1 Kb ladder) was used to allow estimation of the size of DNA bands.

2.2.2.3 Plasmid DNA

Plasmid vectors were routinely used to aid in manipulating DNA fragments for sequencing. These vectors included pGEMT or pGEMT-Easy (Promega), pTZ18R (Fermentas), pGEM 3zf (Promega), and pBluescript (pBS) (Stratagene).

Isolation of plasmid DNA Miniprep

The procedure of Sambrook *et al.* (Sambrook, 1989), was followed with the following modifications. Five millilitres of LBC media were inoculated with a single white colony of DH5α or NM522 cells using a small plastic pipette tip and incubated at 37°C overnight in a rotary shaker at 200 rpm. Cells were collected by centrifugation at 5,000 x g at 4°C for 10 minutes.

QIAprep Spin Miniprep Kit Protocol (using a microcentrifuge)

Isolation of plasmid DNA was undertaken according to the manufacturer's instructions after cells were pelleted. To elute DNA, 50 µl of warmed Buffer EB (10 mM TRIS-HCl, pH 8.5) or H_2O if the DNA was to be used for ligations was added to each QIAprep column.

Miniprep-Manual

Pelleted cells were resuspended in 100 µl GTE, then transferred to a 1.5 ml microcentrifuge tube containing 200 µl of freshly prepared lysis solution (1% SDS, 0.2M NaOH) and mixed thoroughly by inversion. One hundred and fifty microlitres of 3M potassium acetate, pH 4.6 was added to this solution and mixed prior to centrifuging at 13,000 rpm for 10 minutes. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube microcentrifuge tube and 1 ml of ice cold absolute ethanol added, mixed and centrifuged at room temperature at 13,000 rpm for 10 minutes. The supernatant was then discarded, the pellet washed twice with 500 µl of 70% (v/v) ethanol and the DNA pelleted by centrifugation. The pellet was dried in a vacuum centrifuge for 2 min and then dissolved in 50 µl TE buffer. The DNA was stored at -20° C until required.

Plasmid mapping

Long sequences that could not be competed by sequencing from both directions were completed by plasmid mapping. Plasmids were cut using restriction enzymes and religated for sequencing.

Proligo primers

Sequencing primers were produced where restriction digestion, deletion and re-ligation was not possible to continue sequencing of the clone. Primer construction was based upon nucleotide sequences near the end of the incomplete sequence. Sequences were chosen with consideration of the most beneficial melting temperature, length, lack of hairpin production and a lack of duplication within the sequence. These sequences were sent to Proligo Australia Pty Ltd Southern Cross University, Military Road, Lismore NSW 2480 Australia for the primer production.

Full sequences that required plasmid mapping to complete were aligned using Sequencher or undertaken manually, due to numerous repeat regions within the sequences.

2.2.2.4 Restriction enzyme digestion of DNA

DNA was digested by restriction endonucleases from Promega Corporation and Pharmacia Biotech with their appropriate buffers. Typical digestion reactions consisted of approximately 1 µg DNA and 5 to 10 units of restriction enzyme in a total volume of 20 µl. Buffer and temperature conditions were set according to manufacturer's recommendations for individual restriction enzymes. Digests were allowed to proceed for 2 to 3 hrs and terminated by the addition of 5 µl of stop mix (see solutions, Appendix 8). Following digestion, the DNA fragments were resolved by agarose gel electrophoresis. One microlitre of RNAse A(10 mg/ml) was added to restriction enzyme digests of plasmid DNA prepared by the alkaline lysis method (Sambrook *et al.*, 1989).

Producing blunt end DNA

DNA was treated with mung bean nuclease (Promega) following manufacturer's instructions to remove protruding termini.

2.2.2.5 Isolation of DNA from agarose gels

Bands visualised by ethidium bromide under UV light were excised from agarose gels using a sterile scalpel blade. DNA was isolated from gel slices using Qiagen Gel Elution kits according to the manufacturer's instructions or by electroelution.

Electroelution

Dialysis tubing was cut into pieces of convenient length (about 8 cm) and placed in a boiling solution of 2% (w/v) sodium bicarbonate and 1 mM EDTA, pH 8.0 for at least 30 minutes. The tubing was thoroughly washed in double distilled water and then stored in 1 mM EDTA at 4°C. Agarose gel slices containing DNA bands were sealed in a length of dialysis tubing with 200 to 600 µl of 1 x TBE. DNA was eluted from the agarose gel by electrophoresis at 100 V for 30 minutes. Prior to disconnecting the

submarine gel, the electrodes were reversed and the current continued for about 20 seconds. The supernatant was carefully removed from the dialysis tubing and placed in a clean 1.5 ml microcentrifuge tube and the DNA precipitated using ethanol/sodium acetate.

2.2.2.6 Estimation of DNA concentration

DNA samples were analysed on a Beckman DU 650 Spectrophotometer, with the concentration of the sample being estimated using the Warburg and Christian calculation (Warburg and Christian, 1942). Absorbance values are taken at 260 and 280 nm and corrected for the background at 320 nm. The values were then subjected to the following equations developed by Warburg and Christian, based upon absorbencies of yeast enolase and RNA.

Protein concentration $=(1552 \times A_{280}) - (757.3 \times A_{260})$

Nucleic Acid concentration $= (-36.0 \times A_{280}) + (62.9 \times A_{260})$

Results being in micrograms per millilitre. Any dilution factors were taken into account to give the final concentration. When a suitable spectrophotometer was not available DNA concentrations were estimated by comparison to known standards on an agarose gel.

2.2.2.7 Ligation of DNA into plasmid vectors

For ligation reactions a vector:insert molar ratio of 1 to 3, or for small problematic insert 1 to 16 were used in a mixture using a 1 x ligase buffer (Promega) and 6.2 Weiss units of T4 DNA ligase (Promega). A typical ligation reaction consisted of around 50 ng of DNA, 2 µl of 10 x Buffer, 1 µl of T4 ligase, 1 µl vector and 6 µl of water which was incubated at 16°C (sticky end ligation) or 4°C (blunt end ligation) overnight.

Ligation of PCR DNA into pGEM-T

PCR products were routinely subcloned into pGEM-T Easy or pGEM-T (Promega) following manufacturer's instructions.

2.2.2.8 Preparation of competent cells

Bacterial cells were made competent by the use of calcium chloride (Cohen, *et al.* 1972). A glycerol stock of *E.coli* DH5α or NM522 stored at –80°C was streaked onto an LB plate and incubated at 37°C overnight. A single colony from this plate was used to inoculate 5 ml of LB media, which was shaken overnight. Two 1 L flasks containing 250 ml of LB media were individually inoculated with 2.5 ml of the overnight culture and incubated at 37 °C for 3 to 4 hrs (at 220 rpm) in a rotary shaker until the OD_{600nm} of the cultures was between 0.5 and 0.6. The cultures were transferred aseptically into 50 ml Falcon tubes and cooled on ice for about 30 minutes. The cultures were centrifuged at 3,000 x g for 20 min at 4°C. The pellets were resuspended in 25 ml of ice cold $CaCl_2$, stored on ice for 30 min and centrifuged at 3000 x g for 20 minutes. The resuspended bacteria were aliquoted (200 µl) into precooled 1.5 ml microcentrifuge tubes and snap frozen in liquid nitrogen before storing at -80°C.

2.2.2.9 Transformation of competent cells

Plasmids were introduced into *E. coli* cells by chemical transformation, a method based on observations by Mandel and Higa (Mandel and Higa, 1970). Frozen aliquots of competent cells were removed from the -80°C and thawed on ice. DNA was added to the cells, gently mixed and replaced on ice for 20 minutes. The cells were then transferred to a water bath at 42°C for 100 sec then placed back on ice for a further 5 minutes. The cells were then plated directly onto pre-dried and pre-warmed LB plates with appropriate antibiotic. For blue/white screening, 50 µl of an IPTG/X-GAL mix was spread onto the plate. The plates were dried for 5 min in a laminar flow hood then incubated for 12 hrs (DH5α) or 8 hrs (NM522) at 37°C. If DNA was used to transform cells, 200 to 400 ng of DNA were used per 200 to 400 µl of competent cells.

2.2.2.10 Precipitation of DNA

Phenol/chloroform

An equal volume of phenol/chloroform (1:1) solution was added to DNA samples, vortexed for 30 sec then centrifuged at 13,000 rpm for 5 minutes. The top phase was removed and placed into a clean microcentrifuge tube and the DNA precipitated by the addition of 0.1 volumes of sodium acetate pH 5.2 and two volumes of absolute ethanol.

This was incubated at -80°C for half an hour or -20°C overnight. The precipitate was then pelleted at 20,000 rpm for 30 min and the supernatant removed. This pellet was carefully washed twice in 70% ethanol prior to drying in a vacuum centrifuge and redissolved in 10 to 20 µl of buffer (10 mM TRIS-HCl, pH 8.5) or distilled water if DNA was required for ligations.

2.2.2.11 Polymerase Chain Reaction (PCR)

PCR screening of OS cDNA library for an isolated venom protein

Primer construction for the 100 kDa protein isolated from whole venom was based upon inferred nucleotide sequences from the N-terminal and mass spectrometry sequence data obtained; the sequences are summarised below. Primers were deduced from peptide sequences using MacVector™ software and the primer commercially synthesised by Proligo (www.proligo.com). Primer sequences were derived initially from MS peptide 4 and later from N terminal sequences 1 and 2.

Three PCR strategies were used to amplify DNA sequences from the cDNA library. Taq DNA polymerase, with accompanying buffer and $MgCl_2$ were obtained from either Perkin Elmer (Ampli Taq Gold)(Applied Biosystems) and dNTP's were from Progen. Polymerase chain reactions were conducted in a PTC-100™ Programmable Thermal Controller (MJ Research, GeneWorks) or, for protocol optimisation, a ThermoHybaid PCR Express machine when temperature ramping was required. Melting temperature, and reagent conditions were varied to produce optimal results. To minimise the side product and the primer-dimer formation, several sets of primers were tested under different amplification conditions. All polymerase chain reactions were conducted in a total volume of 20 µl, and run with a positive and negative control.

PCR products were subcloned into pGEM-T (InVitrogen), sequenced, and used as a probe to screen cDNA libraries as described. Nucleotide and deduced amino acid sequences were analysed using the MacVector and Sequencher software packages. BLAST searches (Altschul *et al.,* 1997) were performed using the BLAST network service at the NCBI (http://www.ncbi.nlm.nig.gov). Multiple sequence alignments were based on the neighbour-joining method and were generated with Clustal x, Blosum 62, gapped, non redundant database which covers SWISS-PROT.

PCR 1: Primers synthesised from MS peptide 4

Primer sequences were:

MS4 (sense)	5'-GCNGNTTTYTTYCAR-3'
MS4RC (antisense)	5'-YTGRAARAANACNGC-3'
T7	5'TAATACGACTCACTATAGGG-3' and
T3	5'-TAAATTGGGAGTGATTTCCC-3'

In the first round of PCR the primer combinations of MS4 with T7 and MS4RC and T3 were used. Optimal conditions were determined (1.5 mM $MgCl_2$ (Promega), 200 µM dNTP (Promega), 0.25 µM primers, 2 µl of 10x Taq buffer, 0.3 µl Taq (0.25 U/ml, Promega) and 2 µl of cDNA template in 20 µl) using the following protocol.

Stage 1:	94°C 5min
Stage 2:	94°C 1 min
	41°C 1min
	72°C 1 min go to 2 32 times
Stage 3	72°C 10 min

Two distinct bands, three hundred and five hundred base pairs (bp) were detected for each set of primers.

PCR 2: Nested PCR

Primer sequences were:

N-terminal (EPLPLL)(sense)	5' – CCGCGGARCCIYTICCIYTIYTITA - 3'
MS peptide 4 (sense)	5'- GCGGCCGCIGTITTYTTYCA - 3' and
Reverse primer, pBS vector, (antisense)	5'– GGTACCCGGCCCCCCC - 3'

One microlitre of the first round product from N-terminal and reverse primer was used as template in the second round of PCR combining MS peptide 4 and reverse primer. For each PCR round standard conditions were used, except the cycle number was 30 for the first and 40 cycles for the second round. Conditions for this PCR were optimised (1.5mM $MgCl_2$, 200 µM dNTP, 0.25 µM primers, 2µl of 10x Taq buffer, 0.3 µl Taq (0.25U/ml) and 2 µl of cDNA template in 20 µl total volume) and the following protocol used.

Stage 1: Denature 90°C 4 min

Stage 2: Denature 92°C 20 sec

Anneal 55°C 30 sec

Stage 3: Extend 70°C 2 min 30 sec

Five distinct DNA bands were visualised on agarose gel electrophoresis after the second round of PCR. These products were excised from the agarose gel, purified using a Qiaquick gel purification column (Qiagen) and ligated into pGEM-T. These DNA bands were sequenced and used to probe the *O. scutellatus* cDNA library.

PCR 3: primers synthesized from N terminal and MS sequences

Primer sequences were:

Forward primer, from N terminal sequence (2) ATCPQ (sense)

i	5'- GCA TGC ACI TGT CCI CAA- 3'
ii	5'- GCA TGC ACI TGT CCI CAG- 3'
iii	5'- GCA TGC ACI TGC CCI CAA- 3'
iv	5'- GCA TGC ACI TGC CCI CAG - 3'
v	5'- GCA TGC ACI TGI CCI CAI - 3'
N-terminal (EPLPLL)	5'- CCG CGG ARCCIYTICCIYTIYTITA - 3'
MS peptide 4	5'- GCGGCCGCIGTITTYTTYCA-3'
(MS4)	5'-GCNGNTTTYTTYCAR-3'
(MS4RC)	5'-YTGRAARAANACNGC-3'
T3	5'-TAAATTGGGAGTGATTTCCC-3' and
T7	5'-TAATACGACTCACTATAGGG-3'

Reverse primer, pBS vector, upstream from T7 site5'-GGTACCCGGCCCCCCC-3'

Optimisation of conditions for PCR incorporated temperature and magnesium gradients, and variations in the concentration and stocks of dNTP, primer, Taq polymerase and water. Controls using DNA worked, while PCR utilising the cDNA library did not.

2.2.2.12 Sequencing

Due to the difficulty of sequencing DNA within SOLR™ cells, samples were either sequenced using a TempliPhi protocol (Amersham) or subcloned into DH5-α or NM522 cells. From these cells the plasmid could be isolated and sequenced.

DNA Template preparation

Manual Miniprep (Manniatis)

Plasmid DNA prepared by the alkali miniprep method was treated with RNase prior to sequencing. Following removal of RNAse using phenol/chloroform the DNA was precipitated using sodium acetate and ethanol.

Miniprep kit (Qaigen)

DNA isolated using Qiagen Miniprep Kits was sequenced without further treatment.

Templiphi (Amersham)

DNA was amplified using Templiphi (Amersham) according to the manufacturer's instructions.

Sequencing Reaction

Sequences were initially determined using an ABI 310 capillary sequencer with later samples being sequenced in an Amersham MegaBACE 1000. Sequencing samples were prepared using the ABI-Big Dye terminator and ET terminator sequencing kits according the manufacturer's instructions.

2.2.2.13 Analysis of sequence data

Duplicate DNA sequences of mRNA clones from the *O. s. scutellatus* cDNA library were analysed using EditView, MacVector™ 7.0 or Sequencher programs. The translation of the putative protein from the nucleotide sequences was undertaken using either MacVector™ or Sequencher programs. N-glycosylation, potential cleavage and eukaryotic promoter site predictions together with theoretical hydrophobicity, size and pI's were undertaken using Mac Vector™.

Identification of the open reading frame of the sequences was undertaken using MaVector™ or Sequencher software. The identification of an ATG start codon, using a

universal vertebrate codon usage set, supported by either Gribskov, MacVector™ or Staden codon preference plots were used to suggest and confirm an open reading frame.

These open reading frames were often supported with matches from database comparisons. Public database comparisons of *O. s. scutellatus* nucleotide and inferred protein sequences were carried out using the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). The nucleotide (Blastn), translated and protein sequence (Blastx) databases were mined to find homologous sequences.

Where an open reading frame could not be found, but partial sequence matched proteins within the database, different codon usage sets were used. Open reading frames were revealed with some sequences using vertebrate mitochondrial codon usage sets.

Sequence comparisons were performed using the ClustalW alignment program within MacVector 7.0 and at the ExPASy website (http://www.expasy.ch) where domain similarities were also identified.

Chapter 3

Comparison of *Oxyuranus* species venoms

3.1 STORAGE OF VENOMS

Snake venom is a mixture of potent biological enzymes. These enzymes are secreted and stored in the lumen of a gland in venomous snakes (Munekiyo and Mackessy, 1998) only millimetres from the animal's brain. However, apparent autolytic and autopharmacological reactions do not occur. Protective endogenous components have been found in venom (Francis and Kaiser, 1993; Thwin and Gopalakrishnakone, 1998; Valent, *et al.*, 2001; Thwin, *et al.*, 2002) and serum (Hains and Broady, 2000) and other undiscovered protective mechanisms certainly exist as well. Once these biological enzymes are expressed from the animal these many secretory products are active, the end products seen in bite victims as neurotoxic components infiltrating neurons, haemostatic and necrotic factors that cause coagulopathy and dissolving muscle. Nevertheless, the mechanism of animal venoms and their storage *in vivo* is little understood.

Compounding this was the reported degradation of venom after the injection of venom from snakes (Sugihara, *et al.*, 1972), resulting in the common use of lyophilisation to store venoms. Therefore it was important to establish optimal storage conditions for the crude *Oxyuranus* venoms for baseline observations, in the absence of lyophilising facilities, and to form a foundation upon which this project could be based. These considerations were of particular importance when ideal conditions could not be met, such as venom collection in the field

With little information available of venom storage mechanisms, there has not been much resolution of the storage methods or the analysis of these venoms to establish their integrity. Electrophoresis is one of the major techniques available to current biochemical investigation. Its application has been greatly expanded over the past few years due to simplified equipment, which permits quick and precise analyses. The use of electrophoresis has been suggested for use as an auxiliary tool

for an efficient and easy method of venom analysis of species of the Elapidae and Viperidae families (Soares, *et al.*, 1998; Magro, *et al.*, 2001).

One study has demonstrated the stability of venom incorporating enzyme activity and electrophoretic mobilities. Within this study a strong correlation between the reproducible electrophoretic patterns of specific venoms and the integrity of venom proteins was concluded. This study also revealed all enzymes assayed[1] were unaffected by differing storage methods with the exception of activity caused by of L-amino oxidase (Munekiyo and Mackessy, 1998; Hill and Mackessy, 2000). Other reports have also found that the effects of preparatory procedures may have little effect on the stability of the whole venom protein banding patterns after isoelectric focusing (Russell, 1964; Egan and Russell, *et al.*, 1985). Also, that LD_{50} values were largely unaffected by crude or lyophilised venom storage conditions.

Previous electrophoretic studies have indicated that prompt lyophilisation prevented degradation of protein components, maintained biological activities (Russell and Eventov, 1964) and did not produce alterations in electrophoretic mobilities of protein components (Gene, *et al.*, 1985; Gregory-Dwyer, *et al.*, 1986). Yet other reports have stated such treatments can adversely affect electrophoretic mobilities (Willemse and Hattingh, 1985) or enzymatic/biological activities (Villegas, *et al.*, 1993). Further research has also suggested that many biological and enzymatic activities of venom are stable over many years (Russell, *et al.*, 1960; Sugihara, *et al.*, 1972; Egan and Russell, 1984; Russell, *et al.*, 1985), regardless of storage. Therefore it was important to establish an observable and consistent baseline for the storage and handling of *Oxyuranus* venoms from which comparisons could be based.

3.2 RESULTS

The average venom yield of *O. scutellatus* varied from 45 mg for the smaller female snake to 85 mg for the larger male for a single milking, these average

[1] Caseinolytic protease, Thrombin like protease, kallikrein like protease, phospholipase, phosphodiesterase

amounts were reflected with *O. s. canni* with a pooled sample of 100 mg, snake and venom statistics summarized in Table 3.1.

The pH of the snake venoms was determined using Merck pH sticks pH range 1 to 14. Twenty microlitres of whole venom (with no buffer) and 20 µl of venom and water (1:1) were placed onto the pH sticks. Each test showed a pH between 6 and 7.

Table 3.1: Statistics of *Oxyuranus* species contributing to this study.

Species	Sex	Weight (Kg)	Total Length(cm)	Yield (mg)	Notes
OS	M	1.55	193	60 - 110	Housed at Billabong Sanctuary Townsville. Will either be locally caught Cairns animals or captive-bred ex-zoo stock of undeterminable origin.
OS	F	0.75	178	30 - 60	As above
OM	M	0.8	141		As above
OM	F	0.6	149.5	Pooled 15-45	As above
OM	F	0.7	149		
OSC	Pooled	*M 0.50* M 0.82 F 0.94	168 188 205.5	100	Moreguina PNG 2002 Moreguina PNG 2003 Moreguina PNG 2003 GPS coordinates are: 10*0'58.392"S, 148*28'25.644"E using the WGS84 Datum

Weight (kg); Length (cm) is mean snout to tail tip. Average length of snakes are reported of *O. s. scutellatus* 290cm, *O. microlepidotus* 170cm and O. s. canni ; Age (months)

3.2.1 Stability studies of whole venom

1) Effect of time at 25°C upon the stability of venom

There was no change in the banding pattern of proteins from samples incubated at 25°C for 12, 24, 48 and 72 hours as determined by SDS-PAGE (results not shown).

2) Effect of Temperature upon the stability of venom

There was no change in electrophoretic patterning following treatment at temperatures up to 80°C. At 100°C the protein precipitated. After dissolving the precipitate in SDS buffer and visualising the banding pattern using SDS-PAGE and protein staining some bands had become faint or disappeared between the sizes 40 kDa and 66 kDa. This sample revealed banding patterns similar to that seen within the pH study after dilution (Figure 3.1).

3) Effect of pH upon the stability of venom

Venom samples were returned to pH 7.6, aliquots (50 μg) and were loaded and separated on 12% SDS-PAGE and visualised with silver staining. The use of 15% SDS polyacrylamide gels could not resolve the lower molecular weight proteins, thus these were sacrificed to increase resolution of the higher molecular weight proteins (see Figure 3.1). All samples demonstrated the same major banding patterns across all pH values. At least twenty protein bands ranging from 200 - 6.5 kDa were visualised in all sample treatments reflecting the control sample. The control sample was run directly from stored venom and diluted with the same storage buffer to be similar to those samples within the pH test. As the buffer was at pH 7.6, pH 7 was not undertaken in this trial.

The conformity between the venom samples at the varying pH were tested further. The above examination was repeated at pH 6 and 9 using dilutions of venom up to 1 in 10,000. These samples were run in conjunction with differing samples of venom with 1 mM PMSF, 1 mM benzamidine and protease cocktail (1 mg/ml). One microgram of venom samples were separated using 15% reducing SDS-PAGE and were visualised using silver staining (see Figure 3.2).

3.2.2 Electrophoretic analysis

The duplication of venom samples was checked after successive taipan milkings by visualisation of whole venom (80 μg) on 15% SDS-PAGE. No obvious differences of venom patterns between venom 'milking' were discerned (Figure 3.3) or between sexes of the same species. *O. s. canni* venom was pooled over several milkings before analysis thus was not included within this examination. SDS-PAGE showed striking (no pun intended) similarity between banding patterns and elecrophoretic migrations for the most abundant/major proteins visualised between species.

All three species showed complex banding patterns for reduced SDS-PAGE ranging from 120 kDa to 6 kDa, containing an apparent 20 to 30 protein components. Major banding was seen at 120, 100, 80, 66, 45 and 30 kDa (Figure 3.1 and 3.3). Initial visualisation was undertaken using Coomassie blue stain.

However, silver staining was later used to detect the less abundant proteins. Of note was the apparent change in individual protein size on SDS-PAGE dependant on the amount of venom loaded. As it was important to establish accurate protein mass, varying amounts of venom (40, 60 and 80 μg) from *O. s. scutellatus* and *O. microlepidotus* were separated on 15% and 7.5% SDS-PAGE. Venom protein masses were shown to vary slightly depending upon the amount of protein loaded onto the gels (Figures 3.4 and 3.5).

This comparison was continued under reducing (-DTT) and non-reducing (+DTT) conditions (50 and 10 μg)(Figure 3.6 and 3.7). Protein bands of 39 kDa and 45 through to 66 kDa were visualised under non-reducing conditions, while banding from 30 to 35 kDa and 100 kDa were not present within the same samples.

Whole venom visualised on native polyacrylamide gels revealed four broad protein groups and were not used for further comparison between the species. Tricine polyacrylamide gel electrophoresis was used to resolve the small (15-35 kDa) abundant proteins. This method of electrophoresis did not resolve any smaller proteins that could not be seen on 15% SDS-PAGE gel electrophoresis (results not shown) and were not continued.

3.2.3 Separation and identification of venom components

High pressure liquid chromatography (HPLC) was used to separate the three *Oxyuranus* venoms. This was achieved through size exclusion chromatography (SEC), anion exchange and cation exchange chromatography.

3.2.3.1 Size exclusion chromatography (SEC)

SEC from all three snake venoms produced six major protein peaks (summary Figure 3.8), the sizes of the proteins in each of these peaks summarised in Table 3.2. SEC of venom from *O. s. scutellatus* and *O. s. canni* are represented Figure 3.9, *O. s. scutellatus* and *O. microlepidotus* venom and their fractions visualised by SDS-PAGE in Figure 3.10.

Figure 3.1: The effect of pH on the degradation of whole venom (50 ug).
Whole venom (50 µg) of OS, OSC and OM was resolved on a 12% polyacrylamide gel and visualised by silver staining. OS and OSC revealed similar if not identical patterning whilst OM showed similar major banding with distinct differences with lesser expressed proteins.

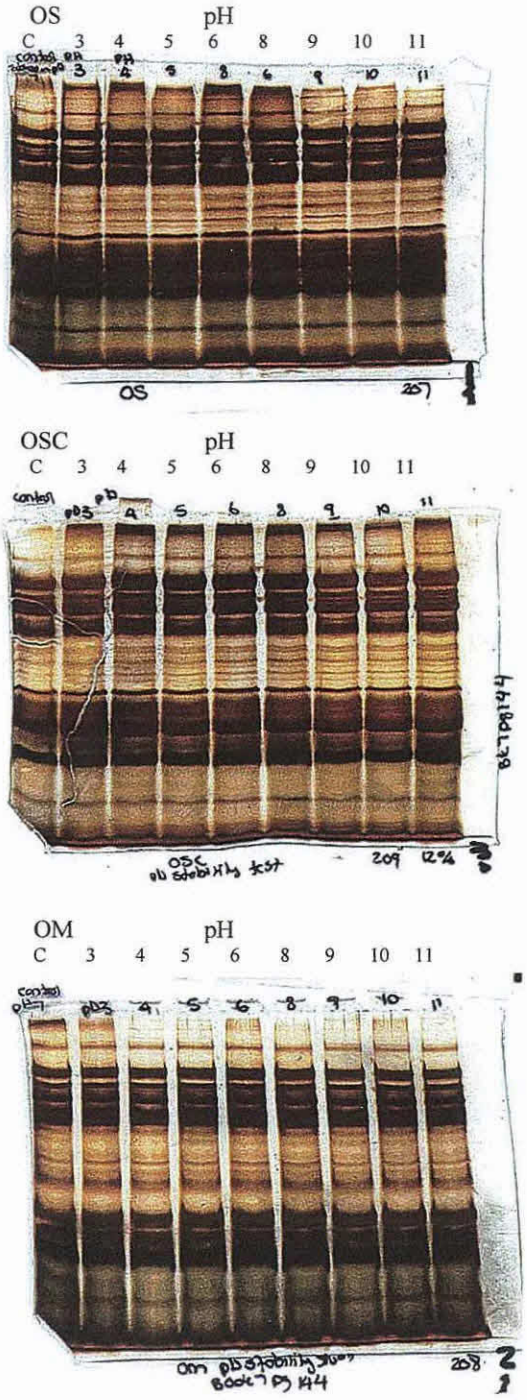


Figure 3.2: Effect of pH on the degradation of whole venom (1 µg).
Whole venom (1 µg) of OS, OSC and OM was resolved on a 15% polyacrylamide gel and visualised by silver staining. OS only pictured.

C:	Control, sample on ice
C pH 7.6:	Sample not pH treated, diluted to copy pH samples
pH 6:	Sample exchanged to pH 6
pH 6 + Ben:	Sample at pH 6 + 1mM Benzamidine
pH 6 +PMSF:	Sample at pH 6 + 1mM PMSF
pH 6 + P. inhib:	Sample at pH 6 + Bacterial Protease Inhibitor Cocktail mixture (2ml/20ml)
pH 9	as above pH 6 samples

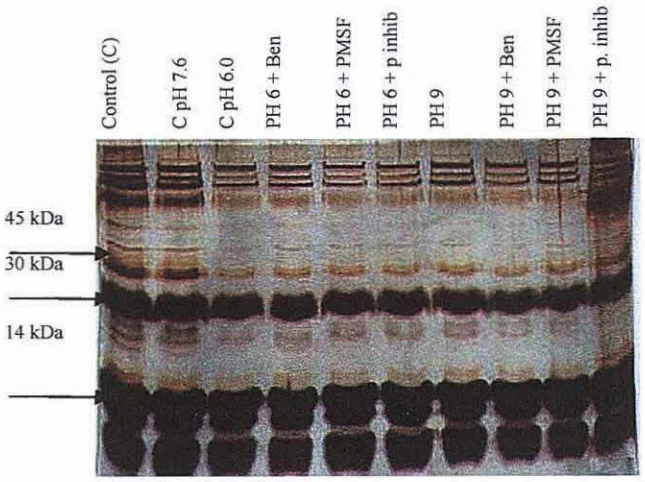


Figure 3.3: Successive venom samples obtained over time.
After each 'milking' whole venom (80 µg) of OS and OM were visualised on 15% SDS-PAGE and stained with Coomassie blue R250.

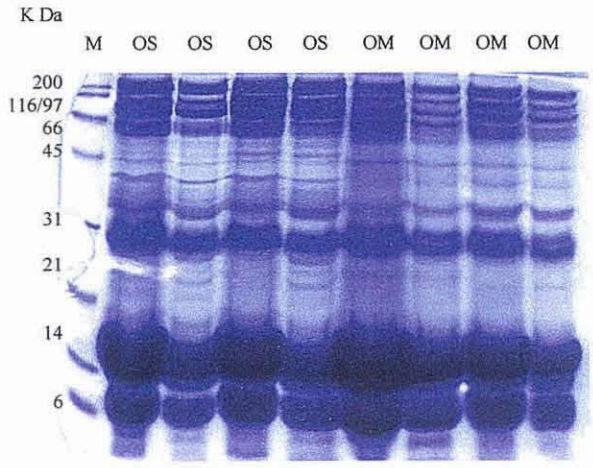


Figure 3.4: The effect of protein amounts (µg) on whole venom electrophoretic patterns.
Different amounts (40, 60, 80 µg) of OS and OM whole venom separated using 15% SDS-PAGE and visualised via silver staining

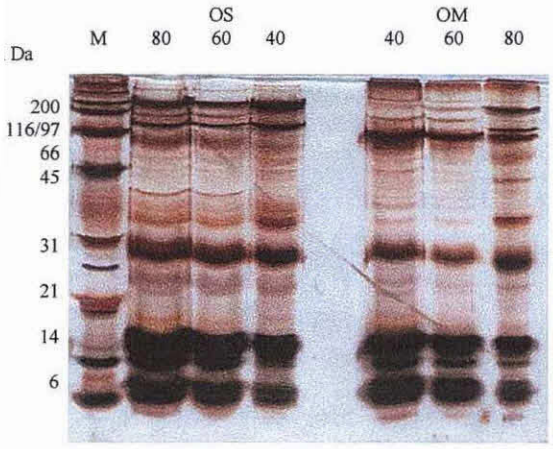


Figure 3.5: The effect of protein amounts (µg) on whole venom electrophoretic patterns.
Different amounts (40, 60, 80 µg) of OS and OM whole venom separated using 7.5% SDS-PAGE and visualised via silver staining

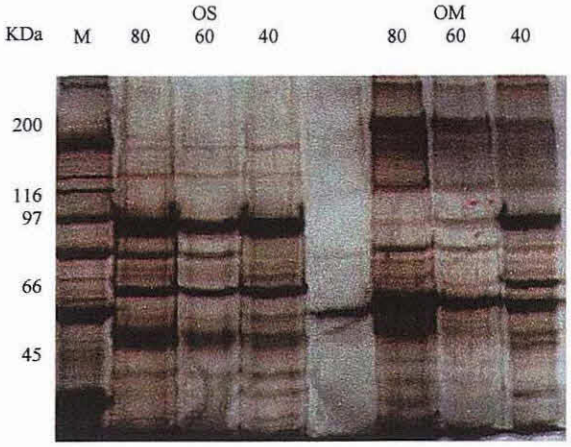


Figure 3.6: Effect of reducing and non-reducing conditions on whole venom electrophoretic patterns.
Reduced whole venom samples (+DTT) of OM, OS and OSC (10 and 50 µg) compared to non-reduced samples (-DTT) of OM, OS and OSC (50 ug) separated using 15% SDS-PAGE and visualised via silver staining

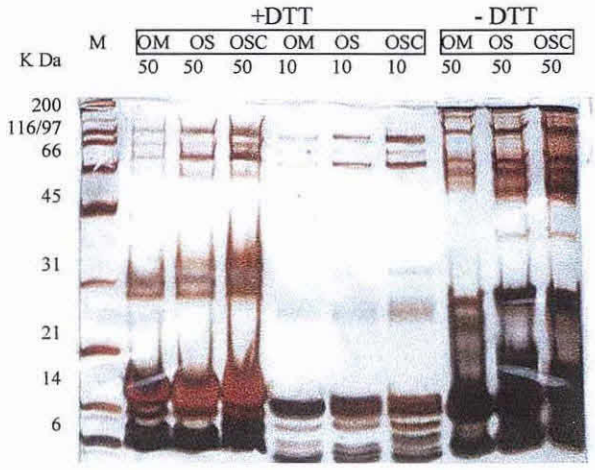


Figure 3.7: Effect of reducing and non-reducing conditions on whole venom electrophoretic patterns.
Reduced whole venom samples (+DTT) of OM, OS and OSC (10 and 50 µg) compared to non-reduced samples (-DTT) of OM, OS and OSC (50 µg) separated using 7.5% SDS-PAGE and visualised via silver staining

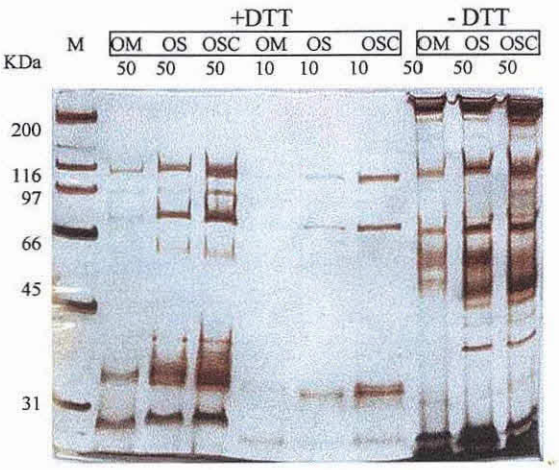


Figure 3.8: Gel filtration Trace of OS venom.
Venom was diluted 1:100 in storage buffer of 50mM HEPES (pH 7.6)+100mM NaCl and fractionated, the Phenomenex BioSep SEC S-2000 column and guard column also equilibrated with this buffer. All samples were loaded via a 1 ml static loop and run at 5 ml/min at 4°C.

The protein fractions eluted were measured at 280nm and patterns analysed on BioRad BioLogic Duo-Flow software. Each of these patterns could have numbered 'tags' manually placed onto each peak. Protein patterns are blue and relate to the absorbance scale on the left of the pattern, also in blue. To the right of this scale in black is the % buffer B. Within this protein pattern is a red line signifying conductivity, reflecting changes in salt concentrations. This scale is seen in red to the right of the pattern. While at the top and bottom of the trace are the fraction numbers collected and time respectively.

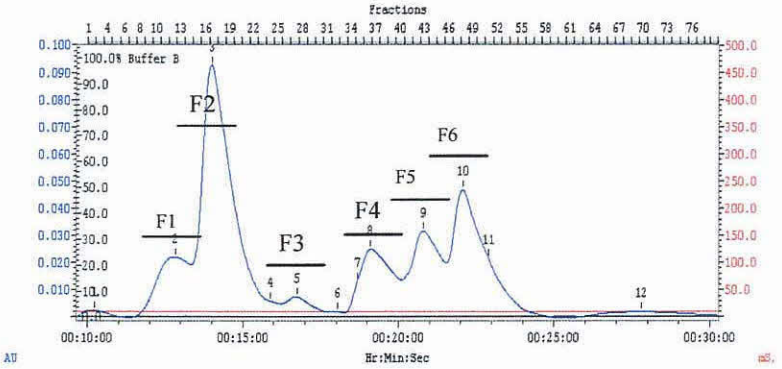


Table 3.2: Summary of the time of elution compared to gel filtration marker (BioRad).
The HPLC separations were compared to 9 mg of Gel Filtration marker (BioRad) which eluted on average at 14.2 min (670 kDa), 15.7 min (158 kDa), 17.4 min (44 kDa), 19.2 min (17 kDa) and 22.9 minutes (1 kDa), respectively.

Fraction	Time the fraction eluted (min)	Size of protein (Da)
F 1	14.8-16	350,000-120,000
F 2	16.5-17.5	100,000-40,000
F 3	19.6-20.2	12,000-9,000
F 4	21.2-21.8	4,500-2,700
F 5	21.8-24.5	2,700-<1,000
F 6	24.5-26	<1,000

Figure 3.9: Comparison of gel filtration profiles of whole venom from OS and OSC.
Crude venom from each species was subjected to gel filtration and the elution profiles analysed using BioRad software. Conditions were per Figure 3.8 with one variation, samples were run at 3 ml/min due to column pressure reaching maximum pressure at 5ml/min.

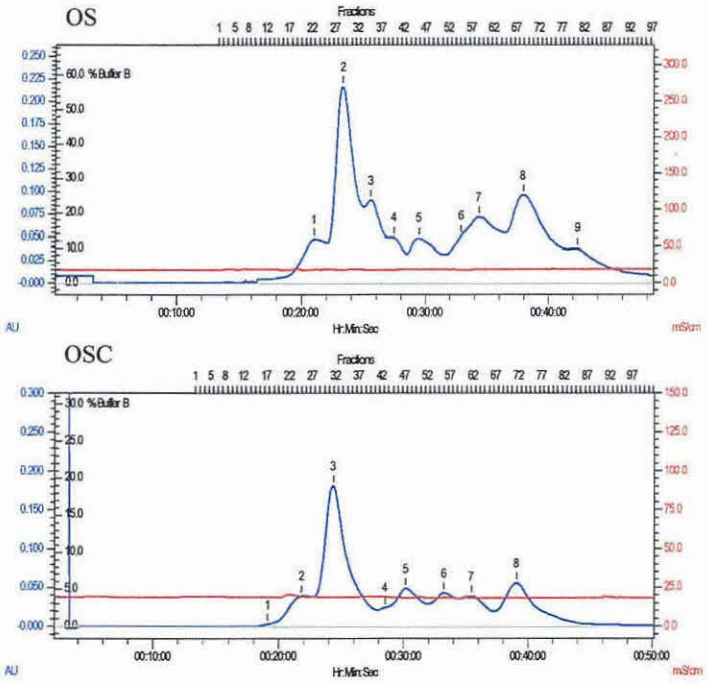
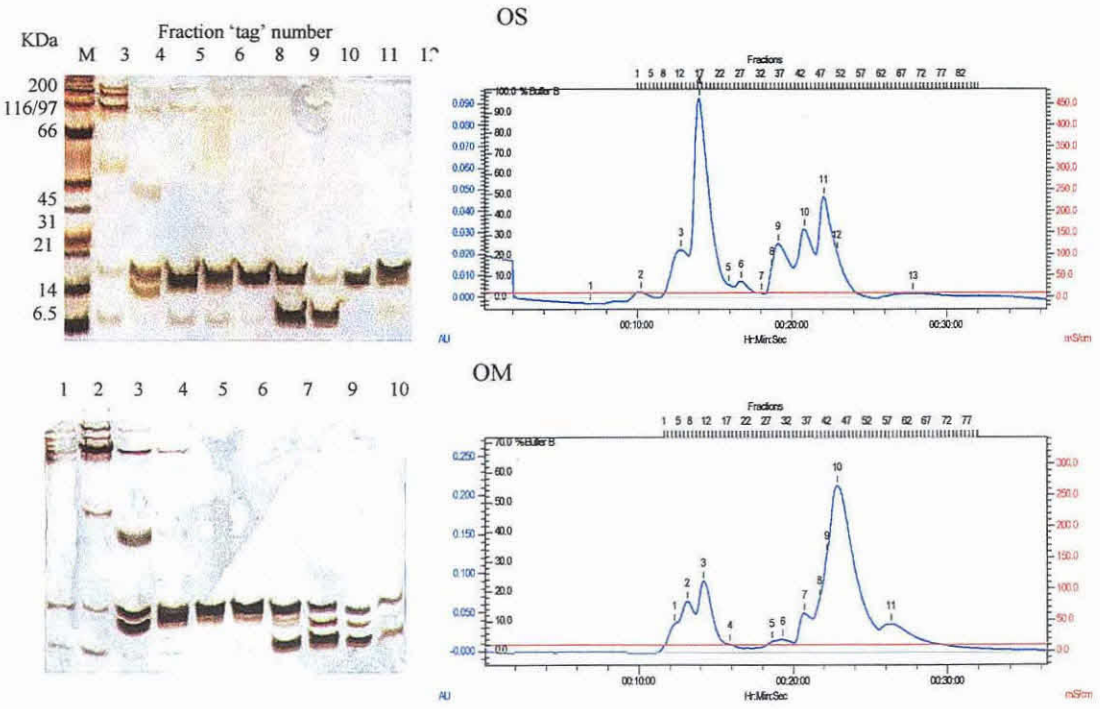


Figure 3.10: Comparison of gel filtration profiles of whole venom from OS and OM.

Crude venom from each species was subjected to gel filtration and the elution profiles analysed using BioRad software. Conditions were per Figure 3.8. Samples of each fraction numbered (15 µl) were separated on 15% SDS-PAGE and visualised using silver stain.



3.2.3.2 Ion Exchange Chromatography

To extend the comparisons of the three species, ion exchange chromatography was used. Anion exchange chromatography revealed profiles with broad peaks with low resolution for all three venoms (Appendix V). Similar to gel filtration samples, SDS-PAGE of these samples revealed similar patterning, though did not reveal minor proteins (results not shown). Due to these problems cation exchange chromatography was used. Initial separation of whole venom (1 mg) revealed similar, well resolved protein peaks for each species.

The pH of the running buffer was decreased from pH 7.6 to 6.0. The resultant protein peaks eluting at pH 6.0 were more defined, with proteins eluting with 16% higher of buffer B (1M NaCl) (Figure 3.11). Protein fractions eluting at differing percentages of Buffer B and are summarised in Table 3.3 and reflect the chromatography profile in Figure 3.11. Subsequent 15% and 7.5% SDS-PAGE gels of proteins within these fractions are shown in Figure 3.12. The majority of proteins for each species eluted between 300 to 600 percent 1M NaCl, with acidic bands eluting in the flow-through and basic proteins eluting with higher percentages of Buffer B. Many proteins of the same size appeared to elute in numerous fractions.

Figure 3.11: Cation exchange chromatography profile of OS whole venom.
One milligram of whole venom was loaded onto a Uno S column using a linear gradient of 0 to 100% Buffer B (1M NaCl), with a flow rate of 0.5 ml/min at 4°C.

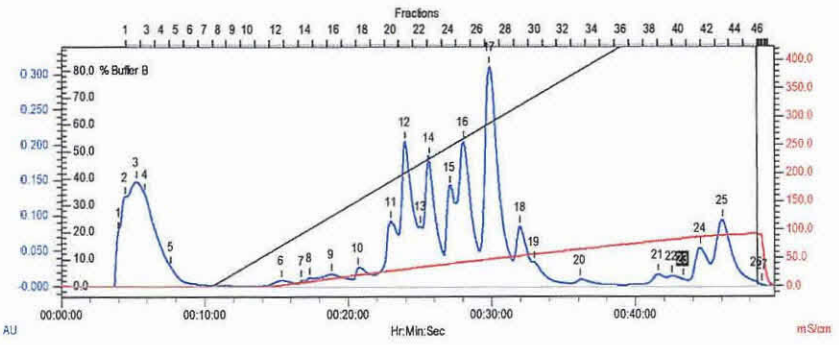
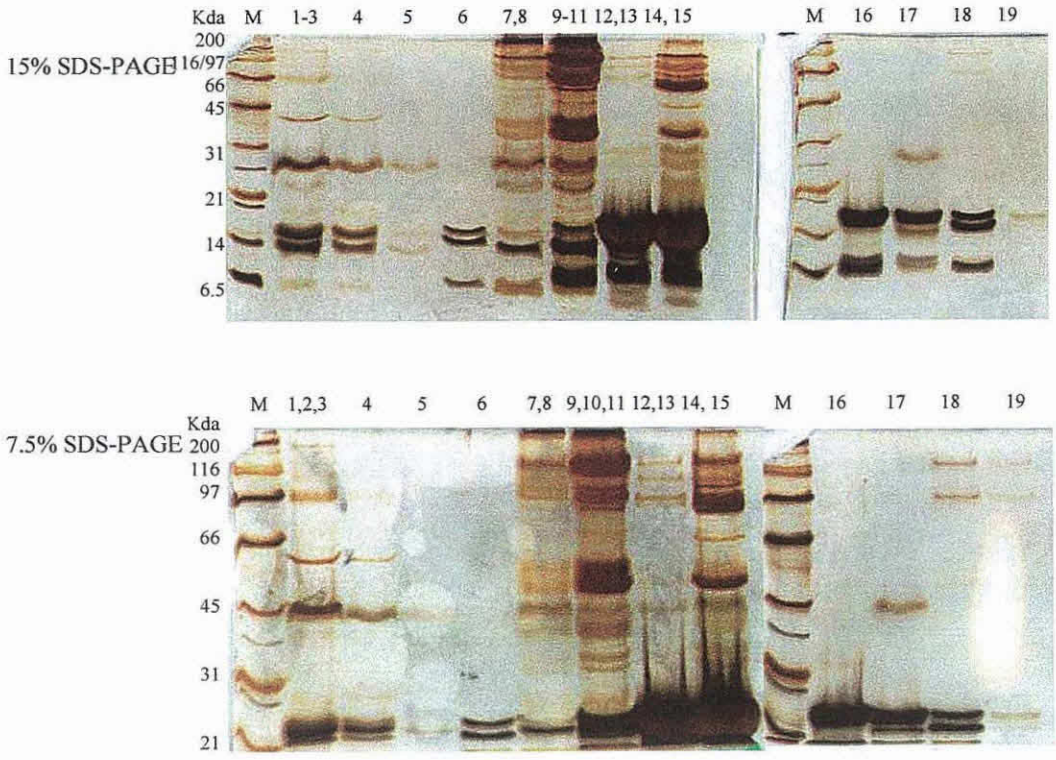


Table 3.3: Fraction number from Figure 18 and the % of Buffer B (1M NaCl) the fraction eluted within.

Fraction no.	% Buffer
1-4	0
5-6	18
7	20
8	22
9	24
10	26
11	28
12	32
13-14	34
15	38
16	44
17	63
18	64
19	78
20	98
21-22	100

Figure 3.12: Fractions from Cation exchange chromatography visualised via SDS-PAGE. One milligram of whole venom was loaded onto a Uno S column using a linear gradient of 0 to 100% Buffer B (1M NaCl), with a flow rate of 0.5 ml/min at 4°C. Samples from fractions were separated on 15 and 7.5% SDS-PAGE and visualised via silver stain.



3.2.4 Two Dimensional Gel Electrophoresis (2DE)

Five to ten micrograms of *O. s. scutellatus* and *O. microlepidotus* crude venom separated using 2DE revealed approximately 100 protein spots which were visualised following silver staining. *O. microlepidotus* appeared to contain more numerous low molecular weight proteins (Figure 3.13). Due to the repetitive similarities of *O. s. scutellatus* and *O. s. canni* venoms throughout prior electrophoretic and chromatographic methods, comparisons between these species was not undertaken. *O. s. scutellatus* and *O. microlepidotus* were very similar with heavy spotting at the acidic molecular mass ranges between 14 kDa and 45 kDa, trains of spots at 30 kDa and the larger proteins above 80 kDa and smears at the low molecular weight basic pI

Choosing a narrower pI range of IPGphor strip separated the same venom proteins though did not reveal more protein spots. Due to the large number of basic proteins visualised via 2DE a basic 11 cm pH 6 - 11 range was utilised (results not shown). Although expanding the range of pI from 6 to 11 increased the length of the smear rather than resolve these protein patterns. Duplicate samples were run on 2DE and differences were apparent between these samples with some protein spots occurring only in some gels of duplicate samples (Figure 3.14).

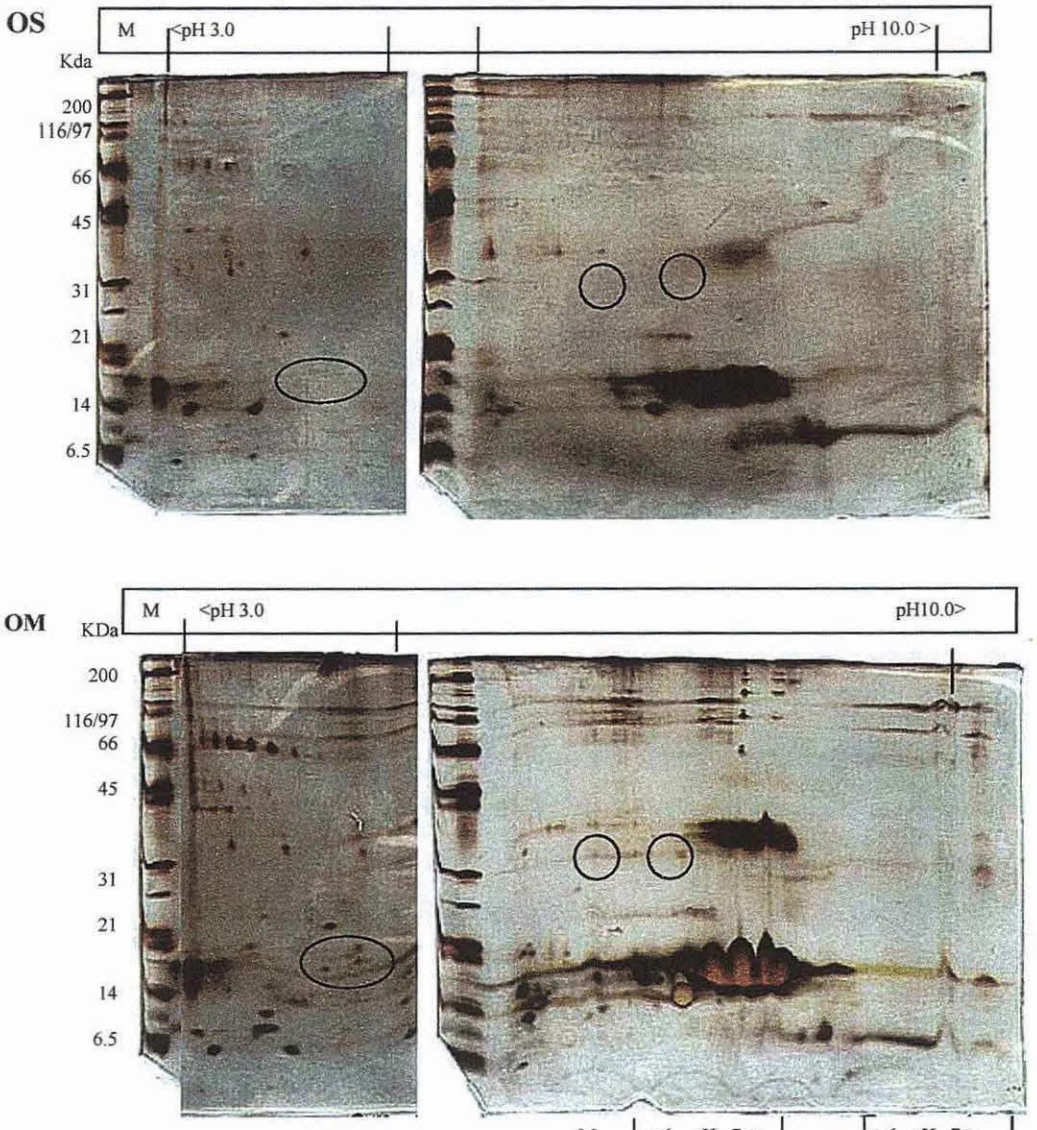
3.2.4.1 Fractionation of whole venom proteins for 2DE

The separation of whole venom on 2DE did not achieve the visualisation of less abundant proteins nor the isolation of individual spots below 14 kDa. Therefore, whole venom was fractionated into proteins greater and lesser than 20 kDa with fractions separated on 2DE. Samples from SEC were visualised on SDS-PAGE to check their integrity.

Once confirmed, SEC fractions 1 and 2 (proteins 300 - 40 kDa), were pooled and concentrated (Ultra free 10,000 MWCO, Millipore). While SEC fractions 4 to 6 (proteins < 40 kDa) were pooled and concentrated (Centricon 3,000 MWCO). These were denoted *O. s. scutellatus* (OS) and *O. microlepidotus* (OM) fraction one (F1 - >20 kDa) and fraction two (F2 - < 20 kDa). Since protein fractions from

cation exchange were visualised with high resolution, samples were concentrated and buffer exchanged into 500 µl of 50 mM MES (pH 6.0).

Figure 3.13: Two dimensional gel electrophoresis of OS and OM whole venom.
OS and OM whole venom was separated on an 11 cm IPG strip pH 3-10. IPG strips were cut to run over two SDS polyacrylamide gels cast in a BioRad Mini Protean II.



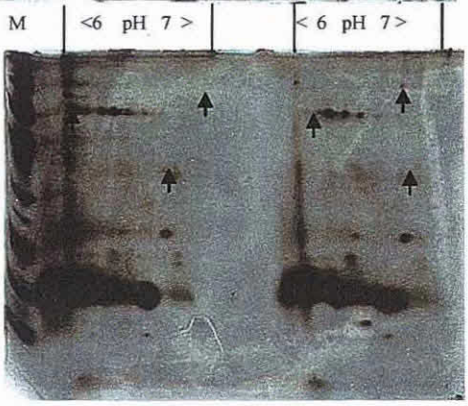
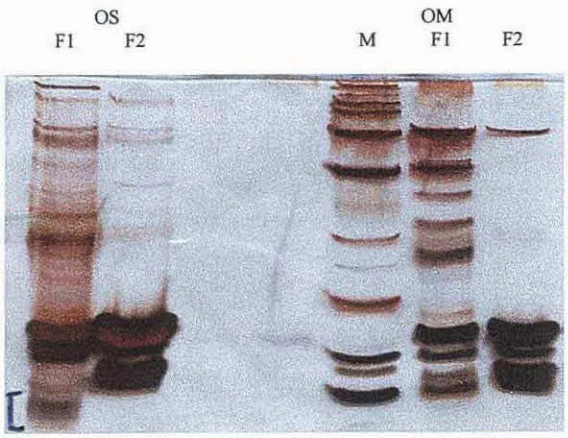


Figure 3.14: Two dimensional gel electrophoresis of duplicate samples from OS whole venom.
OS whole venom was separated on an 11 cm IPG strip pH 6-11.

Degradation was apparent in the OS F1 with many band patterns and smearing of the gel; this was not seen in OM F1 although the large bands of 120, 100 and 80 kDa were very faint where they were previously pronounced (Figure 3.15). Fraction two from both *Oxyuranus* venoms appeared unaffected, although, due to the abundance of proteins at this size it would not be possible to discern if proteins had degraded. When this fractionation was repeated using HEPES (pH 7.6) + 10% glycerol, no degradation was seen (results not shown).

Figure 3.15: The Effect of fractionation of OS and OM whole venom at pH 6.0.
OS and OM whole venom was separated into protein sizes greater than 20 kDa (F1) and less than 20 kDa (F2). Proteins were separated via 15% SDS-PAGE and visualised using silver stain.



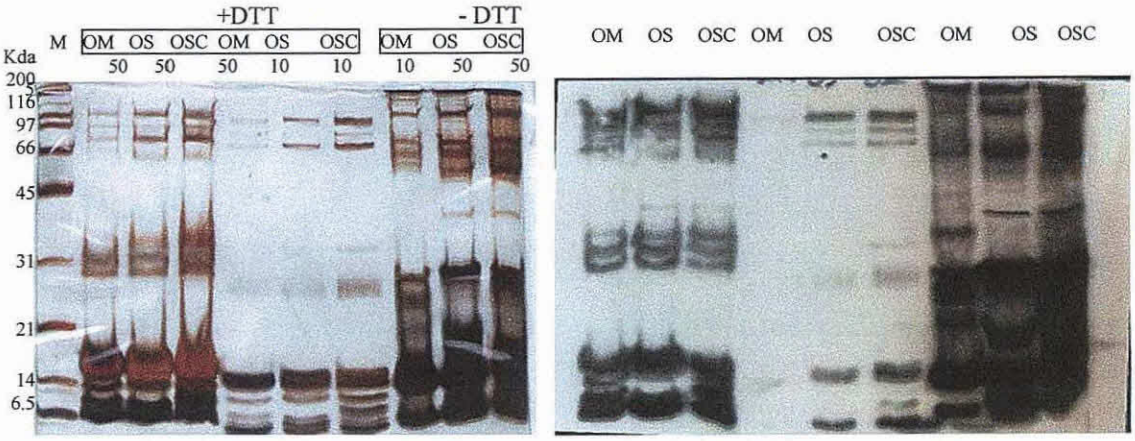
3.2.5 Antivenom binding studies

Whole venom from the three species were separated by 15% SDS-PAGE and duplicate samples were visualised with either silver staining or immunobinding. Antibody binding was detected by ECL (Figure 3.16). All proteins were antigenic with the exception of protein bands from 40 to 60 kDa for all three species under reducing and non-reducing conditions. Higher concentrations of venom proteins followed by immunoscreening did not increase antivenom binding in this size range. The differing sized bands noted in 1D SDS-PAGE of *O. microlepidotus* venom proteins compared to *O. s. scutellatus* were also antigenic.

Due to the overlap of proteins seen in whole venom, fractions from SEC were separated on reducing SDS-PAGE in duplicate, and either immunoscreened or visualised via silver staining (Figure 3.17). The proteins from 60 to 120 kDa were antigenic for all *Oxyuranus* species. Antivenom bound strongly to *O. s. scutellatus* venom proteins across all fractions, and all venom proteins from *O. s. canni* also bound antivenom, although with differing intensities. Of interest was the smaller molecular weight proteins, incorporating many of the phospholipase and neurotoxic

subunits, which were not antigenic between species. Of importance was a lack of antivenom binding within the fractionated *O. microlepidotus* venom. Many of the proteins from fractions two to five, the 5-14 kDa proteins, from *O. microlepidotus* did not bind the antivenom commercially produced from *O. s. scutellatus* venom.

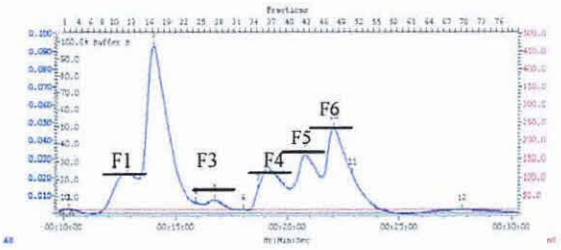
Figure 3.16: Western blot analysis of *Oxyuranus* venom proteins.
Immunobinding study of OM, OS and OSC whole venom separated on 15% SDS-PAGE. Proteins visualised using silver stain or taipan monovalent antivenom to which goat anti horse HRP conjugated antibodies were bound and visualised using ECL.



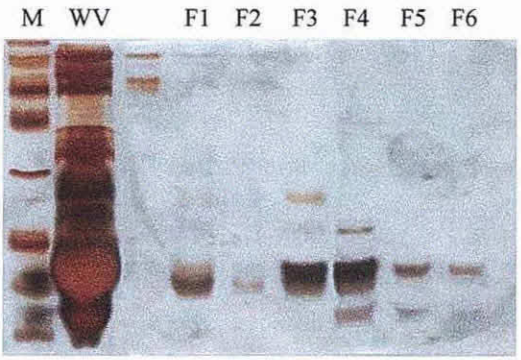
3.2.6 Glycosylation of Venoms

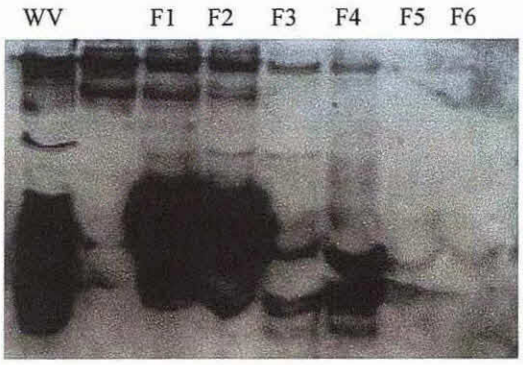
Reflecting the antibody binding to whole venom (Figure 3.16), Concanavalin A also bound to all proteins visualised via SDS-PAGE with proteins from 40 to 60kDa not binding. Separation of whole venom via SEC fractions revealed all proteins larger than 60 kDa were glycosylated while approximately 50% of proteins below 20 kDa were not (results not shown).

Figure 3.17: Immunobinding study of SEC fractions from OS, OSC and OM whole venom. Whole venom from Oxyuranus were separated through gel filtration chromatography and separated on 15% SDS-PAGE. Polyacrylamide gels were either silver stained or immunoscreened with Taipan monovalent antivenom to which goat anti-horse HRP conjugated antibodies were bound and visualised using ECL.

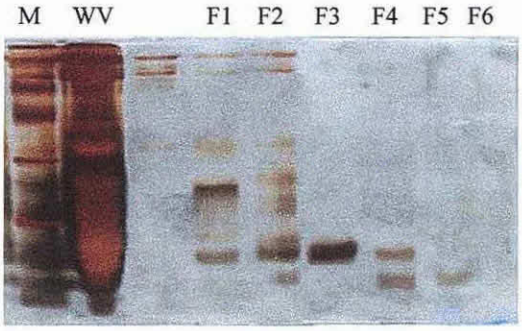


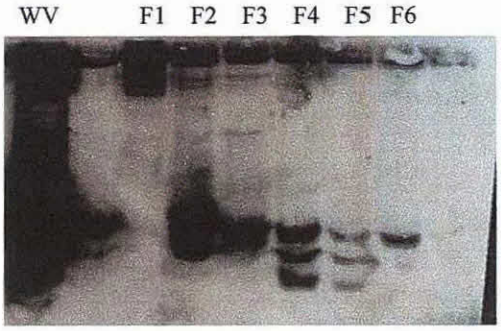
OS



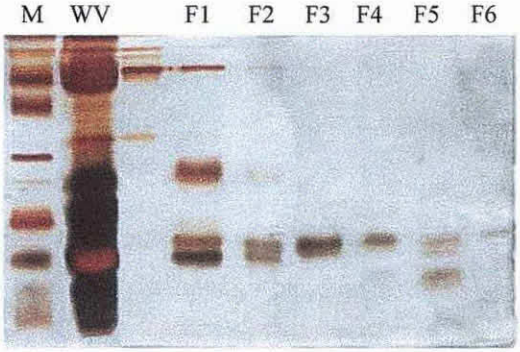


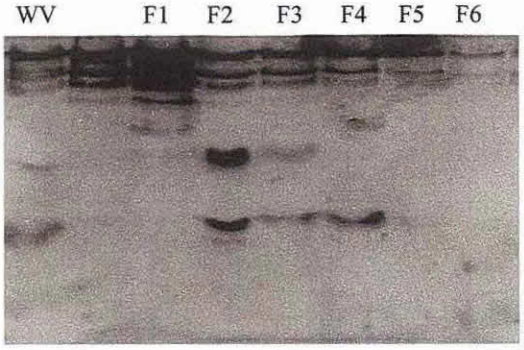
OSC





OM





3.3 DISCUSSION

3.3.1 Details of *Oxyuranus* snakes used within this study

The average venom yield of *O. s. scutellatus* varied from 45 mg for the smaller female snake to 85 mg for the larger male for a single milking, these average amounts were also reflected in *O. s. canni*, with a pooled sample of 100 mg. The average yield of venom is reported as 120 mg (Trinca, 1969). Morrison *et al.* 1982, determined the quantities of venom injected by 11 captive *O. s. scutellatus* varied from, at first strike 20.8 ± 6.4mg, 36.6 ± 12.5 mg at second strike and 46.5 ± 23.1 at third strike (Morrison, *et al.*, 1982). Therefore the venom yields of these snakes were within the expected ranges. The venom yields and protein content are reported to vary with differing methods of extraction (Mackessy, 2002). Consequently, a repetitive method of venom extraction was used, resulting in a viscous sample of 100 to 500 µl, from which reproducible venom profiles were obtained. Large amounts of saliva using this method of extraction resulted in a mucoid sample, necessitating centrifugation of the sample prior to all work. Inhibitors were not added to venom for storage after initial investigations were completed due to the lack of degradation of whole venom observed.

3.3.2 Stability study of whole venom

Venom was subjected to varying conditions to judge the degradation of venom components due to the length of time left exposed to the atmosphere and the stability at differing temperatures and pH. This information would provide a basic comparison between the venoms, and would indicate the optimal handling parameters required for the collection to the storage of venom. Once the venom conditions were established to be consistent and comparable the proteins, or proteome of *Oxyuranus* venoms could be analysed.

The results of these stability studies were displayed via SDS-PAGE, not, as is commonly undertaken, via biological assays due to the extent of varying venom functions. Changes in banding patterns such as lightening of bands or extra lower bands when compared with controls were assumed to be degradation. The potential

weakness of an argument regarding venom stability in *Oxyuranus* venoms might be that without pharmacological studies to verify activity, there might be toxins that maintain structural integrity, but not their function. The lack of apparent autolytic activity, evidenced by consistent venom patterns through electrophoresis and chromatography, indicates these toxins retain their structural properties and, in turn, their functionality.

This hypothesis was substantiated by Munekiyo and in subsequent enzyme assays, where an isolated proteins' lowering or cessation of activity was reflected by a faint or absent SDS-PAGE pattern (Munekiyo and Mackessy, 1998, Chapter 4).

It had been hypothesized that extended exposure to temperatures above freezing, variances in pH and particularly after dilution, would result in the degradation of enzymatic activities. Continuing that these changes were reflected by variations of electrophoretic mobilities and patterning of some venom components (Sugihara, *et al.*, 1972; Gene, *et al.,* 1985). Due to this reported degradation, controls were undertaken including inhibitors to detect autolysis or degradation. PMSF, Benzamidine and Protease inhibitor cocktail were used as inhibitors for some proteases within the venom.

Stability Study: The effect of time, temperature and pH on whole venom

Overall, the whole venom of *Oxyuranus* species was stable, showing little degradation at room temperature for up to 72 hrs and maintaining integrity at temperatures from –80°C to 60°C. Whole venom also revealed repetitive electrophoretic patterns mimicking control patterns from pH 3 to 11.

Starting from the smallest peptides, a high degree of similarity was seen of the lower molecular weight proteins from 6 kDa to 16 kDa when subjected to variations of time, pH and temperature. Due to their abundance within this sample, any degradation of a single protein within this group would not be detected by the methods employed within this study.

Similarly, bands of 110, 100, 80 and 30 kDa showed no change after these studies. The exception to the observed results were bands between 25 kDa to 80

kDa which became more faint than the control in both temperatures over 60°C and when diluted at pH 6.0. This included a minor band at approximately 26 kDa and two major bands at approximately 35 kDa and 66 kDa. The changes noted could be due to degradation or, although every attempt was made to load the same amount of venom there could have been less amount loaded onto the gel. Problematically, due to the requirement to change the venom pH, samples were diluted within the course of this experiment; which may have compounded any degradation.

The overall general lack of degradation indicated there was possibly some endogenous protection within the venom. The storage conditions of whole venom had proven to be robust, work was now undertaken to characterise the three *Oxyuranus* venoms through electrophoresis and fractionation.

3.3.3 Electrophoretic analysis

Basic complexity information from only 0.03 mg to 1 mg of crude venom was obtained from SDS-PAGE, which allowed the visualisation of the three *Oxyuranus* whole venoms after sample treatments under denaturing, reducing and non-reducing conditions. All three species showed banding patterns for reducing SDS-PAGE ranging from 6 kDa to 120 kDa. Samples contained an apparent 20 to 30 protein components, the majority of bands with molecular masses below 20 kDa. However, later chromatography and 2DE analysis followed by protein staining indicated the *Oxyuranus* venoms (using 10 to 200 µg whole venom) could contain up to 100 proteins indicating much greater complexity than suggested by 1D electrophoresis.

Electrophoresis revealed reproducible patterning between the three species, variance within the less expressed proteins of *O. microlepidotus* compared to *O. s. scutellatus* and an extra band at 10 kDa within *O. s. canni*. Under reduced SDS-PAGE the higher molecular weight proteins were similar for all three venoms, with 120, 100, 80, 34 and 30 kDa bands. Some of these abundant proteins correspond with the published proteins previously characterised. The prothrombin activator from *O. s. scutellatus*, scutelarin, is a 250 kDa protein composed of three subunits, 1 x 110 k Da, 1 x 80 k Da and 2 x 30 k Da (the two 30 kDa subunits are linked by disulphide bonds). Other described proteins from *O. s. scutellatus* include taipoxin;

40 kDa (Subunits 14, 13, 12 kDa), Taicatoxin; 49 kDa (17-17, 8-10, 7 kDa), OS1; 14 kDa, OS2; 12 kDa, toxin 1 and 2, both 7,000 Da, phospholipase A_2 (PLA_2) proteins and natriuretic peptides of 6-8 kDa. Due to the abundance of low molecular weight proteins these subunits could not be identified.

Under non reducing conditions, the strong bands of 30, 80 and 100 kDa were not apparent. Banding between 40 to 66 kDa and bands above the highest molecular marker of 200 kDa appearing on these gels. These sizes were extrapolated to be 250 to 300 kDa. Within *O. s. scutellatus*, the 30 kDa band was presumed to incorporate the 60 kDa active subunit of the prothrombin activator with taipoxin (40,668 Da) and taicatoxin (49 kDa). *O. s. canni* proteins were presumed to be identical to *O. s. scutellatus* due to their replica banding patterns with the exception of an extra band at approximately 10 kDa. *O. microlepidotus* contains analogous proteins with *O. s. scutellatus* such as paradoxin, reported similar to taipoxin and similar prothrombin activator. Yet numerous bands between 14 to 30 kDa were abundant in *O. microlepidotus* which was not reflected by the other venoms. This trend was also repeated in later chromatography. Banding patterns of *O. microlepidotus* between 45 and 66 Da were also higher than *O. s. scutellatus* or *O. s. canni.*

The electrophoretic patterns of the venoms varied slightly dependent upon the amount of venom loaded onto the gel. Take for example the protein progression visualized after SDS-PAGE within Figure 3.5. The high molecular weight proteins of *O. s. scutellatus* and *O. microlepidotus* appeared to vary depending upon the amount of venom loaded. This amount of variation was not noted to such a large extent when a similar amount of venom was loaded within a well such as in Figures 3.1 or 3.3. The viscosity of samples may have caused this variation of protein banding. The viscosity may cause inaccuracy with pipetting, hindering accurate dilution of samples. Alternatively this observation may be due to the venom proteins' mechanical interaction with each other while migrating through the gel. Supporting these suggestions was the apparent change in individual protein size dependant on the amount of venom loaded, suggesting dilution problems possibly due to the sample viscosity and migration, not sample degradation, was the cause of the discrepancies.

Subsequent SDS-PAGE, utilising the same sample preparation, demonstrated the return of the diminished proteins while differing proteins, once bold, were now faint bands. This emphasised the variation inherent from venom dilution, loading, casting of gels, and staining. The highly abundant proteins visualised below 20k Da also showed some differentiation after subsequent dilutions. This observation was noted with caution due to the vast quantities of these proteins which may cause distorted running patterns. These proteins stained differently between *O. s. scutellatus* and *O. s. canni* compared to the higher molecular weight proteins indicating possible differences in protein properties.

This trend may have also been reflected with comparative studies undertaken by Sutherland (Sutherland, 1979). Sutherland collected *O. scutellatus* venom samples from differing field locations which, when visualised via 'SDS-PAGE', were shown to contain identical patterning though differing slightly in migration distances within a gel. These variations were interpreted and reported as venom variations between geographical areas.

It may be possible, since a migration variation has now been shown to occur within a single sample, that the differences reported by Sutherland may have been due to the distortion of proteins running within the gel due to glycosylation or volume of proteins. With the exception of an extra band of approximately 10 kDa, reflected within Sutherlands' thesis, it would appear the differences in protein migration of *O. s. scutellatus* and *O. s. canni* in this work were similar to the differences in Sutherlands' report. The venoms may not be different, but the migration of proteins did appear to be dependent upon concentration and conditions.

Thus far, the *Oxyuranus* species showed very similar major protein band patterns, although there were differences in the less expressed protein bands between *O. s. scutellatus* and *O. microlepidotus*. Many of the proteins visualised between *O. s. scutellatus* and *O. s. canni* appeared homologous between species, with *O. microlepidotus* reflecting major similarities under reducing conditions with some banding differences seen. Under closer inspection using denaturing and non reducing conditions *O. microlepidotus* was seen to contain more protein subunits

than *O. s. scutellatus*, and was distinctly different in its minor protein components. Further investigation of the properties of these venoms was undertaken through chromatography to establish further consistency between the species.

3.3.4 Separation of venom components

Separation of each of these species was undertaken to ascertain a more comprehensive characterisation of these venoms through the use of high pressure liquid chromatography (HPLC) size exclusion (SEC) and ion exchange chromatography.

3.3.4.1 Size exclusion chromatography (SEC)

Oxyuranus venom proteins were separated via SEC, allowing the purification of native dominant proteins to remain in their functional complexes. Ion exchange was not used as an initial separation of crude venom due to reported dissociation of some venom components eluting at their respective pI's (Lind and Eaker, 1982).

Venom proteins for each species eluted at the high molecular weight size of 300 kDa, the second fraction at 45 kDa with the majority of proteins eluting as less than 5 kDa. Gel chromatography of the three snake venoms indicated the components of the venoms were similar. However differences in the peak heights, and therefore the amount of protein present in each peak differed between the venoms (Figures 3.9 and 3.10). *O. microlepidotus* revealed similar protein peaks to *O. s. scutellatus*, although extra bands were seen within the low molecular weight proteins eluting late from the chromatography column.

Multiple SEC runs of *O. s. scutellatus*, *O. s. canni* and *O. microlepidotus* were undertaken to check the replication of patterning and traces varied little for each venom. Initial trace patterns of SEC were strong and sharp, but broadened over time suggesting possible mechanical interactions with the column matrix. Visualisation of SEC fractions showed a 'disappearance' of some lower expressed proteins that could not be seen after concentration, suggesting this was occurring. *O. s. canni* venom was markedly more mucoid than the other venoms and this may also account for trace discrepancies observed between *O. s. scutellatus* and *O. s. canni*, since visualisation of fractions using SDS-PAGE revealed identical banding.

Of note with all venom traces was the disparity between, *firstly*, the change in the time of elution between SEC runs, *secondly*, the observed elution time of all protein fractions compared to their size when visualised using SDS-PAGE and *thirdly*, the disappearance of proteins bands.

The change in the time of elution is noted in Figure 3.9 where peak one elutes at 20 minutes while in Figure 3.10 peak one elutes at 10 minutes. This is a reflection of the change in running conditions. Initial running conditions for chromatography was 5 ml/min or at 600 psi, later runs dictated the speed be reduced to 3 ml/min to keep backpressure below maximum. Due to this, all gel filtration was undertaken in 'batches', that is, once the column was equilibrated only two venom samples could be run before broadening of peaks necessitated the column be cleaned and re-equilibrated. Thus only samples run in the same 'batch' were comparatively analysed due to slightly different running conditions.

The *second* discrepancy involved the comparison between the protein sizes of eluted proteins to these sizes visualised on SDS-PAGE. The high molecular weight venom proteins from 300 to 200 kDa visualised by SDS-PAGE broadly correlated to the observed gel filtration marker. Proteins eluting late within SEC, when compared to the gel filtration marker were 1 kDa, yet SDS-PAGE indicated these proteins were 5 kDa. Loading less sample (one quarter the initial sample) onto SEC did not present a better correlation between the gel filtration and protein markers, rather, the less abundant proteins were not visualised.

This anomaly may be attributed to the large quantities and glycosylation of the lower molecular weight proteins. These proteins may be forming their own matrix within the SEC column, changing the charge of the SEC matrix. This may trap proteins via weak ion exchange or through physical entanglement causing the late elution.

The *third* disparity was the inability to identify the less expressed venom proteins observed on 1D SDS-PAGE from whole venom in SEC fractions. An obvious candidate for this predicament would be dilution. However, when the same micrograms of protein from whole and fractionated venom was visualised on SDS-PAGE minor proteins were observed. Degradation was also a candidate, yet

stability studies of had indicated no degradation of samples was observed using dilutions to 1 in 1,000. The concentration of the venom at 100 mg/ml indicated dilutions of 1 in 10 could be used to maintain the protein concentration within the chromatography column manufacturers' guidelines. Despite this, samples were not diluted and stored *en mass*, but were diluted immediately before loading as a precaution against degradation.

Not only were the lower molecular weight proteins appearing to slow elution, proteins appeared to be binding to the column matrix. This suggestion may also resolve the problem why fractions broadened over successive sample loading and the flow rate needed to be slowed. If the high molecular weight proteins were also entangled within the abundant smaller proteins this would help explain why the larger proteins were also found in fractions two and three. The abundance and interactions of the low molecular weight proteins would also explain the varying whole venom protein sizes observed on SDS-PAGE dependent upon the amount loaded

Further methods of fractionation via ion exchanged were employed to attempt to analyse the protein differences between the species.

3.3.4.2 Ion exchange chromatography

The anion exchange column was used to separate *O. s. scutellatus* venom by Folhman in 1976 through to current commercial applications (Marsh, 2002) (Mirtschin, Venom Supplies) for the separation of scutelarin and specific neurotoxins for commercial sale and use. Although this technique has been used previously with this venom it was important to establish a baseline and similarity to literature with the venom used with this project. This method presented broad peaks with low resolution for all three venoms (see appendix V). Again, silver staining after SDS-PAGE could not identify many protein bands after chromatography, reflecting the problems identified using SEC.

Parameters for subsequent chromatography included variations of pH 7.2 through 9.0, slowing the flow rate to 1 ml/min, increasing the salt gradient to twenty column

volumes and initiating a step gradient did not resolve this problem. Although the proteins were not well separated, the prothrombin activator could identified in the anion gradient as published (Walker, *et al.,* 1980).

There were many proteins present in all fractions, specifically, many proteins below 14 kDa. The characterised proteins such as OS1, OS2, toxin 1 and toxin 2 and subunits of proteins such as taipoxin, which have been shown to elute as their individual subunits, within *O. s. scutellatus* venom could only be presumed due to the overlap of proteins and could not be identified through one chromatographic step.

Cation exchange chromatography was undertaken to check if higher resolution could be achieved. Not only did cation exchange chromatography reveal improved resolution compared to anion exchange, this technique resolved a vast array of proteins not visualised using one dimensional gel electrophoresis or gel filtration. Later analysis of glycosylation from cation fractions showed that possibly only 50% of proteins were glycosylated where 100% glycosylation was seen with 1D. To increase the resolution of the elution profile the pH was lowered from pH 7.6 to 6.0. The UnoS cation exchange matrix is SO^{4-}, positive proteins will bind. Decreasing the pH of a solution increases the number of hydrogen ions, thus, more proteins will be protonated and will bind to the negatively charged column. This effect was reflected in the results (Figure 3.11).

Traces at pH 6.0 exhibited sharp resolved protein elution profile. Protein fractions eluted with an average pI of 8 to 10, some with a pI greater than 10.7 (see appendix VI). The four high molecular weight proteins at 120, 100, 80 and 66 kDa were clearly visible in this separation. The subunits of scutelarin (120, 80 and 30 kDa subunits) were not separated by high salt concentrations and, with the exception of the 120 and 42 kDa bands elution of these size bands again appeared in the elution gradient. This suggests differing proteins are present at this molecular weight or there are isomers of these proteins. Two dimensional gel electrophoresis later showed many proteins were composed of trains of spots.

The anion and cation exchange chromatography profiles reflected the similarities of the venoms seen in gel filtration. These profiles also further indicated that the

venoms shared similar compositions. As with the SEC fractionation, this technique also exposed the amount of proteins within the venom sample. This overlap of proteins made the quantification of individual proteins between the species difficult. Several parameters were changed to attempt to isolate proteins. Differing pH buffers helped with resolution, though a change in flow rate, differing micrograms of protein, elution over 10 to 20 column volumes and even step gradients did not achieve the separation of large groups of proteins. Perhaps these proteins were associated with each other, have similar pIs, or, due to the number of proteins, adsorption to the column matrix was causing a change in matrix charge, causing proteins to elute at once. Unfortunately, the initial chromatographic patterns exhibited could not be repeated. Cleaning and re-equilibration the UnoS column, while resulting in identifiable patterns, did not achieve the initial separation pattern.

It was clear from the separation thus far, that possibly hundreds of proteins are present within the venom, more than was first visualised on 1D gel electrophoresis. SDS-PAGE and, with the exception of subtle differences in peak height, gel filtration, anion and cation chromatography had shown *O. s. scutellatus* and *O. s. canni* to be similar if not identical. Whereas *O. s. scutellatus* and *O. microlepidotus*, while mostly similar, contained marked differences between their venom compositions.

3.3.5 Two Dimensional Gel Electrophoresis

Two dimensional gel electrophoresis (2DE) was used to separate whole venom. Although chromatography and SDS-PAGE had proven rewarding for describing the similarities of the three taipan species, it was hoped the limitations of both techniques may be overcome by 2DE to provide a detailed patterning of the three venoms.

The molecular mass distribution of proteins was similar to that found by 1D SDS-PAGE and crude venom was shown to give rise to approximately 100 protein spots. The *Oxyuranus* species showed similar patterns of proteins at 30, 66, 80, 100 and 120 kDa, with heavy spotting at the acidic molecular mass ranges between 14 kDa and 45 kDa and smears at the low molecular weight basic Pi. *O. microlepidotus*

appeared to contain more numerous low molecular weight proteins (Figure 3.13) reflecting the similar gel filtration profiles.

Generally, choosing a narrower pI range of IPGphor strip separated the same venom proteins though not revealing more protein spots. Due to the large number of basic proteins visualised via 2DE a basic 11 cm pH 6-11 range was utilised. Expanding the range of pI from 6 to 11 increased the length of the smear rather than resolve these low molecular weight proteins. Higher reducing environments within the sample (100 mM DTT) also did not resolve these areas.

It was assumed from the accumulated data of 2DE and column chromatography that these basic proteins have similar molecular properties and abundance, which resulted in poor resolution of proteins. Historically, the separation of basic proteins has been difficult and identified basic proteins are underrepresented in proteins databases (Cordwell, 2001). A search for identified snake toxins on the NCBI and SWISS PROT protein databases showed many toxins with basic properties. Thus, this high stain intensity area of basic low molecular mass proteins is likely to be composed of proteins responsible for the toxic properties of venoms from the Elapidae family, the phospholipase A_2s (PLA_2s). The protein spot profile of *Oxyuranus* can be related to other species of the Elapidae family, with the spot locations similar at the lower right corner of the gel. Another similarity between the venoms was bands at neutral pH at 50 to 60 kDa.

The presence of numerous protein spot 'trains' indicated the presence of protein isoforms within all three *Oxyuranus* species. It is unlikely these were cleavage products, as it is doubtful spots would still be so close in pI and molecular weight. It is very common for proteins to be observed as strings of spots on 2D gels. Many organisms studied from bacteria to humans produce somewhere between three and many hundreds of proteins per gene (on average) (Herbert, *et al.,* 2001). This is generally due to posttranslational modifications such as glycosylation, phosphorylation, deamidation, methylataion or due to sequence homologues and protein degradations (Chang and Yang, 1993).

Thus, protein isoforms typically differ slightly in their molecular masses and pI and present themselves as a train of spots that are close together in 2D images. All

these modifications make the protein more acidic, so a string of spots with the 'native version' at the alkaline end and the progressively more modified forms becoming more acidic. It is also common to see an apparent mass increase as these spots become more acidic (especially for glycoproteins) as they do not bind SDS as efficiently. This is reflected in Figure 3.14, with the protein spots visible from 30 kDa and larger.

2DE is a method of protein separation that is fairly rapid and reliable, but basing a comparative analysis that relies exclusively on 2D PAGE suffers from a number of intrinsic limitations. Firstly, the limitation of protein loading means low abundance proteins may not be within the limited dynamic range of the staining protocol, or may be obscured by highly abundant proteins as they are in column chromatography fractions. Secondly, the high abundance of low molecular weight proteins causes focusing difficulties using 2DE (Amersham 2D protocol 2000, Cordwell, *et al.*, 2001). Two-dimensional electrophoresis of whole venom produced significant protein patterns for the major proteins within *Oxyuranus*. Yet duplicate venom samples also revealed differences in protein patterns, causing hesitancy in relying upon the 2DE comparisons between *O. s. scutellatus* and *O. microlepidotus*, whereas the resolution of low abundant proteins was not achieved.

Due to these problems, the separation of the smaller PLA_2s from the larger peptides was undertaken. This separation was not successful using 2DE, although a potential pH dependent protease at pH 6.0 may have been uncovered. It was unknown if the degradation seen in *O. s. scutellatus* F1 was due to pH dependant protease activity, or possibly due to a lack of crowding factors, since a majority of the low molecular weight proteins had been removed. Degradation could have been compounded by both a dilute environment and low pH. Yet sample degradation was not replicated within SEC fractions, or isolating F1 in pH 7.6, indicating the low pH was initiating protein breakdown only when specific groups of proteins were grouped together. Although not within the scope of this work, isolation of a possible pH dependent protease within *Oxyuranus* venom would be an interesting endeavour in further work.

3.3.6 Antivenom binding studies

Immunological techniques using commercially available monovalent taipan antivenom (CSL) were used to demonstrate the similarities and possible differences among *Oxyuranus* venoms. Antigenic comparisons between venoms were aimed at identifying proteins possibly causative of the described variances in toxicity and pharmacology between the three species.

Antivenom and glycosylation studies of whole venom and chromatography samples revealed approximately 50% of the venom proteins were glycosylated whereas antivenom binding studies revealed most proteins from *O. s. scutellatus*, *O. s. canni* and *O. microlepidotus* were antigenic. Of particular interest was the low molecular weight proteins isolated using SEC from 5 to 20 kDa. Antibody binding of these fractions from *O. s. canni* and more markedly from *O. microlepidotus* varied when compared to *O. s. scutellatus* antivenom binding (from which antivenom is produced). Although some peptide subunits have been shown to be non-antigenic, further research into the non-antigenic proteins may indicate potential proteins causative of the differences in pharmacological and toxicity reported between species.

The 'extra' protein bands reported at 10 kDa in *O. s. canni,* and 45 kDa within *O. microlepidotus* bound antivenom. Antivenom was subsequently checked for non-specific binding to carbohydrate (alpha) Mannosyl- and Glucosyl- residues through Concanavalin A conjugated to HRP. Not all proteins binding antivenom bound Concanavalin A (results not shown), indicating glycyl groups were not causing non-specific binding. This implied the differing proteins may be either related proteins or degradation products similar to *O. s. scutellatus* proteins but not, as initially thought, different proteins.

Additionally, all venoms did not bind antivenom between 35 kDa and 60 kDa (Figure 3.16), while immunoscreening of SEC fractions also revealed some low molecular weight proteins may not be antigenic (Figure 3.17). The lack of antigenicity may be due to dilution of the samples through fractionation, causing protein concentrations to fall beyond the range of ECL detection. Silver stain visualises 0.2 – 0.6 ng of protein, while the detection range of ECL varies from 18 –

150 ng of protein, therefore it is possible the protein concentration may have fallen below the threshold for detection via ECL, causing an apparent lack of antigenic proteins. Yet, in Figure 3.17, antigenic protein patterns are seen where silver staining is not apparent, suggesting dilution is not the cause of the lack of antibody binding. Higher concentrations of samples separated and immunoscreened did not change this outcome.

Future work to further the comparison between the three venoms should encompass the isolation of non-antigenic proteins. It may be possible to undertake an immunoprecipitation protocol. Complete antibodies raised against venom could be bound to Protein A or C Sepharose beads. Theoretically, centrifugation of beads results in only non-bound or non-antigenic proteins within the supernatant, from which studies can begin. The CSL antibodies obtained within this report were $(Fab)_2$ fragments, missing much of the Fc portion of the antibody, thus unable to bind to the sepharose beads.