ISOLATION AND CHARACTERIZATION OF HIGH AFFINITY

V_{H}H ANTIBODY FRAGMENTS AGAINST ALPHA-COBRA TOXIN

A Thesis

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF HIGH AFFINITY

VH VH ANTIBODY FRAGMENTS AGAINST ALPHA-COBRA TOXIN

Gabrielle Richard
University of Guelph, 2009

Advisor: Professor J. Christopher Hall

Camelid VHs (also called nanobodies) may provide better treatment for snake envenomation than conventional antivenom antibodies because of their smaller size (~ 16 kDa), better tissue permeability, and lower immunogenicity. In this thesis, a phage-displayed VH library with 4.2 x 10^9 functional clones was constructed from a llama hyperimmunized with crude Thai cobra (Naja kaouthia) venom. After three rounds of panning against α-cobratoxin (α-Cbtx), a potent neurotoxin from N. kaouthia venom, 26 unique clones were found using monoclonal phage ELISA and confirmed by DNA sequencing. Surface plasmon resonance (SPR) analyses showed that the four selected anti-α-Cbtx VH clones had dissociation constants (K_D) in the low nanomolar range (0.4-25 nM) and that these four VHs bound to the same or overlapping epitopes on α-Cbtx. An in vitro muscle twitch assay showed that VH C2 (K_D = 0.4 nM) effectively neutralized the paralytic effects of α-Cbtx at neuromuscular junctions.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>$\alpha$–Cbtx</td>
<td>$\alpha$–Cobratoxin</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Carb</td>
<td>Carbenicillin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>$C_H$</td>
<td>Constant domain of heavy chain</td>
</tr>
<tr>
<td>$C_L$</td>
<td>Constant domain of light chain</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>F(ab$'$)$_2$</td>
<td>Bivalent antigen binding fragment of immunoglobulins</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment of immunoglobulins</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fragment</td>
</tr>
<tr>
<td>fd</td>
<td>Strain of filamentous phage</td>
</tr>
<tr>
<td>FR</td>
<td>Framework region</td>
</tr>
<tr>
<td>GAM-AP</td>
<td>Goat-anti-mouse antibody conjugated to alkaline phosphatase</td>
</tr>
<tr>
<td>GAM-HRP</td>
<td>Goat-anti-mouse antibody conjugated to horseradish peroxidase</td>
</tr>
<tr>
<td>HBS-EP</td>
<td>10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% P-20</td>
</tr>
<tr>
<td>HCAb</td>
<td>Heavy-chain antibody from camellid family</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl $\beta$-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>$k_{off}$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani media / lysogeny broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.5)</td>
</tr>
<tr>
<td>M13</td>
<td>Strain of filamentous phage</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MPBS</td>
<td>Milk phosphate buffered saline</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>o/n</td>
<td>Overnight; 12-16 h</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4)</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with 0.05% Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>$R_{max}$</td>
<td>Maximum response defined as saturation of surface plasmon resonance</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RU</td>
<td>Response unit (in surface plasmon resonance)</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOC medium</td>
<td>Super optimal catabolite repression medium (20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO₄, 10 mM MgCl₂, 20 mM glucose, pH 7.0)</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethyl benzidine</td>
</tr>
<tr>
<td>$V_H$</td>
<td>Variable region of heavy chain of a conventional antibody</td>
</tr>
<tr>
<td>$V_{H'H}$</td>
<td>Variable region of a heavy-chain antibody</td>
</tr>
<tr>
<td>$V_L$</td>
<td>Variable region of light chain of a conventional antibody</td>
</tr>
</tbody>
</table>
1. GENERAL OVERVIEW AND RESEARCH OBJECTIVES

1.1. Introduction

Snake bite is a serious global public health problem especially in tropical and subtropical countries where snakes are abundant and agriculture activities are high (Chippaux, 1998). It is estimated that over 5 million snake bite cases occur worldwide each year, of which 2.6 million cause envenomation, and about 125,000 of these cases result in death (Chippaux, 2006). Despite the number of deaths and injury, Gutiérrez et al. (2006) have categorized snake bite envenomations as a major neglected disease of the 21st century.

*Naja kaouthia* (Thai cobra), a member of the *Elapidae* family, is one of the most venomous and dangerous snakes of Southeast Asia (Viravan et al., 1986). Envenomation by *N. kaouthia* can be manifested by life-threatening neurotoxicity and extensive local tissue necrosis (Viravan et al., 1986). The most toxic component of the *N. kaouthia* venom is \( \alpha \)-cobratoxin (\( \alpha \)-Cbtx; 7.8 kDa), which constitutes about 22-25% of the venom protein (Karlsson, 1979; Kulkeaw et al., 2009). \( \alpha \)-Cbtx is a post-synaptic \( \alpha \)--neurotoxin that binds with high affinity (55 pM) to the nicotinic acetylcholine receptors (nAChRs) at neuromuscular junctions, thereby blocking neuromuscular transmission (Ishikawa et al., 1977; Bourne et al., 2005). Consequently, symptoms including flaccid paralysis and respiratory failure may result which can be followed by death (Minton, 1990).

Since their first development and use in the late 1800s by Calmette, antivenoms are prepared by immunization of an animal (generally a horse) with snake venom followed by antibody purification and processing. Most antivenoms are composed of either whole IgGs (150 kDa), F(\( ab' \))\(_2 \) antibody fragments (100 kDa) or, in some cases,
Fab antibody fragments (50 kDa) (Lalloo and Theakston, 2003). Intravenous administration of antivenom is generally efficacious in treating systemic envenomation (Warrell, 1992 cited in Gutiérrez et al., 2006). However, because of the rapid development of local pathologies and the inability of antivenom antibodies and their respective fragments to reach affected tissues, conventional antivenoms are ineffective in treating local effects on tissues near the snake bite (Gutiérrez et al., 1998; Lalloo and Theakston, 2003). Although many survive envenomation, a large number of victims are left with permanent physical disability as a result of the cytotoxic components of the snake venom (Viravan et al., 1992). Furthermore, high incidences of adverse reactions, such as anaphylaxis and serum sickness, have been associated with administration of conventional antivenoms (Lalloo and Theakston, 2003).

Aside from conventional IgGs, camels and llamas have evolved unique heavy chain IgG immunoglobulins naturally devoid of light chains and the C\textsubscript{H}1 domains (Hamers-Casterman et al., 1993). The antigen binding sites of these heavy chain IgGs are composed of a single variable domain (called \textit{V}\textsubscript{H}Hs), and are the smallest natural antigen binding domain (~16 kDa). \textit{V}\textsubscript{H}H antibody fragments have several attractive properties that may make them better therapeutic reagents for the treatment of snake envenomation; they are relatively non-immunogenic, soluble, stable, and highly tissue penetrable (Conrath et al., 2005; Arbabi-Ghahroudi et al., 1997; Cortez-Retamozo et al., 2002 and 2004). Owing to their low molecular mass, \textit{V}\textsubscript{H}H antibody fragments permeate tissue compartments more readily than conventional antibody fragments (Cortez-Retamozo et al., 2002 and 2004) and, therefore, may protect victims from the tissue-damaging effects of venom toxins. Furthermore, because of their small size and high
homology to the human V_{H}3 gene family (Vu et al., 1997), V_{H}Hs may produce less adverse reactions in patients than conventional antivenoms. For these reasons, V_{H}H-based antivenoms may represent a safer and more efficacious treatment for snake bite envenomation than conventional IgG, F(ab')_{2} or Fab antivenoms.

1.2. Research objectives

Previously in our laboratory, three V_{H}Hs specific for α-Cbtx were isolated from a naïve llama (Lama glama) phage-displayed V_{H}H library (Stewart et al., 2007). However, the affinities of these naïve V_{H}Hs were too low (i.e. uM range) for therapeutic efficacy. Since antibody affinity maturation occurs via repeated animal exposure, it was hypothesized, in this current research, that high affinity V_{H}Hs specific for neutralization of α-Cbtx could be isolated from an immune library. To investigate this, the main objectives of this research were:

\( i) \) To construct an immune V_{H}H library from a llama hyper-immunized with *N. kaouthia* venom,

\( ii) \) To isolate V_{H}H clones with high affinity and specificity to α-Cbtx by phage-display technology, and

\( iii) \) To determine, by performing an *in vitro* muscle-twitch assay, if the isolated V_{H}Hs binders are capable of neutralizing the action of α-Cbtx at neuromuscular junctions.
2. LITERATURE REVIEW

2.1. Introduction

Snake venoms are composed of a rich mixture of toxins and enzymes that display different pharmacological properties. The venom of *Naja kaouthia* (Thai cobra) is especially rich in α-neurotoxins and cytotoxins. Consequently, envenomation by *Naja kaouthia* (Thai cobra) often results in severe systemic neurotoxicity and local pathologies.

Conventional antivenoms, prepared as intact IgG, F(ab’)₂, or Fab immunoglobulin fragments from horses immunized with venom, are generally efficient for the treatment of systemic envenomation. Although many survive envenomation, many are left with permanent physical disability because conventional antivenom antibodies poorly distribute in deep tissues to neutralize venom components. Furthermore, conventional antivenoms often elicit adverse reactions such as anaphylaxis or serum sickness in patients. In this thesis work, we proposed that V₅H₅-based antivenom may serve as an alternative treatment for snake envenomation because of their small size, tissue permeability and low immunogenicity.

This chapter is a literature review that covers four main topic areas. In the first section, venomous snakes with particular attention to *N. kaouthia* and its venom components will be reviewed. Since α-cobratoxin (α-Cbtx) is the most lethal neurotoxin of this venom and the antigen chosen for this thesis work, the second section will discuss its structure and mode of action. The third section will introduce conventional antivenoms and discuss problems associated with these. Lastly, V₅H₅s and their unique
properties that make them good potential therapeutic reagents will be discussed in the last section of this literature review.

2.2. Venomous snakes and *Naja kaouthia*

2.2.1. Incidence of snake bites

Envenomation induced by snake bites represents a serious public health problem, especially in tropical and subtropical countries where snakes are abundant and agriculture and forestry are the primary activities (Chippaux, 1998). It is estimated that over 5 million snake bite cases occur world-wide each year, of which 2.7 million cause envenomation, and about 125,000 of these cases result in death (Chippaux, 2006; Table 1). Although many people survive envenomation, a large number of victims develop severe local tissue damage that may lead to permanent physical disability (Gutiérrez *et al*., 2006). Since most snakebite victims are young male agricultural workers, their disability has serious social and economical impacts, especially for developing countries.

Table 1. Annual world-wide incidences of snake bites (Chippaux, 2006).

<table>
<thead>
<tr>
<th>Region</th>
<th>Population (x 10^6)</th>
<th>Number of bites</th>
<th>Number of envenomation</th>
<th>Number of deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Europe</td>
<td>750</td>
<td>25,000</td>
<td>8,000</td>
<td>30</td>
</tr>
<tr>
<td>Middle East</td>
<td>160</td>
<td>20,000</td>
<td>15,000</td>
<td>100</td>
</tr>
<tr>
<td>USA-Canada</td>
<td>270</td>
<td>45,000</td>
<td>6,500</td>
<td>15</td>
</tr>
<tr>
<td>Latin America</td>
<td>400</td>
<td>300,000</td>
<td>150,000</td>
<td>5,000</td>
</tr>
<tr>
<td>Africa</td>
<td>750</td>
<td>1,000,000</td>
<td>500,000</td>
<td>20,000</td>
</tr>
<tr>
<td>Asia</td>
<td>3,000</td>
<td>4,000,000</td>
<td>2,000,000</td>
<td>100,000</td>
</tr>
<tr>
<td>Oceanie</td>
<td>20</td>
<td>10,000</td>
<td>3,000</td>
<td>200</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5,100</strong></td>
<td><strong>5,400,000</strong></td>
<td><strong>2,682,500</strong></td>
<td><strong>125,345</strong></td>
</tr>
</tbody>
</table>
2.2.2. Venomous snakes

All venomous snakes belong to the *Colubroidea* superfamily which includes four families: *Elapidae* (Elapids), *Viperidae* (Viperids), *Atractaspidae* (Atractaspis) and *Colubridae* (Colubrids) (Chippaux, 2006). This superfamily consists of over 2,300 snake species, 600 of which are venomous, and 250 of the latter can cause human envenomation (Ménez, 2003). Elapids, Viperids and Atractaspis consist of venomous species only, while Colubrids consists of venomous and non-venomous species (Chippaux, 2006).

Vipers and Elapids cause the largest number of snake bites and mortalities (Gutiérrez *et al.*, 2007). Cobras (*Naja* sp.) and kraits (*Bungarus* sp.), which are both Elapids, are responsible for the most severe cases of snake bite envenomations in Asia (Warrell, 1995 in Gutiérrez *et al.*, 2007). Meanwhile, saw-scaled vipers (*Echis*) from northern Africa and lance-headed pit vipers (*Bothrops atrox* and *B. asper*) from South and Central America (Warrell, 1995, Fan *et al.*, 1995, and Gutiérrez, 1995 in Gutiérrez *et al.*, 2007) cause the highest numbers of bites and mortalities in these respective regions.

2.2.3. Biochemical composition of snake venom

Snake venoms are chemically complex mixtures of enzymes, toxins and other small molecules whose function are to immobilize, to kill and/or to facilitate digestion of the prey. The composition of snake venom differs among species and even among individuals of the same species, thereby producing different pathological effects on the victim. Venoms of *Elapids* are rich in α-neurotoxins and cytotoxins (cardiotoxins), often
resulting in neurotoxicity and local tissue damage to snake-bite victims (Chippaux, 2006). The venom of vipers, on the other hand, is rich in heamorrhagins and cytotoxins, which cause haemorrhages and severe local effects (e.g. necrosis) to snake-bite victims (Chippaux, 2006). The venom of Atractaspids contains enzymes and toxins (sarafotoxins) that have strong cardiovascular effects that result in death within an hour of the bite (Chippaux, 2006). Bites from these snakes also cause local symptoms (e.g. swelling, necrosis) (Chippaux, 2006). Finally, the venom of Colubrids contains toxins that act on blood coagulation and haemorrhagic factors, which often cause severe haemorrhages and renal failure (Ménez, 2003).

2.2.4. *Naja kaouthia* (Thai cobra)

*Naja kaouthia*, an Elapid, is the most dangerous snake of Thailand, causing the highest mortality and morbidity due to snake envenomation (Viravan *et al*., 1986). It is also known as the Thai cobra, the monocled cobra, and the monocellate cobra and was previously known as *Naja naja siamensis* (Figure 1). *N. kaouthia* is also present in Burma, Malaysia, Bengal, Cambodia, Vietnam, and Northeast India (Wüster, 1996).

2.2.4.1. TOXICITY OF N. KAOUTHIA VENOM

The venom glands of *N. kaouthia* have the capacity to store about 140 to 400 mg of dry venom (Chippaux, 2006). Although quite variable, most snakes inject between 10 to 50% of the venom in the gland (Chippaux, 2006). The venom of *N. kaouthia* is very potent with LD$_{50}$ values of 0.7 mg/kg (Mukherjee and Maity, 2002) and 0.4 mg/kg (Chotwiwatthanakun *et al*., 2001; Chippaux, 2006) when intravaneously and
intraperitoneally administered to mice, respectively. In comparison, the venoms of *Bungarus caeruleus* (common krait), *N. haje* (Egyptian cobra), *Bathrops lanceolatus* (Martinique lancehead; pitviper), have LD$_{50}$ (mice) values of 0.1, 0.8, and 15.0 mg/kg, respectively (assuming mice weigh 20 g) (Chippaux, 2006).

![Figure 1](image1.png)

**Figure 1.** *N. kaouthia’s* frontal view (a) and dorsal view (b) with its head raised and hood open (Wüster, 2003). The single O-shape on the hood is unique to *N. kaouthia*.

### 2.2.4.2. COMPOSITION OF N. KAOUTHIA VENOM

$\alpha$-Neurotoxins and cytotoxins are abundant in cobra venoms (Chippaux, 2006). Many other proteins with diverse structure and function are also present in cobra venoms. In fact, as many as sixty different peptides can be found in the venom of *N. kaouthia* (Kulkeaw *et al.*, 2007). Nevertheless, many components share the same biological functions and are classified into about 12 structural types (Kulkeaw *et al.*, 2007).
The venom of *N. kaouthia* is especially rich in α-neurotoxins (Karlsson *et al*., 1971), phospholipases A₂ (Mukherjee, 2007), cytotoxins (Kumar *et al*., 1997), and cobra venom factor (Eggertsen *et al*., 1981) all of which display different pharmacological properties. Other less abundant protein constituents present in this venom include metalloproteinases (Ito *et al*., 2001), myotoxin (Harvey *et al*., 1994), oxoglutarate dehydrogenase (Kulkeaw *et al*., 2007), and natrin (Kulkeaw *et al*., 2009). Table 2 provides a list of the currently known protein constituents of the *N. kaouthia* venom along with their synonym names, accession number, MW, toxicity and principal biological function, if known.

### 2.2.4.2.1. NEUROTOXINS

The venom of *N. kaouthia* is especially rich in α-neurotoxins which are the most toxic components of snake venoms (Tsetlin, 1999). α-Neurotoxins are post-synaptic neurotoxins that bind to the nicotinic acetylcholine receptors (nAChR) at neuromuscular junctions (Chippaux, 2006). Since these toxins block access of ACh to its receptor, neurotransmission is inhibited and symptoms including flaccid paralysis and respiratory failure may result which can be followed by death (Minton, 1990).

α-Neurotoxins are low-molecular mass toxins of about 6-7 kDa and share structural homology with ‘three-finger fold’ (TFTs) toxins (Chippaux, 2006). Neurotoxins are classified into two groups based on their chain length: Short- chain α-neurotoxins are composed of 60-62 amino acid residues and contain four disulfide bridges (Tsetlin, 1999). Long-chain α-neurotoxins are composed of 70-74 residues and contain five disulfide bridges (Chiou *et al*., 1989).
2.2.4.2.1.1. **Long-chain α-neurotoxins**

The most abundant and lethal component of the *N. kaouthia* venom is α-cobratoxin (α-Cbtx) (See Section 2.3), which constitutes about 23-25% of the total venom proteins (Karlsson, 1979; Kulkeaw *et al*., 2009). α-Cbtx exhibits its lethality by blocking nicotinic acetylcholine receptors (nAChRs) at neuromuscular junctions (muscle-type) and neuronal synapses (α7) in the brain (Teerapong *et al*., 1978; Tsetlin and Hucho, 2004).

2.2.4.2.1.2. **Short-chain α-neurotoxins**

The venom of *N. kaouthia* contains at least five short-chain α-neurotoxins (Table 2). Like α-Cbtx, these target muscle-type nicotinic acetylcholine receptors at neuromuscular junctions; however, with much lower affinity than α-Cbtx (Chippaux, 2006).

2.2.4.2.2. **CYTOTOXINS**

Several cytotoxins have been isolated from the venom of *N. kaouthia*. Although cytotoxins share structural homology (TFTs) with short-chain α-neurotoxins, their biological function differs considerably (Kini, 2002). Some cytotoxins, also known as cardiotoxins, can cause cardiac arrest by increasing heart rate (Dufton and Hider, 1988). Other cytotoxins non-selectively disrupt cell membranes, thereby killing many different cell types (Inoue *et al*., 1987).
2.2.4.2.3. PHOSPHOLIPASES A₂

Most snake, scorpion and bee venoms contain phospholipases A₂ (PLA₂) (Joubert and Taljaard, 1980a). Phospholipases A₂ disrupt the integrity of cells by hydrolyzing cellular and subcellular membrane phospholipids (Joubert and Taljaard, 1980a). More specifically, they catalyze the hydrolysis of fatty acid ester bonds at position 2 of 1,2-diacyl-sn-3-phosphoglycerides to produce free fatty acids and lysophospholipids. Phospholipases A₂ constitutes about 22% of the total N. kaouthia venom protein (Kulkeaw et al., 2009). Phospholipases A₂ and metalloproteinases are the main components of the N. kaouthia venom responsible for local necrosis (Gutiérrez and Rucavado, 2000). Furthermore, N. kaouthia phospholipases A₂ also have potent anticoagulant activities (Mukherjee, 2007).

2.2.4.2.4. COBRA VENOM FACTOR

About 18% of the N. kaouthia venom protein consists of cobra venom factor (Kulkeaw et al., 2009). Cobra venom factor (CVF), a structural and functional analog to human complement component C3b, is unique to cobra venom and is involved in depleting serum complement (Utkin and Osipov, 2007; Eggertsen et al., 1981; Chippaux, 2006).

2.2.4.2.5. HAEMORRHAGIC METALLOPROTEINASE

Kaouthiagin is a zinc-dependant haemorrhagic metalloproteinase that has been isolated from the venom of N. kaouthia (Ito et al., 2001). Kaouthiagin inhibits blood coagulation by binding and cleaving the von Willebrand factor (VWF) (Ito et al., 2001). VWF is a plasma protein required for platelet and collagen aggregation during platelet
plug formation at the site of vascular injury. Therefore, cleavage of VWF results in increased bleeding.

2.2.4.2.6. OTHER VENOM COMPONENTS

The venom of *N. kaouthia* also contains minor components that are either non-toxic or have low toxicity. Cysteine rich secretory proteins (CRIPs) are present in this venom (Osipov et al., 2005; Table 2). Their significance in cobra snake venoms is still largely unknown since they are relatively non-toxic and do not exhibit any enzymatic activity (Utkin and Osipov, 2007). Other components include nerve growth factor (Hogue-Angeletti et al., 1976), and L-amino acid oxidase which inhibit platelet aggregation (Sakurai et al., 2001). For more details on minor components of cobra venom please refer to Utkin and Osipov (2007).
Table 2. *N. kaouthia* venom components.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>MW (Da)</th>
<th>Toxic Dose LD50</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Cobratoxin (P01391)</td>
<td>7820</td>
<td>0.1 mg/kg i.v.</td>
<td>Inhibits neuromuscular transmission by binding to nicotinic acetylcholine receptors at neuromuscular junctions.</td>
<td>Karlsson, 1973</td>
</tr>
<tr>
<td>Cobrotoxin (P60771)</td>
<td>9262 (precursor)</td>
<td>0.325 mg/kg i.p.</td>
<td>As above</td>
<td>Meng <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>Cobrotoxin II (P82849)</td>
<td>6862</td>
<td>Unknown</td>
<td>As above</td>
<td>Cheng <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Cobrotoxin-b (P59275)</td>
<td>6944</td>
<td>400 mg/kg i.p.</td>
<td>As above</td>
<td>Meng <em>et al.</em>, 2002; Cheng <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Cobrotoxin-c (P59276)</td>
<td>6859</td>
<td>80 mg/kg i.p.</td>
<td>As above</td>
<td>Meng <em>et al.</em>, 2002; Cheng <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Short neurotoxin I (P14613)</td>
<td>6983</td>
<td>Unknown</td>
<td>As above</td>
<td>Chiou, S.H., <em>et al.</em>, 1989</td>
</tr>
<tr>
<td>Protein Name</td>
<td>MW (Da)</td>
<td>Toxic Dose LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
<td>--------------</td>
<td>---------</td>
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<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td><strong>Phospholipase A2 isozyme 1</strong> (P00596)</td>
<td>16, 271</td>
<td>10 mg/kg i.v., mouse</td>
<td>Catalyzes the hydrolysis of the acyl group attached to the 2-position of 3-sn-phosphoglycerides</td>
<td>Joubert and Taljaard, 1980a; Chuman et al., 2000</td>
</tr>
<tr>
<td>NnkPLA-I, CM-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phospholipase A2 isozyme 2</strong> (P00597)</td>
<td>16, 016</td>
<td>4.4 mg/kg i.v., mouse</td>
<td>As above</td>
<td>Joubert and Taljaard, 1980a; Chuman et al., 2000</td>
</tr>
<tr>
<td>NnkPLA-II, CM-III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytotoxin 1</strong> (P60305)</td>
<td>6701</td>
<td>1.3 mg/kg, i.v.</td>
<td>Cytolytic activity</td>
<td>Joubert and Taljaard, 1980b; Fryklund and Eaker, 1975; Ohkura et al., 1988</td>
</tr>
<tr>
<td>Cardiotoxin F-8, CTX1, CM-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytotoxin 2</strong> (P01445)</td>
<td>6745</td>
<td>1.2 mg/kg i.v.</td>
<td>As above</td>
<td>Joubert and Taljaard, 1980b</td>
</tr>
<tr>
<td>Cytotoxin CM-7A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytotoxin 3</strong> (P01446)</td>
<td>6708</td>
<td>1.2 mg/kg i.v.</td>
<td>As above</td>
<td>Joubert and Taljaard, 1980b; Ohkura, et al., 1988</td>
</tr>
<tr>
<td>Cytotoxin CM-7, CX3, CT3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cytotoxin IV</strong> (P60303)</td>
<td>6739</td>
<td>1.48 mg/kg i.p.</td>
<td>As above</td>
<td>Ohkura et al., 1988; Chiou et al., 1989</td>
</tr>
<tr>
<td><strong>Cytotoxin 5</strong> (P24779)</td>
<td>6646</td>
<td>Unknown</td>
<td>As above</td>
<td>Ohkura et al., 1988</td>
</tr>
<tr>
<td>Cytotoxin II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein Name</td>
<td>MW (Da)</td>
<td>Toxic Dose LD$_{50}$</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
<td>---------</td>
<td>----------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Cytotoxin like basic protein (P14541)</td>
<td>6977</td>
<td>Unknown</td>
<td>Low cytotoxic activity</td>
<td>Inoue et al, 1987</td>
</tr>
<tr>
<td>Hemorrhagic metalloproteinase- disintegrin kaouthiagin (P82942)</td>
<td>44493</td>
<td>Unknown</td>
<td>Cleaves the von Willebrand factor in humans, thereby inhibiting platelet aggregation during hemorrhages</td>
<td>Ito et al., 2001</td>
</tr>
<tr>
<td>Cobra venom factor (Q91132)</td>
<td>149,000</td>
<td>Unknown</td>
<td>Compliment-activating factor of venom</td>
<td>Eggertsen et al., 1981; Fritzinger et al., 1994; Kock et al., 2004</td>
</tr>
<tr>
<td>CVF. Complement C3 homolog</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscarinic toxin-like protein 1 (P82462)</td>
<td>7361</td>
<td>Unknown</td>
<td>Binds weakly to muscarinic acetylcholine receptor</td>
<td>Kukhtina et al., 2000</td>
</tr>
<tr>
<td>MTLP-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscarinic toxin-like protein 2 (P82463)</td>
<td>7293</td>
<td>Unknown</td>
<td>As above</td>
<td>Kukhtina et al., 2000</td>
</tr>
<tr>
<td>MTLP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscarinic toxin-like protein 3 (P82464)</td>
<td>7615</td>
<td>Unknown</td>
<td>As above</td>
<td>Kukhtina et al., 2000</td>
</tr>
<tr>
<td>MTLP-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak toxin CM-9a (P25679)</td>
<td>7438</td>
<td>82 mg/kg i.v.</td>
<td>Binds weakly to the nicotinic acetylcholine receptor</td>
<td>Joubert and Taljaard, 1980c cited in the Universal Protein Resource website (UniProt).</td>
</tr>
<tr>
<td>Protein Name</td>
<td>MW (Da)</td>
<td>Toxic Dose LD$_{50}$</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>---------</td>
<td>----------------------</td>
<td>------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Weak tryptophan-containing neurotoxin (P82935)</td>
<td>7613</td>
<td>Approximately 300 less potent than α-Cbtx and NT 2</td>
<td>Binds weakly to the nicotinic acetylcholine receptor</td>
<td>Utkin et al., 2001</td>
</tr>
<tr>
<td>WTX</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine-rich venom protein 23 (P84808)</td>
<td>23621</td>
<td>Non-toxic</td>
<td>Unknown</td>
<td>Osipov et al., 2005</td>
</tr>
<tr>
<td>CRVP-23k</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine-rich venom protein 24 (P84803)</td>
<td>24080</td>
<td>As above</td>
<td>As above</td>
<td>Osipov et al., 2005</td>
</tr>
<tr>
<td>CRVP-24k</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine-rich venom protein 25 (P84805)</td>
<td>24093</td>
<td>As above</td>
<td>As above</td>
<td>Osipov et al., 2005</td>
</tr>
<tr>
<td>CRVP-25k</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2.4.3. CLINICAL SYMPTOMS OF N. KAOUTHIA ENVENOMATION

Envenomation by N. kaouthia is usually manifested by profound neurotoxicity and extensive local tissue necrosis (Warrell, 2005). General systemic symptoms usually include nausea, vomiting, malaise, abdominal pain, weakness, drowsiness and prostration (Warrell, 2005).

2.2.4.3.1. SYSTEMIC EFFECTS

Systemic pathologies are an immediate life-threat and are caused by the action of post-synaptic neurotoxins targeting neuromuscular junctions, thereby causing progressive flaccid paralysis (Gutiérrez et al., 2006). Flaccid paralysis affects skeletal and respiration muscles, while cardiac and smooth muscles are not affected. Paralysis becomes an immediate life-threat when bulbar and respiratory muscles are affected (Warrell, 2005). Ptosis (“heavy” eyelids) is generally the first sign of systemic envenomation. Other signs and symptoms of neurotoxicity include drowsiness, difficulty swallowing, drooling of saliva, dysphonia, diplopia, partial ophthalmoplegia, limb paralysis, and the appearance of a ‘broken neck’ (Warrell, 2005). As paralysis progresses, complete ophthalmoplegia may develop as well as loss of deep-tendon reflexes, coma, respiratory paralysis and death (Minton, 1990).

2.2.4.3.2. LOCAL EFFECTS

In some cases, local tissue damage and necrosis dominate the clinical signs of N. kaouthia envenomation. In fact, local necrosis appears to be more extensive in victims of N. kaouthia envenomation than with other Naja species (Gutiérrez et al., 2006). Other
local signs of envenomation include fang marks, local pain, local bleeding, swelling, blistering, edema, lymph node enlargement, and tissue necrosis (Warrell, 2005). Phospholipases A₂ and metalloproteinases are the main components of the *N. kaouthia* venom responsible for local necrosis (Gutiérrez *et al.*, 2000). Figure 2 shows wounds following a bite from *N. kaouthia*; local tissue swelling and damage are quite apparent.

![Figure 2](www.kingsnake.com/elapids/warning1.htm)

**Figure 2.** a) Local swelling, blistering, early necrosis (Warrel, 2005), and b) extensive tissue damage following a bite by *N. kaouthia* (www.kingsnake.com/elapids/warning1.htm).

### 2.2.4.4. TREATMENT OF *N. KAOUTHIA* ENVENOMATION

Passive immunotherapy using conventional antivenoms remains the only specific treatment for severe envenomation by *N. kaouthia* (See Section 2.4). Two antivenom manufacturers produce equine monovalent antivenoms against *N. kaouthia*: “The Thai Red Cross Society” (Bangkok, Thailand) and the “Thai Government Pharmaceutical Organization” (Chippaux, 2006).
2.3. α-Cobratoxin

2.3.1. Nomenclature

Although α-cobratoxin is the most commonly used name for this toxin, it has synonymous names in the literature including: Long neurotoxin I, Neurotoxin 3, NK3 and α-Cbtx (UniProt entry code: P01391; Table 2).

2.3.2. Structure of α-Cbtx

2.3.2.1. PRIMARY STRUCTURE

α-Cbtx is a long-chain α-neurotoxin (See section 2.2.4.2.1.1) composed of 71 amino acid residues with 5 disulfide bridges: Cys-3/Cys-20, Cys-14/Cys-41, Cys-26/Cys-30, Cys-45/Cys-56, and Cys-57/Cys-62 (Karlsson, 1973). It has a molecular mass of 7831 Da. The primary sequence and disulfide bridges of α-Cbtx are depicted in Figure 3.

Figure 3. Amino acid sequence of α-Cbtx, a long chain neurotoxin, from *N. kaouthia* snake venom (Karlsson, 1973). The five disulfide bridges are depicted with lines.

2.3.2.1.1. HOMOLOGY TO OTHER LONG-CHAIN NEUROTOXINS

A protein blast search revealed that α-Cbtx has high identity with other long chain α-neurotoxin from other *Naja* species. Figure 4 shows a multiple sequence alignment of the proteins that display the highest identity to α-Cbtx.
**Figure 4.** Multiple sequence alignment of $\alpha$–Cbtx with other long-chain $\alpha$-neurotoxins from others cobra (Naja) species.
2.3.2.2. SECONDARY AND TERTIARY STRUCTURES

Neurotoxins are a well-studied class of proteins. In fact, the three-dimensional (3D) structures of over 80 α-neurotoxins and cytotoxins (cardiotoxins) from snake venoms have been determined by X-ray crystallography and/or NMR. Moreover, the 3D structure of α-Cbtx has been resolved at 2.8 Å (unrefined) by Walkinshaw et al. (1980), refined at 2.4 Å by Betzel et al. (1991) and solved by NMR by Laplante et al. (1990) and Le Goas et al. (1992). Interestingly, neurotoxins and cytotoxins share a common polypeptide fold known as a “three-finger toxin fold (TFT-fold)” (Kini, 2002; Tsetlin, 1999).

2.3.2.2.1. THREE-FINGER TOXIN (TFT) – FOLD

Three-finger toxins are relatively flat proteins that have a disc-like shape with one concave face and one convex face (Walkinshaw et al., 1980). The secondary structures of these toxins are dominated by β-structures, including one double and one triple-stranded anti-parallel β-sheet, β turns and random coils. Three hairpin-like loops (Loops I, II and III) emerge from the globular core which resembles three fingers emerging from the palm of a hand. The core is hydrophobic and knotted together by four disulfide bridges. Figure 5 shows the refined structure of α-Cbtx illustrating the three-finger fold and overall flatness of the toxin (Protein Data Bank code; 2CTX; Betzel et al., 1991). α-Cbtx interacts with AChR with the tips of Loops I and II, and the C-terminal tail, mostly through hydrophobic and aromatic interactions (Bourne et al., 2005).
Figure 5. Structure of α-Cbtx (resolved at 2.4-Å by Betzel et al., 1991) (PDB code: 2CTX), depicting the backbone, secondary structures, and disulfide bridges. (a) α-Cbtx viewed from the concave face, showing the three-finger fold. (b) 90° rotation of (a), demonstrating the overall flatness of the three-finger fold. The program PyMol was used for visualization of this protein.
2.3.2.2. STRUCTURAL DIFFERENCES BETWEEN LONG- AND SHORT-CHAIN α-NEUROTOXINS

As previously stated, long-chain and short-chain α-neurotoxins share the three-finger toxin fold. However, superimposition of α-Cbtx with the short-chain erabutoxin from the sea snake *Laticauda semifasciata* reveals some important local structural differences (Figure 6).

![Backbone superimposition of long chain α-Cbtx (red) from *N. kaouthia* and short chain erabutoxin (green) from the sea snake *Laticauda semifasciata* (Antil et al., 1999). Disulfide bridges are indicated in yellow.](image)

**Figure 6.** Backbone superimposition of long chain α-Cbtx (red) from *N. kaouthia* and short chain erabutoxin (green) from the sea snake *Laticauda semifasciata* (Antil et al., 1999). Disulfide bridges are indicated in yellow.

The key structural differences that discriminate long-chain from short-chain α-neurotoxins include: a shorter Loop I, a longer Loop II, an extra C-terminal tail, and a fifth disulfide bridge at the tip of Loop II of α-Cbtx. This disulfide bridge allows the tip of Loop II to fold into two distorted, right-handed helical turns, creating a bulging tip (Betzel et al., 1991). This disulfide bridge and residues located at the tip of Loop II are pharmacologically important since they allow long-chain α-neurotoxins (e.g. α-Cbtx) to bind to the neuronal α7 subunits of the nicotinic acetylcholine receptor with high affinity.
(Servent et al., 1997). Furthermore, these subtle structural differences allow long-chain α-neurotoxins to bind with higher affinity to muscle-type nAChRs when compared with short-chain α-neurotoxins.

2.3.3. Mode of Action of α-Cbtx

α-Cbtx is an antagonist molecule that competes with acetylcholine (ACh) for binding to the nicotinic acetylcholine receptor (AChR) (Antil et al., 1999).

2.3.3.1. NEUROMUSCULAR TRANSMISSION – A BRIEF OVERVIEW

Skeletal muscle fibres are innervated by long motor nerve fibres. When an action potential reaches the end of a motor neuron, calcium channels open allowing high concentrations of Ca\(^{2+}\) to enter inside the nerve terminal (Guyton and Hall, 2000; Figure 7). This in turn triggers the release of acetylcholine (ACh) from the pre-synaptic cleft. Acetylcholine diffuses into the synaptic space to reach acetylcholine-gated ion channels which are embedded in the motor end plate (post-synaptic cleft) of muscle fibers. Binding of two ACh molecules to the ACh receptor causes the channel to open and a large number of cations, mostly Na\(^+\), enter the muscle cell down their concentration gradients. This creates an end plate potential inside the muscle fibre which ultimately results in muscle contraction.
2.3.3.2. NICOTINIC ACETYLCHOLINE RECEPTOR

Nicotinic acetylcholine receptors can adopt three different conformation states: resting (closed, non-conducting state), activated (open, conducting state), or desensitized (Hogg et al., 2005). Agonist ligands (e.g. acetylcholine) stabilize the open conformation, whereas antagonist ligands stabilize the resting conformation. Binding of α-Cbtx causes major conformational changes of the receptor which stabilizes the resting state (Bourne et al., 2005).

Nicotinic AChRs are classified into two categories based on their location and subunit composition: muscle-type and neuronal type. Muscle-type nAChRs are found in the neuromuscular junctions of somatic muscles and are composed of two α1, one β1, one
δ, and one γ subunits. Neuronal-type nAChRs are located in the central and peripheral nervous system and are composed of five α subunits (α7, α9 or α10) or a combination of α and β subunits (Antil-Delbeke et al., 2000).

2.3.3.3. NEUROMUSCULAR BLOCKADE BY α-NEUROTOXIN

Short-chain and long-chain α-neurotoxins have the same mode of action: they block neuromuscular transmission (Chippaux, 2006). Binding of α-neurotoxins cause major conformational change to the receptor which stabilizes the resting (closed) state of the channel (Bourne et al., 2005). Furthermore, bound α–neurotoxins prevent access of ACh to its binding pocket (Bourne et al., 2005). As a result, neuromuscular transmission is inhibited and symptoms of flaccid paralysis result.

2.3.3.3.1. BLOCKADE BY LONG-CHAIN VERSUS SHORT-CHAIN α-NEUROTOXIN

There are two important pharmacological differences between long-chain and short-chain α-neurotoxins. First, long-chain α-neurotoxins (e.g. α-Cbtx) bind with higher affinity to the nicotinic AChRs than do short-chain α-neurotoxins (Chippaux, 2006). Therefore, long-chain α-neurotoxins are more neurotoxic than short-chain α-neurotoxins (Refer to Table 2 - Toxicity). Secondly, long-chain α-neurotoxins bind both muscle-type and α7 neuronal-type nicotinic AChRs, while short-chain α-neurotoxins only bind the former.
2.4. Antivenom Therapy

2.4.1. Brief historical background

In 1895, Albert Calmette, a French physician and immunologist, demonstrated that serum from an immunized horse could treat human envenomation following a cobra bite (Calmette, 1895). This led him to develop the first commercial equine antivenom in 1898 for Vietnam (Lalloo and Theakston, 2003). Soon thereafter, antivenoms against snake, scorpions and spiders were produced in many parts of the world (Russell, 1988). Today, animal-derived antivenoms remain the only specific treatment for envenomation.

2.4.2. Preparation of conventional antivenoms

Methodologies in antivenom preparation have changed little since its discovery by Calmette over a century ago (Lalloo and Theakston, 2003). An animal, generally a horse or in some cases a sheep, is immunized over the course of several months with increasing doses of a single type of venom to produce monovalent antivenom, or with a mixture of different venoms to produce polyvalent antivenoms (Theakston et al., 2003). In response to the foreign molecules, the animal’s immune system generates neutralizing antibodies (IgGs) specific to the venom components. After reaching an appropriate antibody neutralizing titre, blood is collected from the animal, and antibodies (IgGs) are purified and processed as outlined in Figure 8. Most antivenoms consist of purified whole IgG (150 kDa), F(ab’)2 fragments (100 kDa) obtained by pepsin digestion of IgG or, in some rare cases, Fab fragments (50 kDa) prepared by papain digestion of IgG (Lalloo and Theakston, 2003). Smaller fragments are thought to be less immunogenic and thought to
have a pharmacokinetic profile that more closely matches that of venom toxins. These antibodies or fragments can be administered intravenously to an envenomed patient to neutralize the activity of the snake venom toxins.

<table>
<thead>
<tr>
<th>Screening production animals for antibody titer production</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG concentration [(NH₄)₂SO₄/NaSO₄ precipitation]</td>
</tr>
<tr>
<td>Enzyme digestion (pepsin→ F(ab)₂; papain→ Fab)</td>
</tr>
<tr>
<td>Caprylic acid stabilization</td>
</tr>
<tr>
<td>Ion exchange (removes Fc)</td>
</tr>
<tr>
<td>Affinity purification (concentrates venom-specific IgG)</td>
</tr>
<tr>
<td>Pasteurisation (10 hr at 60°C)</td>
</tr>
<tr>
<td>Endotoxin exclusion (below 0.5 μg/kg/dose)</td>
</tr>
<tr>
<td>Lyophilisation</td>
</tr>
</tbody>
</table>

**Figure 8.** Summary of methodologies for antivenom production (Theakston *et al.*, 2003).

2.4.3. Immunization of animals for antivenom production

Although the first commercial antivenom was produced over a century ago (Calmette, 1894), antivenom production against snake venom, and in particular against *N. kaouthia* venom, has been problematic (Pratanaphon *et al.*, 1997). For example, only about 20% of the horses immunized with *N. kaouthia* venom produced sufficient
neutralizing antibodies for antivenom production. Furthermore, in some cases, up to 600 mL of antiserum was required for the treatment of *N. kaouthia* envenomation (Viravan *et al.*, 1986). This low yield has resulted in both limited supply and high cost of snake antivenoms (*Sriprapat et al.*, 2003). More recently, Chotwiwatthanakun *et al.* (2001) developed an immunization strategy that generated a 4-fold increase in antivenom potency. Their strategy consists of hyper-immunizing horses with crude venom(s) according to a “low dose, low volume, multi-site” protocol emulsified in Freund’s complete adjuvant for the first immunization and emulsified in incomplete adjuvants for booster immunizations.

2.4.4. Adverse reactions to antivenoms

Antivenoms often elicit adverse reactions in patients. There are two main types of adverse reactions to antivenom treatment: early and late reactions. *Early reactions* usually occur within the first few hours after administration of antivenom and symptoms include itchy scalp, urticaria, dry cough, fever, nausea, vomiting, abdominal colic, diarrhoea and tachycardia (Warrel, 2005). Although rare, life-threatening anaphylactic shock, a type I hypersensitivity reaction, may develop in patients that have had a previous exposure to horse (or sheep) proteins and consist of bronchospasm, angioneurotic edema and hypotension (Warrel, 2005).

The mechanisms responsible for the onset of early adverse reactions remain largely unknown. However, most cases are not truly ‘allergic’ reactions since they are not caused by circulating IgE (Lalloo and Theakston, 2003; Warrel, 2005). Instead, most early reactions are believed to occur via complement activation caused by IgG or residual Fc aggregates, and to a lesser extent, by pyrogenic (endotoxin) contaminants in the
antivenom preparation (Lalloo and Teakston, 2003; Theakston et al., 2003; Warrel, 2005).

_Late adverse reactions_, also known as serum sickness, may develop 1-12 days (Warrell, 2005) after administration of antivenom (Warrell, 2005). Serum sickness is a type III hypersensitivity reaction in which the patient develops antibodies against foreign (equine) antivenom proteins (Lalloo and Theakson, 2003). Symptoms include fever, itching, urticaria, arthralgia, lymphadenopathy, and proteinuria (Lalloo and Theakson, 2003).

### 2.4.5. Pharmacokinetics of antivenom antibodies

Due to their different molecular mass, IgG, F(ab’)2 and Fab antivenoms display different pharmacokinetic characteristics including volume of distribution, distribution half-life, elimination half-life and clearance (Gutiérrez et al., 2003). Whole IgG (150 kDa) molecules, which have the largest molecular mass, have the lowest volume of distribution in the body (Covell et al., 1986). As a result, IgG antivenoms ineffectively neutralize locally-acting toxins in deep tissue compartments (Gutiérrez et al., 2003). On the other hand, an important advantage of IgG molecules is that they have a prolonged elimination half-life compared to F(ab’)2 and Fab molecules. Since IgG molecules circulate in the body for a longer period of time they can neutralize toxins that reach the circulation at a later stage during envenomation (Covell et al., 1986).

The pharmacokinetic profile of Fab antivenoms differs remarkably from IgG antivenoms. Owing to their small molecular mass, Fab fragments readily diffuse into a larger volume of distribution and reach deep tissue compartments at a faster rate than IgG antivenoms (Covell et al., 1986; Scherrmann, 1994). Since the pharmacokinetic profile
of Fab antivenoms more closely resembles those of low molecular mass venom toxins, it has been proposed that they are better suited for envenomation therapy. Despite their improved tissue permeability, their clinical use has been limited by two major problems. First, because of their small size, Fab fragments are rapidly catabolised and eliminated by the kidneys (Lalloo and Theakston, 2003). As a result, the tissue concentration of Fab antivenoms is markedly decreased (Riviere et al., 1997). Furthermore, if Fab antivenoms are eliminated from the body before venom toxins reach circulation, signs of recurrence of evenomation after initial successful therapy may arise (Lalloo and Theakston, 2003). To compensate for this rapid renal clearance, it has been proposed that Fab antivenoms should be administered as multiple consecutive dosages (Abdel Latif et al., 2003).

Most antivenom producers prepare F(ab’)₂ antivenoms, which have intermediate volume of distribution and elimination half-life relative to IgG and Fab (Covell et al., 1986).

2.4.6. Other animals as sources of antivenoms

Most antivenom producers use horses for antivenom production because they are docile, yield large volume of serum, and can handle large doses of venom which enable the production of neutralizing antibodies (Lalloo and Theakston, 2003). However, there are disadvantages to using horses for antivenom production. Individuals that are already sensitized to horse proteins might develop early adverse reactions to equine antivenom therapy (Lalloo and Theakston, 2003). Furthermore, horses are difficult to handle and do not thrive in tropical regions where snakebite is endemic (Lalloo and Theakston, 2003).
Other animals including donkeys, cattle, goat, rabbits, ducks, hens and even camelids have been used to raise antivenom; however, there are no commercial antivenoms available using these animals as sources.

2.4.7. Using camelids for antivenom production

The possibility of using camelids has recently been suggested for antivenom production (Meddeb-Mouelhi et al., 2003; Harrison et al., 2006; Stewart et al., 2007). In addition to conventional IgGs, camelids (camels, llamas, alpacas) have evolved a unique class of immunoglobulins naturally devoid of light chains called heavy-chain antibodies (HCAbs) (Hamers-Casterman et al., 1993). In one report, camels immunized with scorpion (Androctonus australis hector) venom neurotoxins generated high antibody titres in the same range as those in horse sera (Meddeb-Mouelhi et al., 2003). Furthermore, both polyclonal sera and purified HCAbs were capable of neutralizing lethal neurotoxins from this venom in mice (Meddeb-Mouelhi et al., 2003). Another study showed that sera from camels and llamas immunized with Echis ocellatus (viper) venom could prevent the development of local haemorrhagic lesions induced by this venom in mice experiments (Harrison et al., 2006). Furthermore, camelid IgG have been shown to be less immunogenic compared to horse and sheep IgGs (Herrera et al., 2005). Therefore, camelid antivenoms may be less likely to induce adverse reactions compared to conventional antivenoms (Herrera et al., 2005; Laloo and Theakston, 2003). These studies are encouraging for the use of camelids for antivenom production.
2.4.8. Using recombinant antibodies for antivenom development

The advent of recombinant antibody technology has created the possibility of rationally designing novel antibody fragments that could be used for the treatment of envenomation. Since conventional antivenom antibodies poorly neutralize venom toxins located in deep tissues, small recombinant antibody fragments (e.g. scFvs and V\textsubscript{H}Hs), which are highly tissue permeable, have been explored in recent years for use in antivenom preparation (Lafaye \textit{et al.}, 1997; Stewart \textit{et al.}, 2007; Kulkeaw \textit{et al.}, 2009). Nevertheless, antivenoms composed entirely of small antibody fragments would likely have low therapeutic efficacy because these fragments are too rapidly cleared from the body (Gutiérrez \textit{et al.}, 2007). Alternatively, antivenoms prepared with a mixture of high molecular mass antibodies (IgG; F(\text{ab}’)\textsubscript{2}) and low molecular mass antibody fragments (Fab; scFv; V\textsubscript{H}H) may offer better treatment for envenomation (Gutiérrez \textit{et al.}, 2003, Gutiérrez \textit{et al.}, 2007; Harrison \textit{et al.}, 2005; Stewart \textit{et al.}, 2007). This type of antivenom would not only allow the rapid neutralization of toxins in tissue compartments by small fragments, but also ensure that significant concentrations of the antibodies (IgG, F(\text{ab}’)\textsubscript{2}) remain in circulation long enough to neutralize toxins that reach the circulation later in the course of envenomation, thereby preventing recurrence of adverse effects.

2.5. Antibody Engineering

The vertebrate immune system has evolved to make antibodies (immunoglobulins; Ig) against virtually any type of biological or chemical compound. Following immunization, B cells that carry surface antibodies that recognize and bind a specific antigen will expand by clonal proliferation and evolve into cells that produce
soluble antibodies. Repeated immunization generally generates antibodies with higher affinity and specificity towards the antigen.

2.5.1. Conventional IgG

Immunoglobulin G (IgG) is the most abundant class of antibodies in serum and accounts for about 80% of total-serum antibodies (Kuby, 2007). While their specificity may differ extensively, the structure and composition of IgGs are highly conserved among vertebrate species. Whole IgG molecules are composed of two identical heavy (H) chains (~ 55 kDa) and two identical (L) light chains (~22 kDa) which are joined together by disulfide bonds to form a tetrameric molecule of about 150 kDa (Kuby, 2007) (Figure 9). The heavy chains fold into three constant domains, the C_H1, C_H2 and C_H3, and one variable domain, the V_H, which is located at the amino terminal of the polypeptide. The light chains fold into two domains; a constant light chain (C_L) and a variable light (V_L) domain. There are six complementarity-determining regions (CDRs; hypervariable regions), three provided by each of the V_H and V_L domains, that contribute to the antigen-binding site.

2.5.2. Heavy-chain Antibodies (HCAbs)

Aside from conventional IgGs, species of Camelidae (camels, llamas, alpacas) produce functional heavy-chain antibodies (HCAbs) that are naturally devoid of light chains and the C_H1 domains (Hamers-Casterman et al., 1993). Thus, in contrast to conventional IgGs, the antigen-binding sites of these HCAbs are reduced to a single variable domain, referred to as V_HH domain (Figure 9) (Section 2.5.3.1). Despite their apparent limited antigen-binding surface, these HCAbs retain antigen recognition with
moderate to high affinity and specificity (Arbabi-Gahroodi et al., 1997; Lauwereys et al., 1998). About 75% of the total-serum antibodies from camel constitute HCAbs while llamas (*Lama glama*) have about 45% (van der Linden et al., 2000).

![Diagram of antibody fragments](image)

**Figure 9.** Schematic representation of a conventional IgG, a camelid heavy-chain antibody (HCAb) and a HCAb variable domain (V\_H\_H).

### 2.5.3. Antibody fragments

In 1975, Nobel Prize winners Köhler and Milstein pioneered mouse hybridoma technology which rapidly launched a new era of monoclonal antibody technology for experimental research, therapeutic and monitoring applications (Köhler and Milstein, 1975). In the mid 1980s, new technologies, including chimerization and humanization, emerged to ameliorate the inherent immunogenicity associated with murine mAbs, which then lead to the approval of many therapeutic antibodies (Brekke and Sandlie, 2003). Since this time, antibody engineers have dissected whole IgGs into various antibody fragments, including V\_H\_s, scFvs, Fabs, F(ab')\_2s, and also used them as building
blocks to construct multivalent/multispecific reagents with desired properties (Holliger and Hudson, 2005).

The following section reviews unique properties of camelid V\textsubscript{H}Hs with emphasis on their use as therapeutic reagents.

2.5.3.1. **CAMELID V\textsubscript{H}HS**

The antigen-binding domain of HCAbs (Section 2.5.2) is formed by a single domain known as variable domain of the heavy chain of heavy chain antibodies (V\textsubscript{H}H) (Hamers-Casterman et al., 1993). When expressed as soluble fragments, these fragments are also referred to as single-domain antibodies (sdAbs) or Nanobodies\textregistered and are the smallest natural Ab-based binding domains with a molecular mass of \(~16\) kDa (Figure 9). Camelid V\textsubscript{H}Hs display several unique biochemical properties that distinguish them from other antibody fragments including V\textsubscript{H}s, scFvs and Fabs. They are relatively non-immunogenic, soluble, stable (heat and pH), and highly tissue penetrable (van der Linden et al., 1999; Arbabi-Ghahroudi et al., 1997; Cortez-Retamozo et al., 2002 and 2004). Some of the key physico-chemical features that give them their claimed advantageous characteristics will be discussed below with specific emphasis on their potential as therapeutic reagents.

2.5.3.1.1. **INCREASED SOLUBILITY**

One of the most important characteristic that distinguishes camelid V\textsubscript{H}Hs to conventional V\textsubscript{H}S is their increased solubility (Vu et al., 1997). Conventional V\textsubscript{H} residues that interact with the V\textsubscript{L} domain are extremely well conserved and hydrophobic (Vu et al., 1997). Recombinant expression of conventional V\textsubscript{H}, without the V\textsubscript{L} domain,
exposes this hydrophobic patch and accounts for the poor solubility observed for \( V_{H} \) (Conrath et al., 2005). However, the hallmark feature that discriminates camelid \( V_{H} \)s from \( V_{H} \)s is that they have substituted these solvent-exposed, hydrophobic residues with more hydrophilic ones, thereby making the surface more hydrophilic and soluble. These substitutions are located in framework region 2 (FR2) and include Val37Phe (or Tyr), Gly44Glu (or Gln), Leu45Arg and Trp47Gly (Leu or Phe) (Vu et al., 1997; Harmsen et al., 2000).

2.5.3.1.2. TISSUE PENETRATION

Due to their low molecular mass (~16 kDa), \( V_{H} \)s have an improved biodistribution profile and permeate tissue compartments more readily than conventional antibody fragments (Cortez-Retamozo et al., 2002 and 2004). In one report, when a \( V_{H} \) was intravenously injected into tumor-bearing transgenic mice, it was able to locate and bind to its antigen which was located in deep-tissues (Cortez-Retamozo et al., 2002). In another report, \( V_{H} \)s were selected to transmigrate the human blood-brain-barrier (BBB) in an \textit{in vitro} model, and following intravenous administration were shown to accumulate in the brain of mice (Muruganandam et al., 2002). Hence, \( V_{H} \)s may be valuable reagents for cancer diagnosis and therapy, for the treatment of neurodegenerative diseases (e.g. Alzheimer’s disease), and other disorders.

2.5.3.1.3. TARGETING CRYPTIC EPITOPES / ENZYME INHIBITOR

Like conventional \( V_{H} \)s, camelid \( V_{H} \)s are composed of four framework regions (FR-1 to -4) and three complementarity determining regions (CDR -1 to -3), which are folded into the typical immunoglobulin fold (Chothia et al., 1998). The CDR-3 loop of
camelid V_{H}Hs, which is important for antigen recognition, is on average longer (17 amino acid residues) than that of humans (12 residues) and mice (9 residues) V_{H}Hs (Vu \textit{et al.}, 1997). This extended flexible CDR3 loop has the capacity to form fingerlike extensions to reach into cavities of antigens (e.g. active site of enzymes, receptor clefts) that are inaccessible to conventional antibodies. Accordingly, some V_{H}Hs have been shown to act as potent enzyme inhibitors (Desmyter \textit{et al.}, 1996). Moreover, Lauwereys \textit{et al.} (1998) have demonstrated that when the antigen is an enzyme, about 50% of the HC Abs interact with the enzyme cleft. Hence, these studies show that V_{H}H have tremendous potential for therapeutic applications relating to inactivation of enzymes.

2.5.3.1.4. LOW IMMUNOGENICITY

When creating therapeutic reagents, it is important to ensure that the reagent is safe and will not elicit adverse reactions in humans. Compared to most mammalian antibodies, camelid HC Abs are relatively non-immunogenic to humans and mice (Cortez-Retamozo \textit{et al.}, 2002; Cortez-Retamozo \textit{et al.}, 2004; Herrera \textit{et al.}, 2005). Furthermore, V_{H}Hs are believed to be relatively non-immunogenic to humans because of their small size and also their high homology to the human V_{H}3 gene family (Vu \textit{et al.}, 1997). Therefore, administration of camelid V_{H}Hs to human hosts is likely safe and should not elicit serious adverse reactions.

2.6. Summary

Snake venoms consist of a rich mixture of toxins and enzymes that display different pharmacological properties. The venom of \textit{Naja kaouthia}, an Elapid common in Southeast Asia, is especially rich in \(\alpha\)-neurotoxins and cytotoxins. As a result,
envenomation by *N. kouathia* often results in severe systemic neurotoxicity and extensive local tissue damage.

Since its discovery in the late 1800s, passive immunotherapy using conventional antivenoms remains the only specific treatment for envenomation. Although conventional antivenoms help increase survival rate, there are several problems associated with their use including *i)* poor neutralization of toxins in deep tissues, *ii)* adverse reactions to antivenoms (anaphylaxis, serum sickness), and *iii)* their limited supply (Lalloo and Theakston, 2003). There is a need for more efficacious, safer, and widely distributed therapeutics for snake envenomation.

In recent years, camelid derived V\textsubscript{H}H antibody fragments have been suggested for antivenom development. Their tissue permeability, ease of genetic manipulation, low inherit immunogenicity, small size, high solubility and stability, and ability to target novel epitopes make V\textsubscript{H}Hs attractive therapeutic reagents. Moreover, because of their extreme stability and small size, V\textsubscript{H}Hs can be administered to subjects intravenously, orally, or topically (Ablynx.com). Therefore, V\textsubscript{H}Hs may represent an attractive alternative to the treatment of snake envenomation.
3. ISOLATION AND CHARACTERIZATION OF HIGH AFFINITY V_{H}H ANTIBODY FRAGMENTS AGAINST α-COBRA TOXIN

3.1. Introduction

Aside from conventional IgGs, camels and llamas have evolved unique heavy chain IgG immunoglobulins naturally devoid of light chains and the C_{H}1 domains (Hamers-Casterman et al., 1993). The antigen binding sites of these heavy chain IgGs are composed of a single variable domain (called V_{H}Hs), and are the smallest natural antigen binding domain (~16 kDa). V_{H}H antibody fragments have several attractive properties that may make them better therapeutic reagents for the treatment of snake envenomation; they are relatively non-immunogenic, soluble, stable, and highly tissue penetrable (Conrath et al., 2005; Arbabi-Ghahroudi et al., 1997; Cortez-Retamozo et al., 2002 and 2004). Owing to their low molecular mass, V_{H}H antibody fragments permeate tissue compartments more readily than conventional antibody fragments (Cortez-Retamozo et al., 2002 and 2004) and, therefore, may protect victims from the tissue-damaging effects of venom toxins. Furthermore, because of their small size and high homology to the human V_{H}3 gene family, V_{H}Hs may produce less adverse reactions in patients than conventional antivenoms (Vu et al., 1997). For these reasons, V_{H}H-based antivenoms may represent a safer and more efficacious treatment for snake bite envenomation than conventional IgG, F(ab')_{2} or Fab antivenoms.

Previously in our laboratory, a naïve llama (Lama glama) phage-displayed V_{H}H library was used to select V_{H}Hs against α-cobratoxin (α-Cbtx), a potent α–neurotoxin toxin of the venom of Naja kaouthia (Stewart et al., 2007). However, the affinities of the isolated naïve V_{H}Hs were too low (low μM range) for therapeutic efficacy. Since it is
well known that higher affinity antibodies can be isolated from an immune library, we immunized a llama (*Lama glama*) with crude *N. kaouthia* venom in this current research. After detecting convIgG and HCAb responses against *N. kaouthia* venom components and purified α-Cbtx, an immune phage-displayed *V*~hH~ library was constructed from blood lymphocytes harvested on day 110 post-immunization. Thereafter, three rounds of panning were done to isolate high affinity *V*~hH~ binders to α-Cbtx by phage-display technology, followed by an analysis of the *V*~hH~ coding sequences. After soluble expression and purification of selected *V*~hH~ binders, kinetic measurements were determined by surface plasmon resonance (SPR). Furthermore, SPR experiments were performed to determine if the *V*~hH~ clones target the overlapping or non-overlapping epitopes on α-Cbtx. Lastly, an *in vitro* muscle twitch assay was done to determine if the isolated C2 *V*~hH~ binder is able to effectively neutralize the paralytic effects of α-Cbtx at neuromuscular junctions.

### 3.2. Materials And Methods

#### 3.2.1. Toxins

*N. kaouthia* venom was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY), while purified α-Cbtx was purchased from Latoxan (Valence, France), both in a lyophilized form. Note that although Latoxan spells their α-cobratoxin product with an ‘o’, it is the same toxin as α–cobratoxin (α–Cbtx).
3.2.2. Llama immunization

After collection of pre-immune sera (day -7), a one-year-old male llama \textit{(Lama glama)} was immunized on days 0, 14, 35, 56, 75 and 103 with increasing amounts (i.e. 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 mg, respectively) of crude \textit{N. kaouthia} venom. The venom [dissolved in phosphate buffered saline (PBS), pH 7.4] was emulsified in an equal volume of TiterMax\textsuperscript{TM} Classic Adjuvant (Sigma-Aldrich, Oakville, ON, Canada) for the first three immunizations, and emulsified with an equal volume of Freund’s Incomplete Adjuvant (Sigma-Aldrich, Oakville, ON, Canada) for subsequent immunizations. The immunogen was administered subcutaneously at three different locations (0.25 mL/site) (i.e., one site near the neck and two in the hind quarters), which was similar to scheme described by Chotwiwatthanakun \textit{et al.} (2001). Immune bleeds (~100 mL) were withdrawn from the jugular vein on days 21, 42, 63, 82 and 110. Serum was collected by centrifuging at 2,700 X g for 10 min, aliquoted and stored at -20ºC until required for use. Whole llama blood was also collected, at days 82 and 110, in a glass vacuum bottle containing 1-2 mg/mL of EDTA (dipotassium salt) to prevent coagulation. Thereafter, peripheral blood leukocytes were recovered from 1.5 mL aliquots of this whole blood, lysed as described in the QIAamp RNA Blood Mini Kit (Qiagen), and stored at -80ºC until required for RNA isolation.

3.2.3. Polyclonal immune response

The polyclonal immune responses against \textit{N. kaouthia} venom components and \(\alpha\)-Cbtx were monitored over the course of the llama immunization by indirect enzyme-linked immunosorbent assay (ELISA). Wells of a Reacti-Bind\textsuperscript{TM} maleic anhydride-
activated polystyrene microtitre plate (Pierce Biotechnology, Rockford, IL) were coated with 1 µg/mL of crude *N. kaouthia* venom or with 2.5 µg/mL of α-Cbtx (100 µL/well; PBS, pH 7.4) overnight (o/n) at 4ºC. Negative background control wells were not coated with the antigen (PBS only). Wells were washed 3x with 200 µL of PBS (pH 7.4) to remove unbound antigen and then blocked o/n with 300 µL of 4% MPBS [4% (w/v) milk powder in PBS, pH 7.4]. Llama polyclonal serum from days -7, 21, 63, 110 was diluted by a serial two-fold dilution starting with a 1:50 dilution, added to the wells (100 µL/well), and incubated with gentle shaking at room temperature (RT). After 1.5 hr incubation, serum samples were removed and the wells were washed 3x with 200 µL of PBS-T (PBS plus 0.05% (v/v) Tween-20). Goat anti-llama IgG-heavy and light-chain conjugated to HRP (horseradish peroxidase) (Bethyl Lab Inc, 6 Montgomery, CA) diluted 1:2000 in 4% MPBS was added to the wells (100 µL/well) and incubated for 1 hr at RT with gentle shaking. Wells were washed 3x with 200 µL of PBS-T, and then developed with 100 µL/well of TMB substrate (3,3',5,5'-tetramethyl benzidine; Pierce, Rockford, IL). After 10 min, the reactions were neutralized with 1.5 M H₂SO₄ (100 µL/well) and the level of binding was determined spectrophotometrically at 450 nm.

3.2.4. Serum fractionation

After detecting a polyclonal immune response, it was necessary to determine if there was also a specific HCAb immune response against α-Cbtx. Llama sera were fractionated into HCAbs and convIgGs using protein G chromatography as described by Hamers-Casterman *et al.* (1993) with minor modifications. Four mL of sera from days 21 and 110 post-immunization and day -7 (pre-immune negative control) were dialyzed o/n against PBS (pH 7.4) using dialysis tubing with a 12-14 kDa MW cutoff. Dialyzed
sera were diluted 10-fold in PBS (final volume 40 mL) and loaded onto a 5-mL protein G column (HiTrap, GE Healthcare) using an ÄKTA FPLC system (GE Healthcare). After washing the column with PBS (pH 7.4), the HCAb fraction G1 was first eluted using 0.1M citrate buffer (pH 3.5) and then the convIgG fraction G2 was eluted using 0.1M glycine-HCl (pH 2.3). After elution, fractions were immediately neutralized with 1M tris-HCl (pH 8.8), dialyzed o/n against PBS (pH 7.4), filtered through 0.22 μm and stored at 4ºC. The purity of convIgG and HCAb fractions was determined by standard SDS-PAGE and Western blotting. Protein concentrations were spectrophotometrically measured at wavelength of 280 nm.

3.2.5. Heavy-chain Ab immune responses against α–Cbtx

The HCAb immune response against α–Cbtx was assessed by indirect ELISA, as described in Section 3.2.3, with the following exception: Instead of using polyclonal serum, HCAb fractions G1 were titrated against microtitre plate-immobilized α–Cbtx (2.5 μg/mL).

3.2.6. V_H library construction

After detecting a HCAb-positive immune response against α–Cbtx from fractionated sera, the V_H library was constructed following the methods of Arbabi-Ghahroudi et al. (1997) with minor modifications. Total RNA was extracted from one aliquot (1.5 mL) of lysed leukocytes (Refer to Section 3.2.2) from day 110 using the QIAamp RNA Blood MiniTM kit (Quiagen). Subsequently, this RNA (2 μg/8 uL) was used as template for the synthesis of the first strand cDNA using the First-Strand cDNA synthesis kit (GE Healthcare) and random hexamers [pd(N)6] as primers. Two cDNA
synthesis reactions were carried out and pooled. The cDNA concentration was not quantified.

The general strategy to amplify the immunized \( V_H \) gene repertoire is summarized in Figure 10. The first round of PCR reactions used framework-1 specific sense primers (MJ1.2.3 Back; Table 3; Arbabi-Gharouhdi \textit{et al.}, 1997) and CH2-specific anti-sense primers (CH2 and CH2B3; Table 3; Arbabi-Gharouhdi \textit{et al.}, 1997), thereby amplifying the \( V_H-H2 \) and \( V_H-H1-H2 \) regions of HCAb and convIgG genes, respectively. To optimize the amplification of the \( V_H \) gene segment, small-scale test PCR reactions were first carried out with various amounts of cDNA (0.5-3 \( \mu \)L) and MgCl\(_2\) (0.5-3.0 mM). The optimal conditions for the amplification using the CH2 primer used 3.0 \( \mu \)L of cDNA and 1.5 mM MgCl\(_2\) and and 2.5 units of proofreading Taq polymerase (Expand high-fidelity Taq DNA polymerase; Hoffmann-LaRoche Ltd.) (See Appendix 1; Figure A1.1). The PCR cycling parameters were as follows: 94°C for 5 min (\textit{Taq} hot start); 30 cycles of: 94°C for 45 sec, 57°C for 45 sec, 72°C for 1.5 min; 72°C for 7 min; 4°C ∞.

The optimal conditions for the amplifications using the CH2B3 primer used 3.0 \( \mu \)L of cDNA and 0.5 mM MgCl\(_2\) (See Appendix 1; Figure A1.2). The PCR cycling parameters were as follows: 94°C 5 min (\textit{Taq} hot start); 6 cycles of: 94°C for 45 sec, 57°C for 45 sec, 54°C for 45 sec, 72°C for 1.5 min; 24 cycles of: 94°C for 45 sec, 57°C for 45 sec, 72°C for 1.5 min; 72°C for 7 min; 4°C ∞.

Ten 50-\( \mu \)L PCR reactions of both primer sets (CH2 and CH2B3) were carried out with 5 pmol of the respective primers, 0.25 mM dNTPs and 2.5 units of \textit{Taq} DNA polymerase (Hoffmann-La Roche Ltd.; Mississauga, ON). The 10 respective PCR
products were pooled and electrophoresed on a 2% agarose gel to separate the $V_{H}H$-$C_{H}2$ (~600 bp) band from the $V_{H}$-$C_{H}1$-$C_{H}2$ (~900 bp) fragment. The $V_{H}H$-$C_{H}2$ (~600 bp) band was excised and purified using the Qiagen QIAquick Gel Extraction kit (QIAGEN Inc., Mississauga, ON, Canada).

Subsequently, the amplified $V_{H}H$-$C_{H}2$/-$C_{H}2B3$ products were used as template DNA for nested PCRs using primers specific for the extremities of framework-1 (MJ7; sense) and framework-4 (MJ8; anti-sense; Table 3; Arbabi-Gharouhdi et al., 1997), resulting in an amplified $V_{H}H$ fragment without the $C_{H}2$ gene segment. These primers also introduced Sfi I restriction sites (5’- GCCNNNN^NGGCC -3’; See Table 3 underlined sequences) at the 5’ and 3’ end of the $V_{H}H$ sequence. To optimize the $V_{H}H$ gene amplification, small-scale test PCR reactions were performed with various amounts of $V_{H}H$-$C_{H}2$ or $V_{H}H$-$C_{H}2B3$ as template DNA (1-3 µL) and MgCl$_2$ (0.25-1.5 mM), and with different annealing temperatures. The optimal conditions for the amplifications of the $V_{H}H$ gene segment used 5 ng of $V_{H}H$-$C_{H}2$ or 10 ng of $V_{H}H$-$C_{H}2B3$ amplicons as template DNA and 1.0 mM MgSO$_4$. Twenty 50-µL PCR reactions for both sets were done with 3.5 pmol of MJ7 and MJ8 primers, 0.25 mM dNTPs and 2.5 units of proofreading Taq DNA polymerase (Expand high-fidelity Taq DNA polymerase; Hoffmann-LaRoche Ltd.). An aliquot (~ 3 µL) from each PCR product was run on a 1% agarose gel to confirm the amplified product was of the correct size for a $V_{H}H$ (~ 450 bp). All the PCR products were pooled and purified using MinElute spin columns (MinElute PCR Purification Kit; QIAGEN Inc., Mississauga, ON, Canada).
Figure 10. Summary of the V\textsubscript{H} cloning strategy.

The V\textsubscript{H} gene repertoire (10 µg) was digested with S\textit{i} I (New England Biolabs, Ipswich, MA, USA) at 50°C for 24 hr. After digestion, a small aliquot was analyzed on a 1% agarose gel to confirm it was the proper size for a V\textsubscript{H} fragment. The V\textsubscript{H}-S\textit{i} I insert was purified using MinElute spin columns.

Twenty µg of pMED1 phagemid vector (Arbabi-Ghahroudi et al., 2009; Figure 11) were digested with S\textit{i} I for 24 hr at 50°C, purified using QIAquick PCR purification kit, and double-digested with \textit{Pst} I (Roche) and \textit{Xho} I (Roche) for 5 hr at 37°C. The
digested vector was purified with the *QIAquick PCR purification kit* and concentrated by standard ethanol DNA precipitation.

**Table 3. Nucleotide sequences of primers used** for the construction of the phage-displayed *V_{H}H* library. Source of primers: Dr. Mehdi Arbabi-Ghahroudi.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJ1 Back</td>
<td>5′-GCCCCAGCGGCCTGCTGGGAKTCTCTGGGGA-3′</td>
</tr>
<tr>
<td>MJ2 Back</td>
<td>5′-GCCCGAGCCGGCCATGGCCAGGTAAAGCTGGAGGAGTCCTGGGGA-3′</td>
</tr>
<tr>
<td>MJ3 Back</td>
<td>5′-GCCCGAGCGGCTGGCCAGGCTCAGGTACAGCTGGTGGAGTCTGTGGGAGTCT-3′</td>
</tr>
<tr>
<td>CH2</td>
<td>5′-CGCCATCAAGGTACCAGTGTGA-3′</td>
</tr>
<tr>
<td>CH2B3</td>
<td>5′-GGGGTACCTGTCATCACGGACCAGCTGA-3′</td>
</tr>
<tr>
<td>MJ7</td>
<td>5′-CATGTGATAGACTCGCAGGCCCAGCCGGCCATGGGCAATGGCC-3′</td>
</tr>
<tr>
<td>MJ8</td>
<td>5′-CATGTGATAGATTCCCTGGCGGCTGGCTGAAGAGACCGTGACCTGG-3′</td>
</tr>
<tr>
<td>PN2</td>
<td>5′-CCCTCATAAGCGTAACGATCT-3′</td>
</tr>
<tr>
<td>M13RP</td>
<td>5′-CAGGAAACAGTATGAC-3′</td>
</tr>
</tbody>
</table>

*M = A + C, K = G + T, S = G + C.*

Several test ligations/transformations were done to optimize the *V_{H}H* cloning (% insert) and transformation efficiencies. After optimization, twenty-seven small-scale ligation reactions were serially done with 200 ng vector, 75 ng of *V_{H}H-Sfi I* insert, 1 µL of T4 DNA ligase (Promega, Madison, WI), 1 µL of buffer at 16°C for 16 hr. The ligation material was pooled, purified using spin columns provided in the *QIAquick PCR purification kit*, and eluted in a total volume of 200 µL ddH2O.

Four uL of the *pMED1-V_{H}H* ligated product were transformed into 50 µL of prepared electrocompenent *E. coli* TG1 at 1200 V, 25 µF and 200 Ω using a Gene Pulser Xcell™ electroporator (Bio-Rad Laboratories, Mississauga, ON, Canada) and 0.1-cm electroporation cuvettes (Bio-Rad Laboratories, Mississauga, ON, Canada). Immediately after transformation, the cells were transferred to 1 mL of pre-warmed SOC medium, and incubated with shaking for 1 hr at 37°C. A total of 50 transformations were done. After
the 1 hr of incubation, the fifty 1-mL cultures were pooled. To determine the size of the library, a small aliquot (10 µL) of transformed cells was serially diluted (10^-4, 10^-5 and 10^-6) and plated onto 2xYT agar plates containing ampicillin (100 µg/mL) and 1% (w/v) glucose (2xYT/Amp/1% glucose). The culture was centrifuged at 3,000 g for 20 min and resuspended in 500 mL of 2xYT/Amp/2% glucose. The library was amplified o/n at 37°C at 220 rpm. The next morning, the library was centrifuged as described above and resuspended in 100 mL of 2xYT/Amp/2% glucose with glycerol (30% final concentration). The amplified library was aliquoted (~5.0 x 10^9 bacterial cells/aliquot; 3.5 mL), and stored at -80°C until required for use.
**Figure 11.** pMED1 vector map. The multiple cloning site (MCS) is shown below the map displaying the PelB signal peptide, restriction sites, and purification/detection tags. (Arbabi-Ghahroudi et al., 2009).
3.2.7. Determining the quality of the V\textsubscript{H}H library

The quality of the V\textsubscript{H}H library was estimated based on library size, insert ratio, and diversity of the V\textsubscript{H}H sequences. The size of the V\textsubscript{H}H library was estimated by counting the number of independent clones, from the serial dilution, after it was spread onto 2xYT/Amp/1\% glucose agar plates (Refer to Section 3.2.6) and multiplying it by the dilution factor and the total culture volume (50 mL).

The success of cloning the V\textsubscript{H}H insert (~400 bp) into the phagemid vector was determined by screening fifty random library colonies (from the 10\textsuperscript{-6} titer plate) by colony PCR. The M13RP and PN2 primers (Table 3) were used, which are situated upstream and downstream of the multiple cloning site and separated by ~350 bp in an ‘empty’ vector. Therefore, an amplified product of 750 bp (400 bp + 350 bp) indicates that an insert, having correct size for a V\textsubscript{H}H, was cloned into the vector. The PCR cycling parameters were as followed: 94\(^\circ\)C for 5 min; 30 cycles of: 94\(^\circ\)C for 30 sec, 55\(^\circ\)C for 30 sec, 72\(^\circ\)C for 1 min; 72\(^\circ\)C for 7 min; 4\(^\circ\)C \(\infty\).

To assess the library diversity, 20 clones positive for an insert of the size of a V\textsubscript{H}H were sequenced (Laboratory Service Division (LSD), University of Guelph). Their predicted amino acids were aligned and analyzed to determine the diversity of the V\textsubscript{H}H sequences.
3.2.8. Selection by phage display

3.2.8.1. RESCUING THE PHAGE LIBRARY

A 3.5-mL aliquot from the amplified V_H library stock, representing about 5.0 x 10^9 cells, was thawed on ice and inoculated into 300 mL of pre-warmed 2xYT supplemented with 1% glucose and 100 µg/mL ampicillin. The culture was grown at 37°C at 220 rpm. When the optical density at 600 nm (OD_{600}) reached 0.45, about 10^{12} plaque forming units (pfu) of M13KO7 helper phage (New England Biolabs, Ipswich, MA), representing a phage to cell ratio of about 20:1, were used to infect the cells (bacteria). The culture was incubated at 37°C for 30 min without shaking to allow infection, and further incubated at 220 rpm for 1 hr. The infected cells were centrifuged at 3,300 g for 10 min, resuspended into 200 mL of 2xYT supplemented with 0.1% glucose, 100 µg/mL ampicillin and 50 µg/mL kanamycin, and grown o/n at 30°C at 220 rpm.

The o/n culture was centrifuged at 3,300 g, 4°C, for 30 min and the supernatant was transferred into six (~ 36 mL each) 50-mL Falcon tubes. To precipitate the phage, ~9 mL of PEG/NaCl (1:5 volume; 20% polyethylene glycol 6000, 2.5 M NaCl) was added and the contents were mixed gently by inverting the tubes several times. The tubes were incubated on ice for about 1.5-2 hr and centrifuged at 10,000 g, 4°C, for 30 min. After decanting the supernatant and drying the remaining PEG/NaCl, the six white precipitated phage pellets were redissolved in a total final volume of 2 mL of sterile PBS (pH 7.4) (1 mL for round 2 and round 3 of panning). To further remove bacterial cell debris, the sample was centrifuged a second time for 15 min, as described above.
To determine the input phage titre, a 10-µL phage aliquot was used to infect 90 µL of exponentially growing *E. coli* TG1 cells at 37°C without shaking for 15 min. After infection, a serial dilution (from $10^{-2}$ to $10^{-12}$) was prepared, spread on 2xYT/Amp agar plates and incubated o/n at 32°C. The next day colony forming units were counted to calculate the phage titre (input).

### 3.2.8.2. PANNING

For round 1 panning, one well of a Reacti-Bind™ maleic anhydride-activated polystyrene microtitre plate (Pierce Biotechnology, Rockford, IL) was coated with sterile PBS (pH 7.4; 100 µL) and a second well was coated with 40 µg of α-Cbtx (diluted in sterile PBS, pH 7.4; 100 µL). For rounds 2 and 3 panning, the coating concentration of α-Cbtx was decreased to 20 and 5 µg, respectively. After o/n incubated at 4°C, wells were washed 3x with 200 µL PBS and blocked with 300 µL of 4% MPBS at 37°C for 2 hours. During this time, 100 µL of amplified phage (Refer to Section 3.2.8.1) and 100 µL of 8% MPBS (1:1 phage:blocking agent ratio) were combined in a 0.5 mL tube and pre-incubated with rotation for 1 hr at RT. After the blocking incubation was complete, the wells were washed 5x with PBS (300 µL). For subtractive panning of plastic binders, 100 uL of the pre-incubated phage were first incubated in the PBS coated well for 1 hr at 37°C. After incubation, the content of the well was transferred to the α-Cbtx coated well and incubated for 2 hr at 37°C. Unbound phages were removed by washing 5, 8 and 12x with PBST (200 µL) for panning rounds 1, 2 and 3, respectively, and then washed 2x with PBS (200 µL). To elute bound phages, 200 µL of 100 mM triethylamine (TEA) was added to the well and incubated at RT for 10 min. For the last 2 min of this incubation, the content of the well was stirred by pipetting up and down several times. Eluted phages
were transferred to a microcentrifuge tube and vortexed with 400 µL of 1 M tris-HCl (pH 7.4) to neutralize the TEA. Eluted phage (600 µL) were used to infect 1.4 mL of exponentially growing E. coli TG1 without shaking for 30 min at 37°C. After infection, a 10 µL aliquot of infected E. coli was used to make a serial dilution (from 10^{-2} to 10^{-6}) to determine the phage titre (output). The remaining culture was spread on a 2xYT/Carb/1% glucose agar plate and incubated o/n at 32°C. The next morning, the cells were scrapped from the plate using a plastic loop and 2 mL of 2xYT/Carb/15% glycerol. A 100 µL aliquot of these cells were inoculated into 50 mL of 2xYT/Carb/1% glucose for the production of phage for the next round of panning. (The remaining cells were stored at -80°C). When the absorbance reading reached 0.4, 10^{11} pfu of M13KO7 helper phage was added to 10 mL of the E. coli culture. The growing of the culture and phage harvesting were performed as previously described in preparation for the next round of panning.

3.2.8.3. POLYCLONAL PHAGE ELISA

The progress of selection against immobilized α-Cbtx was monitored by performing an ELISA using polyclonal phage from each round of panning (round 1, round 2 and round 3) as well as with the unselected library phage. Wells from a Reacti-Bind™ maleic anhydride-activated polystyrene microtitre plate (Pierce Biotechnology, Rockford, IL) were coated o/n with 100 µL of α-Cbtx (1 µg/mL) at 4°C. Wells were washed 3x with 200 µL of PBS (pH 7.4) to remove unbound antigen, and then blocked with 300 µL of SuperBlock (Pierce Biotechnology, Rockford, IL) o/n at 4°C. Amplified eluted phage (100 µL; 10^{11} cfu/mL) from each round of panning or phage from the unselected library (100 µL; 10^{11} cfu/mL) were added to blocked wells and incubated for 2
hr at 37°C. After washing the wells 5x with PBST, bound phage were detected by using 100 μL of anti-M13 IgG conjugated to HRP (GE Healthcare, Baie d'Urfé, QC, Canada) diluted 1:5000 in SuperBlock. After 1 hr incubation at RT with gentle shaking, the wells were washed 5x with 300 μL of PBST. The reactions were developed with 100 μL of TMB substrate (Pierce Biotechnology, Rockford, IL) for about 15 min and then neutralized with 1.5 M H₂SO₄ (100 μL). The level of binding was determined spectrophotometrically at 450 nm.

3.2.8.4. MONOCLONAL PHAGE ELISA

A total of 46 colonies (from the titre plate) from the third round of panning were screened for α–Cbtx binding by monoclonal phage ELISA. Colonies were grown in a 96-well culture plate (Corning Incorporated Life Sciences, Acton, MA) containing 100 μL/well of 2xYT supplemented with ampicillin (100 μg/mL) and 1% glucose. After incubation at 37°C for 16 hr with shaking (220 rpm), 2 μL of each o/n culture was transferred into 200 μL of 2xYT supplemented with ampicillin and 1% glucose. Culture plates were incubated at 37°C for 2 hr with shaking. After incubation, each culture was infected with 10¹⁰ helper phage (M13KO7) and incubated at 37°C for 15 min without shaking and then for 1 hr with shaking (250 rpm). The culture plate was centrifuged at 1800 rpm for 10 min at 4°C, and the supernatant was carefully removed and discarded. Cell pellets were resuspended in 200 μL of 2xYT containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL), and grown o/n with shaking (250 rpm) at 30°C. After centrifugation of the plate for 30 min as described above, 50 μL supernatant (which contain the phage-displayed V₄H) were collected and used for monoclonal phage ELISA.
Reacti-Bind™ maleic anhydride-activated polystyrene microtitre plates (Pierce Biotechnology, Rockford, IL) were coated with α-Cbtx (1 μg/mL), blocked with SuperBlock, and washed as described previously (Refer to Section 3.2.8.3). Phage supernatant (50 μL) and SuperBlock (50 μL) were added to wells and incubated at 37°C for 2 hr. Wells were washed and bound phage was detected as described in Polyclonal phage ELISA Section 3.2.8.3.

Clones with absorbance (450 nm) readings greater than 0.3 background were sequenced using the universal M13RP primer at the Laboratory Division Services (University of Guelph).

3.2.9. Antibody Characterization

3.2.9.1. SUBCLONING V<sub>H</sub> CODING SEQUENCES INTO pMED2 EXPRESSION VECTOR

Preliminary expression and purification results showed that C2 and C43 clones expressed at low levels in pMED1 phagemid vector. Therefore, these V<sub>H</sub> coding sequences were subcloned into the SfiI restriction sites of the expression vector pMED2 (kindly provided by Dr. Mehdi Arbabi-Ghahroudi). Proteins expressed from both pMED1 and pMED2 vectors contain a His<sub>6</sub> tag for purification.

3.2.9.2. EXPRESSION OF A-CBTX V<sub>H</sub>H BINDERS

For soluble expression of V<sub>H</sub>Hs, purified recombinant C2-pMED2, C19-pMED1, C20-pMED1 and C43-pMED2 constructs were electroporated (Refer to 3.2.6.) into E. coli strain HB2151, a non-suppressor strain. Single colonies were picked and transferred into 5 mL of 2x YT starter culture supplemented with 75 μg/mL Carb and 1% (w/v)
glucose. Cultures were grown o/n at 37°C while shaking at 220 rpm. For large-scale expression, 1 mL of starter culture was transferred into 1 L of 2x YT medium supplemented as described above and grown at 37°C while shaking at 220 rpm until the OD$_{600}$ reached 0.6-0.7. The cell pellets were collected by centrifuging at 3,000 g and resuspended in 1 L of 2x YT medium supplemented with 0.1% (w/v) glucose and 75 μg/mL Carb. To induce soluble V$_{H}$H expression, IPTG (1 mM final conc.) was added to the cultures. Cultures were grown at 26°C for 24 hr while shaking at 220 rpm. The induced cultures were centrifuged at 8,000 g for 15 min at 4°C. The harvested cells were resuspended in 100 mL of ice-cold lysis buffer (50 mM tris-HCl at pH 8.0, 25 mM NaCl, 2 mM EDTA), and stored at -20°C until needed for protein extraction.

3.2.9.3. **PURIFICATION OF V$_{H}$HS**

V$_{H}$Hs were purified from the periplasmic fractions of *E. coli*. The induced cell pellets that were stored in lysis buffer (100 mL) were taken out of -20°C freezer (Refer to *Section 3.2.9.2*) and 1 mL of 100 mM protease inhibitor phenylmethylsulphonyl fluoride (PMSF; 1 mM final conc.; Sigma-Aldrich, Oakville, ON) and 200 µL of 1M dithiothreitol (DTT; 2 mM final conc.; Bioshop, Burlington, ON) were immediately added. The frozen suspension was thawed at RT with occasional shaking. To lyse the cells, 5 mL of freshly prepared lysozyme (100 µg/ml final conc. from 3 mg/mL aqueous solution; Roche, Indianapolis, IN) were added to the thawed cells. The suspension was incubated at RT for 30-50 min with occasional shaking. When the suspension became viscous 300 µL of DNase I (Sigma, Sigma-Aldrich, Oakville, ON; 15units/µl stock in 1 M MgCl$_2$) was added and the lysate was incubated at RT until the suspension became watery (ca. 20-30 min). The lysate was centrifuged at 12,000 rpm for 20 min at 4°C to
separate the soluble and insoluble fractions. Centrifugation was repeated with the soluble fraction until it became clear. The fraction which contained soluble V₁Hs was dialyzed o/n against PBS (pH 7.4) containing 1 mM EDTA. Samples were filtered through a 0.22 μm sterile filter (Millipore, Nepean, ON). V₁Hs were purified by standard immobilized metal affinity chromatography (IMAC) using a 5 mL HisTrap™ HP nickel affinity column (GE Healthcare).

Western blots were done to confirm expression and purification. The tagged V₁Hs were detected using anti-penta His monoclonal antibody (Qiagen, Mississauga, ON) diluted 5,000 fold, and goat anti-mouse mAb conjugated to alkaline phosphatase (GAM-AP) diluted 5,000 fold. Membranes were washed and developed with alkaline phosphatase substrate (1-Step NBT/BCIP; Pierce Biotechnology, Rockford, IL). Fractions containing V₁H were dialyzed o/n against SPR analysis buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA; HBS-E) with a 3,500 MW cutoff. Absorbancies were measured at 280 nm (A₂₈₀), and V₁H concentrations were estimated using an extinction coefficient based on the predicted amino acid sequence of each V₁H calculated at http://ca.expasy.org/tools/protparam.html. The extinction coefficients for the purified clones were: C2, 2.1 (mg/mL); C19, 1.7 (mg/mL); C20, 1.7 (mg/mL); C43, 2.1 (mg/mL). The purified V₁Hs were stored at 4ºC.

3.2.9.4. KINETICS ANALYSIS BY SURFACE PLASMON RESONANCE (SPR)

Binding kinetic experiments were performed by SPR using a Biacore 3000 instrument (GE Healthcare). Approximately 137 resonance units (RUs) of α-Cbtx were immobilized using standard amine coupling onto a research grade CM5 sensor chip (Biacore Inc.) in 10 mM acetate buffer. Prior to SPR analysis, V₁H samples purified
from periplasmic fractions of *E. coli* (Refer to Section 3.2.9.3) were subjected to Superdex 75 gel filtration chromatography (GE Healthcare) to isolate monomers from aggregates. V$_H$H monomers were passed over the sensor chip coated in HBS-EP running buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P-20 (GE Healthcare)]. The four V$_H$Hs (C2, C19, C20 and C43) were injected at concentrations ranging from 2.5 to 30 nM, 6.25 to 150 nM, 1 to 32 nM and 5 to 160 nM, respectively. All experiments were conducted at RT at a flow rate of 40 μL/min.

### 3.2.9.5. EPITOPE MAPPING USING SPR

Conventional epitope mapping methods, like radioimmunoassays (RIA) and enzyme immunoassays (EIA), are time-consuming and require labeling of the antibody or antigen. To circumvent labeling, SPR analysis was used to determine if the α-Cbtx binders target the same or different epitopes. α-Cbtx was immobilized to the sensor chip as described in Section 3.2.9.4 and then saturated with C19 or C43. After saturation, the ability of the second α-Cbtx binder to simultaneously bind the complex was monitored. To ensure the surface capacity (Rmax) was reached, α-Cbtx binders C2, C19, C20 and C43, were injected at 1 μM, 600 nM, 500 nM and 2 μM, respectively, as determined by the kinetic analysis results (Refer to Table 6). All assays were done at RT with a flow rate of 40 μL/min in HBS-EP running buffer. An RU signal for the second V$_H$H approaching that of the first V$_H$H indicates simultaneous binding of the V$_H$Hs in the same amount.
3.2.10. *In vitro* α-Cbtx neutralization assay

The diaphragm-phrenic nerve preparation is a widely used nerve–muscle preparation and was used to investigate the neutralization efficiencies of isolated V₃H clones to α-Cbtx at neuromuscular junctions. The assay was conducted in a manner similar to that described by Bulbring (1946). Briefly, the left hemi-diaphragm with the attached phrenic nerve was carefully excised from male Sprague Dawley Rats (175-250 g), and mounted on a special tissue holder immersed in a 50-mL Schuler organ bath containing Krebs-Henseleit solution. The incubation bath was maintained at 37ºC and fed constantly with 95% O₂-5% CO₂ by bubbling the gas through the solution. The preparation was attached via a thread suture to a Harvard isometric transducer for recording of contractions on a Biopac Systems MP150. The diaphragm contracted in response to “direct” stimulation using a set of parallel electrodes, which also serve to anchor the diaphragm, or to “indirect” stimulation via a second set of electrodes which stimulates the phrenic nerve to release neurotransmitter.

The phrenic nerve was continuously stimulated with supramaximal square wave pulses (0.25 ms) at a frequency of 0.1 Hz., followed by three 3-sec periods, at 30 sec intervals, at frequencies of 25, 50 and 100 Hz., to evoke twitch and tetanic responses respectively, using a Grass® S88 stimulator. This protocol was repeated every 15 min in the presence or absence of neurotoxin with appropriate changes of bath fluid. In this case the tetanic response to 100 Hz was measured and expressed as area/volt-sec by the Biopac software program.

Purified V₃H C2 (100 nM) was pre-incubated with α-Cbtx (50 nM) for 60 min at RT prior to adding to the tissue bath. The assay was also performed with 50 nM of α-
Cbtx (without V\text{H}H) and served as a negative control. As a positive control, the tissue preparation was stimulated without the presence of α-Cbtx and V\text{H}H C2.

3.3. RESULTS

3.3.1. General health of the immunized llama

The llama, immunized with 0.25-1.5 mg of crude \textit{N. kaouthia} venom, did not suffer from visible symptoms of cobra envenomation. Systemic neurotoxicity and local tissue damage (e.g. necrosis) at the site of injection were not observed for any of the injections administered. Local inflammation and formation of granulomas were not more severe than those caused by other non-toxic immunogens (as per Karen Gourlay; Wyldewodd Farm, Freelton, ON, Canada).

3.3.2. Polyclonal sera response against \textit{N. kaouthia} venom and α-Cbtx

Polyclonal sera responses against crude \textit{N. kaouthia} venom and purified α–Cbtx were monitored by indirect ELISA and detected with goat anti-llama IgG-heavy and light-chain conjugated to HRP (Bethyl Lab Inc, 6 Montgomery, CA) (Figure 12). A good immune response were detected against \textit{a}) \textit{N. kaouthia} venom, and \textit{b}) α–Cbtx, were detected by Day 21 and did not increase much throughout the remainder of the immunization schedule (Day 110).
Figure 12. ELISA using llama polyclonal sera, taken at days -7 (▲), 21 (♦), and 110 (■) after the first immunization, and immobilized a) crude N. kaouthia venom and b) α-Cbtx.
3.3.3. Serum fractionation

After detecting a positive llama polyclonal immune response against α-Cbtx (See Section 3.2.3), it was necessary to determine if there was also a specific HCAb immune response. Llama sera were fractionated into HCAb and convIgG as described by Hamers-Casterman et al. (1993). Fractions were run by standard 12% SDS-PAGE to confirm their presence and purity (Figure 13; not all data shown).

**Figure 13.** SDS-PAGE of HCAbs and convIgG fractions. Lane 1: protein ladder; Lane 2: HCAb (fraction G1) in reducing buffer; Lane 3: convIgG (fraction G2) in reducing buffer.

Under denaturing conditions, convIgGs (MW ~150 kDa) reduce into two heavy-chains of ~50 kDa (Lane 3, upper band) and two light-chains of ~25 kDa (Lane 3, lower
band). Meanwhile, because of the absence of the C_H1 domain, HCAbs reduces into two lighter heavy-chains of ~46 kDa (Lane 2). No light chains (MW~25 kDa) were detected in the HCAb fractions which indicates that they were not contaminated with convIgGs. Likewise, convIgG fractions were not contaminated with detectable HCAbs.

3.3.4. HCAb immune response against α-Cbtx

The binding activity of HCAb (fraction G1) to immobilized α-Cbtx (2.5 μg/mL) was analyzed by indirect ELISA. As revealed by the ELISA (Figure 14), a good HCAb response was detected from days 21 and 110.

![Figure 14. ELISA showing the HCAb binding activity to immobilized α-Cbtx. HCAbs were fractionated from sera collected on days -7 (▲), 21 (♦), and 110 (■) after the first immunization.](image)

3.3.5. Construction of phage-displayed V_HH library

The positive HCAb immune response against α–Cbtx (See Section 3.3.4) confirmed the suitability of engineering a phage-displayed V_HH library. The library was constructed using total RNA isolated from peripheral blood lymphocytes harvested on day 110. After
synthesis of the first strand of cDNA, the V_{H} gene repertoire was amplified as described in Section 3.2.6. Figure 15 shows the amplification of the HCAb V_{H}H-C_{H}2/ C_{H}2b3 (~620 - 670 bp) and the conventional V_{H}-C_{H}1-C_{H}2 hinge region (~850 - 900 bp) products using MJ1.2.3. forward primers with C_{H}2 or C_{H}2B3 reverse primers. The V_{H}H-C_{H}2 (Figure 15a) and V_{H}H-C_{H}2B3 (Figure 15b) products were extracted from the gels to purify them from conventional V_{H}-C_{H}2-C_{H}1 products.

**Figure 15.** Agarose gels (1%) showing the amplified V_{H}H-C_{H}2 (~620 - 670 bp; see arrow) and conventional V_{H}-C_{H}1-C_{H}2 (~850 - 900 bp) hinge regions from the cDNA library using MJ1.2.3. sense and with a) C_{H}2 or b) C_{H}2B3 anti-sense primers. The V_{H}H-C_{H}2(B3) products (~600 bp; see arrow) were purified from the gel.

Subsequently, the V_{H}H-C_{H}2 and V_{H}H-C_{H}2B3 products were used as template DNA for the nested PCR using MJ7 and MJ8 primers. The resulting amplified V_{H}H product (Figure 16; Lane 2, ~450 bp) no longer had the C_{H}2 gene segment and was also flanked by Sfi I restriction sites.
The \( V_H \) gene library was digested with \( Sfi \) I restriction enzyme and then cloned into the pMED1 vector as described in Section 3.2.6. The ligated pMED1-\( V_H \) product was pooled and transformed into \( E. \ coli \) to maintain and propagate the library.

3.3.6. Determining the quality of the \( V_H \) library

The functional size and quality of the \( V_H \) library was estimated based on the number of independently transformed clones, insert ratio (%), and diversity and quality of the \( V_H \) sequences.

The total size of the \( V_H \) library was approximated by titering the transformed library onto 2x YT/Carb/1% glucose plates. Since 100 colonies grew on the \( 10^6 \) titer plate from a 50 mL library culture stock, the library was estimated to have \( 5.0 \times 10^9 \) (100 \( \times \) \( 10^6 \) \( \times \) 50) individual clones. However, counting the number of individual clones overestimates the functional size of the library because it does not account for clones that
harbour an ‘empty’ phagemid vector. Therefore, 50 random library clones (from the $10^6$ titer plate) were screened by colony PCR. Forty-two clones were positive for an insert the size of a $V_H$ (Figure 17; not all data shown). Therefore, about 84% of the library clones harbour a $V_H$ insert.

![PCR screening of fifty random library colonies using M13RP and PN2 primers. Fragments of ~350 bp represent clones with an ‘empty’ vector, while fragments of ~750 bp represent clones harboring a $V_H$. A total of 42 of the 50 colonies screened (84%) harboured an insert the size of a $V_H$ gene segment (not all data shown).](image)

**Figure 17.** PCR screening of fifty random library colonies using M13RP and PN2 primers. Fragments of ~350 bp represent clones with an ‘empty’ vector, while fragments of ~750 bp represent clones harboring a $V_H$. A total of 42 of the 50 colonies screened (84%) harboured an insert the size of a $V_H$ gene segment (not all data shown).

Thereafter, twenty clones positive for a $V_H$-size insert were sequenced (LSD, University of Guelph) to determine the quality and heterogeneity of the library. Sequence analyses revealed that all the clones contained llama $V_H$ signature residues at positions 37 (Phe or Tyr), 44 (Glu or Gln), 45 (Arg) and 47 (Leu, Phe or Gly) which are located in the framework region 2 (Kabat numbering system; Kabat and Wu, 1991) (Vu *et al.*, 1997). Furthermore, comparison of the predicted amino acid sequences revealed 100% diversity among the clones (Figure 18). While each clone contained unique CDR regions, the most variability in terms of amino acid composition and length (7-20 a.a) was observed in CDR3.
Based on the system of Harmsen et al. (2000) to classify $V_{\text{H}}$ subfamilies, 8 clones belonged to $V_{\text{H}}$-1, 4 clones belonged to $V_{\text{H}}$-2 and the remaining 8 clones belonged to $V_{\text{H}}$-3 (Figure 18). None of the clones belonged to $V_{\text{H}}$-4; however, this subclass is usually less frequently found (Harmsen et al., 2000). Nevertheless, the $V_{\text{H}}$ library constructed reflects a good representation of the $V_{\text{H}}$ subfamilies and thus has good diversity.

The functional size of the $V_{\text{H}}$ library was therefore estimated by multiplying the number of individual clones ($5.0 \times 10^9$ clones) by the insert ratio (84%) by the diversity of the coding sequences (100%). Thus, the size of the $V_{\text{H}}$ library was estimated to be $4.2 \times 10^9$ functional clones. This is a relatively large $V_{\text{H}}$ library and was considered ready for panning.

It is also important to note that about 15% of the clones sequenced contained a frameshift mutation (FSM), while none of the clones had a premature amber stop codon mutation.
Figure 18. Predicted amino acid sequence alignment of the CDR regions of 20 clones from the unselected V_{H}H library. Each clone had unique CDR regions with the CDR3 being the most diverse in terms of amino acid composition and length. The library is, therefore, said to be ‘100% diverse’. The brackets with numbers indicates which V_{H}H subfamily each clone belong to (i.e., V_{H}H-1, V_{H}H-2, V_{H}H-3 or V_{H}H-4) (Harmsen et al., 2000). “C” followed by a number (e.g. C20), shown along left side of the figure, designates the name of the clone.

3.3.7. Panning against α-Cbtx

Three rounds of panning were performed to select α-Cbtx V_{H}H binders as described in Section 3.2.8.2. In the first round of panning, 40 µg of α-Cbtx was coated onto the well and 5 washes were done to remove non-specific binders. This resulted in a high percent recovery (2%) of phages from round 1 (Table 4). Because the percent recovery is an indication of the stringency of selection, the stringency was further increased in the second round by decreasing the concentration of α-Cbtx (20 µg/well) and
by increasing the number of washes. As a result, the output titre of the second round was substantially decreased from 700 to $8.2 \times 10^6$ cfu (Table 4). Again, the stringency was further increased in the 3rd round of panning as previously described, while the output titre from the 3rd round of panning increased to $40 \times 10^6$ cfu.

Table 4. Polyclonal phage titres of three rounds of panning against immobilized $\alpha$-Cbtx.

<table>
<thead>
<tr>
<th>Round</th>
<th>Input ($x 10^{10}$ cfu)</th>
<th>Output ($x 10^6$ cfu)</th>
<th>(Output/Input)*100 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>700</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>8.75</td>
<td>8.2</td>
<td>0.009</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>40</td>
<td>0.007</td>
</tr>
</tbody>
</table>

3.3.8. Polyclonal phage ELISA

The progress of selection against immobilized $\alpha$-Cbtx (1 $\mu$g/mL) was monitored by performing an ELISA using polyclonal phage from each round of panning (round 1, round 2 and round 3) and with the unselected library phage as a control (Figure 19). After three rounds of panning, the ELISA signal was at least 4 and 10 times greater than that from round 2 and the unselected library, respectively, indicating enrichment for clones specific to $\alpha$–Cbtx.
Figure 19. The progress of selection against α–Cbtx was monitored by polyclonal phage ELISA. Aliquots of eluted phages (amplified; 100 µL of 10^{11} cfu/mL) from each round of panning were incubated with immobilized α–Cbtx (1 µg/mL) and detected with anti-M13 antibody conjugated to horseradish peroxidase (see Materials and Methods, Section 3.3.8.3.). Absorbance values taken at 450 nm are the means of triplicates after subtraction of background (no α–Cbtx).

3.3.9. Monoclonal phage ELISA

To identify specific α-Cbtx V₄H binders, individual phage clones from the 3rd round of panning were screened by monoclonal phage ELISA. A total of 46 clones were picked at random (from a titre plate) and screened against immobilized α-Cbtx. Bound phages were detected with anti-M13 IgG conjugated to HRP. Clones with an absorbance reading > 0.3 were considered positive for α-Cbtx binding. About 60% of monoclonal phages (27/46) showed specific binding to α-Cbtx (Figure 20; not all data shown).
Figure 20. Monoclonal phage ELISA of clones selected from the 3rd round of panning. Monoclonal phage (amplified; 100 µL) were incubated with immobilized α–Cbtx (1 µg/mL) and detected with anti-M13 antibody conjugated to horseradish peroxidase (see Materials and Methods, Section 3.2.8.4). Absorbance values at 450 nm are the mean of triplicates with background (no α–Cbtx) subtracted.

3.3.10. Sequence analysis of round 3 anti-α-Cbtx V\textsubscript{H} clones

A total of 27 clones considered positive for α-Cbtx binding by monoclonal phage ELISA were sequenced at the Laboratory Services Division (LSD; University of Guelph). Analysis of the coding sequences and predicted amino acid compositions revealed that the 3rd round of panning generated several unique α-Cbtx binders. Of all the clones sequenced, only two clones, C15 and C46, shared 100% identity. All other clones were unique with at least one different amino acid substitution; thus, many clones shared high identity as revealed by a multiple sequence alignment (MSA) of the predicted amino acid
sequence (Figure 21). Moreover, based on CDR homology and CDR3 length, two distinct groups of α-Cbtx binders are apparent, hereafter named Cluster I and Cluster II (see Figure 21).

The nine clones (C33, C46, C15, C7, C13, C19, C34, C31, C20) that form Cluster I are characterized by a CDR3 length of 17 amino acid residues with the following consensus motif: GSV(V/L/I)SY(E/V)TGNYEPS(N/D)Y. All these binders have identical CDR2 regions (FISSGGRSKYTDSVK), and a well conserved CDR1 (consensus motif: G(D/S)ISSFN(A/G)MG).

In contrast to Cluster I, clones that form Cluster II (C2, C29, C43, and C42) have a shorter CDR3 region with 14 amino acid residues, which has the following highly conserved consensus motif: EGVRYGDSWYDG(D/V)Y. Like Cluster I, clones among Cluster II, share an identical CDR2 with the sequence VITNGNSPNYADSVKG. Furthermore, all the clones from this group have a conserved CDR1 with the sequence GSISSIYAMG, except C43 which has a unique CDR1.

Sequences were further analyzed to confirm they contained llama V₇H signature residues. In conventional V₇Hs, residues Val37, Gly44, Leu45 and Trp47 are extremely well conserved and interact with the V₇L domain (Vu et al., 1997). However, in the absence of a light chain, camelid V₇Hs constitutively substitute these solvent-exposed residues with more hydrophilic ones. As shown in Figure 21, all the anti-α-Cbtx clones have Val37→Tyr(Y), Gly44→Gln(Q), and Leu45→Arg(R) substitutions confirming they are in fact V₇Hs. Interestingly, Trp47 which is normally substituted by Phe (for V₇H-1), by Leu (V₇H-2; Cluster I) or by Gly (V₇H-3 and -4) in llama V₇Hs (Harmsen et al., 2000) is substituted by a Val(V) in clones of Cluster II. The occurrence of a valine
residue at this position is extremely rare in llama V<sub>H</sub>Hs (Harmsen <i>et al.</i>, 2000). Nevertheless, all the α–Cbtx binders isolated (Cluster I and Cluster II clones) from this immunized library belonged to V<sub>H</sub>H subfamily 2 (V<sub>H</sub>H-2).

Although the anti-α-Cbtx V<sub>H</sub>Hs from both Cluster I and Cluster II display a relatively long CDR3 loop (17 and 14 residues, respectively), none possess additional cysteine residues that could form an additional disulfide bridge to help stabilize the long CDR3 loop. However, these extra cysteine residues are usually a characteristic of V<sub>H</sub>H subfamilies 3 and 4 (Harmsen <i>et al.</i>, 2000).

Several positive binders detected by the monoclonal phage ELISA (Figure 20) contained premature amber (TAG) stop codon mutations. Amber (TAG) stop codon mutations in the V<sub>H</sub>H coding sequence are represented by a red asterisk (*) in Figure 21. Most of these mutations occurred in framework region 1 (FR1), more specifically at the first amino acid of the V<sub>H</sub>H. Furthermore, many clones that showed binding by monoclonal phage ELISA contained a frameshift mutation (FSM). Most of these FSM were caused by 1-2 missing nucleotides immediately upstream of the first amino acid of the V<sub>H</sub>H.
Figure 21. Predicted amino acid sequence alignment of anti-α-Cbtx V_H binders isolated from the 3rd round of panning. The clones were categorized with either Cluster I or Cluster II based on their sequence homology. Residues are numbered according to the Kabat numbering system (Kabat and Wu, 1991). The dots in the sequences indicate amino acid identity that is the same as in C33 (Cluster I) or C2 (Cluster II). All clones belong to V_H Subfamily 2 (Harmsen et al., 2000). An asterisk (*) represents an amber stop codon (TAG) mutation.
3.3.11. Anti-α-Cbtx V<sub>H</sub>H clones selected for further characterization

Four α-Cbtx V<sub>H</sub>H clones with high absorbance values as determined by ELISA (data not shown) were selected for further characterization; two from Cluster I (C19 and C20) and two from Cluster II (C2 and C43). As shown in Table 5, C2 and C43 differ by nine amino acid residues which are located in FR1 and CDR1. C19 and C20 differ by 10 amino acid residues which are located in different regions.

<table>
<thead>
<tr>
<th>Table 5. Predicted amino acid sequences of the α-Cbtx V&lt;sub&gt;H&lt;/sub&gt;H binders selected for further characterization.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V&lt;sub&gt;H&lt;/sub&gt;H Name</strong></td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>C2</td>
</tr>
<tr>
<td><strong>V&lt;sub&gt;H&lt;/sub&gt;H Name</strong></td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>C2</td>
</tr>
<tr>
<td>C43</td>
</tr>
<tr>
<td>C19</td>
</tr>
<tr>
<td>C20</td>
</tr>
<tr>
<td><strong>V&lt;sub&gt;H&lt;/sub&gt;H Name</strong></td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>C2</td>
</tr>
<tr>
<td>C43</td>
</tr>
<tr>
<td>C19</td>
</tr>
<tr>
<td>C20</td>
</tr>
</tbody>
</table>

3.3.12. Large-scale expression and purification of selected V<sub>H</sub>H clones

SDS-PAGE (data not shown) and Western blot analysis (Figure 22) showed that the four selected clones (C2, C19, C20 and C43) were successfully expressed in 1-L E. coli cultures, and could be purified from the periplasmic fractions using IMAC. Based on the predicted amino acid composition along with the His<sub>6</sub> detection/purification tag, the theoretical MWs of the V<sub>H</sub>Hs were predicted to be ~16.6 kDa. With the exception of C20, two bands
migrating close to each other were detected around the expected position. The nature of the second band was not further investigated. However, Dr. Serge Muyldermans (Vrije Universiteit Brussel, Brussels, Belgium), who also observed similar results and did further investigations on them, suggested that these different band patterns may be caused by different configurations of the \( V_\text{H} \) protein (personal communication).

Expressed concentrations of \( V_\text{H} \) were similar among the clones at 12-18 mg per 1 L, which was a sufficient yield for characterization by ELISA, SPR analysis, and \textit{in vitro} neutralization assays.

![Western blot](image)

**Figure 22.** Western blot of \( V_\text{H} \)s C2, C19, C20 and C43 purified from the periplasmic fractions of \textit{E. coli} using IMAC. \( V_\text{H} \)s were detected with anti-penta His mAb and goat anti-mouse mAb conjugated to alkaline phosphatase.

### 3.3.13. Kinetic analysis

The affinities of the four selected \( V_\text{H} \) clones for \( \alpha \)-Cbtx antigen were measured by surface plasmon resonance (SPR). The overlaid sensorgrams are shown in Figure 23. Rate and affinity constants were determined by global fitting (black lines in Figure 23) of the data set with a one-to-one interaction model. Kinetic rate constants are summarized in Table 6. All four \( V_\text{H} \) clones exhibited high affinity (i.e. low nM) to immobilized \( \alpha \)-Cbtx. In particular, \( \alpha \)-
Cbtx had the highest affinity to C2 with a sub-nanomolar $K_D$ value of 0.4 nM. The lower $K_D$ of C2 is mostly due to its slow dissociation rate constant of $10^{-4}$ s$^{-1}$, compared to $10^{-2} – 10^{-3}$ s$^{-1}$ for the other clones (Table 6). C20 had the fastest association rate constant; however, its dissociation was faster (i.e. greater) than that of C2. C19 and C43 had 60-fold and 25-fold lower affinity than that of C2, respectively.
Figure 23. Surface plasmon resonance analysis of immobilized α-Cbtx vs. anti-α-Cbtx V_{H}H clones a) C2, b) C19, c) C20, and d) C43.
Table 6. Equilibrium dissociation constants (K_Ds), association (k_on) and dissociation (k_off) rate constants for the interaction of α-Cbtx with the V_H clones as determined by surface plasmon resonance.

<table>
<thead>
<tr>
<th>V_H</th>
<th>K_D (nM)</th>
<th>k_on (M⁻¹s⁻¹)</th>
<th>k_off (s⁻¹)</th>
<th>R_max (RU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>0.4</td>
<td>5.2 x 10⁵</td>
<td>1.9 x 10⁻⁴</td>
<td>133-178*</td>
</tr>
<tr>
<td>C43</td>
<td>24</td>
<td>2.9 x 10⁵</td>
<td>6.8 x 10⁻³</td>
<td>143</td>
</tr>
<tr>
<td>C19</td>
<td>25</td>
<td>5.8 x 10⁵</td>
<td>1.0 x 10⁻²</td>
<td>188</td>
</tr>
<tr>
<td>C20</td>
<td>1</td>
<td>1.8 x 10⁶</td>
<td>1.7 x 10⁻³</td>
<td>218</td>
</tr>
</tbody>
</table>

*The surface capacity dropped over the course of the analysis.

3.3.14. Epitope mapping by SPR

SPR was used to determine if the V_H clones target non-overlapping epitopes on α–Cbtx. Figure 24 shows the overlaid sensograms for sequential injection of two α–Cbtx binders over time. α–Cbtx was first immobilized on the surface of a sensor chip by standard amine coupling. When 600 nM of C19 (Figure 24a) or 2 μM of C43 (Figure 24b) was injected over immobilized α–Cbtx, the RU signal sharply increased to approach the surface capacity (R_max value; See Table 6). This indicates that the respective epitope on α–Cbtx was saturated with the first V_H (C19 or C43). After saturation, the second V_H clone (Figure 24a: C2, C20 or C43; Figure 24b: C2, C19 or C20) was injected (See arrow) to determine whether it could simultaneously bind the complex. The increase in RU measurement observed after the arrow in Figure 24 is proportional to the amount of binding of the second V_H. If the second V_H clone targets a different non-overlapping epitope, then the RU signal should increase by about the surface capacity (R_max) obtained in the kinetic analysis (See Table 6; R_max value). However, if the α-Cbtx binders recognize a common, overlapping or adjacent epitope, then little change in RU will be observed.
After saturation of α-Cbtx with C19, some binding was observed with C2 and C20; however, the signal monitored was minimal and only increased by 19 and 33 RUs, respectively (See Appendix 2). C19 almost completely blocked the binding of C43 to α-Cbtx.

a) 600 nM C19 followed by 2 μM C43, 500 nM C20 and 1 μM C2

b) 2 μM C43 followed by 600 nM C19, 500 nM C20 and 1 μM C2

Figure 24. Epitope mapping using surface plasmon resonance. After saturation of α-Cbtx with a) C19 or b) C43, the three other V_{H} clones were individually injected (arrows). The increase in RU is directly proportional to the amount of binding to α-Cbtx.

As shown in Table 6, C43 has the lowest surface capacity with 143 RUs. When α–Cbtx was first saturated with C43 (Figure 24b), further binding with C2, C19 and C20 was detected. However, the signal only increased by 23, 21 and 25 RUs, thus indicating little binding (See Appendix 2).
The sequential injection of different $V_H$H combinations revealed that the $\alpha$–Cbtx binders recognize a common, overlapping or adjacent epitopes. None of the binders target a discrete epitope that is not at least affected by steric hindrance. Therefore, it appears that $\alpha$–Cbtx cannot be simultaneously bound by more than one of these $V_H$Hs.

3.3.15. *In vitro* neutralization assay

The ability of $V_H$H C2 to attenuate the neuromuscular paralytic effect of $\alpha$-Cbtx was investigated by performing an *in vitro* neutralization assay. When 100 Hz was applied to the diaphragm-nerve preparation without the presence of $\alpha$-Cbtx or $V_H$H C2, the diaphragm muscle was able to contract and produced an area under the curve (AUC) of 3.46 millivolts second (mV sec) (Figure 25a). However, addition of $\alpha$-Cbtx (50 nM) decreased muscle contraction by about 74% (AUC = 0.91 mV sec) of the control, thereby indicating muscle paralysis (Figure 25c). The addition of $V_H$H C2 (100 nM) neutralized the paralytic effect of $\alpha$-Cbtx at neuromuscular junctions (AUC = 3.47 mV sec).
Figure 25. Attenuation of $\alpha$-Cbtx inhibition of the tetanic response to phrenic nerve stimulation by $V_{II}H$ C2. Tetanic response in an untreated preparation (a), following $\alpha$-Cbtx 50 nM plus $V_{II}H$ C2 100 nM (b), and following 120 min later $\alpha$-Cbtx 50 nM without $V_{II}H$ (c). $\alpha$-Cbtx was co-incubated with $V_{II}H$ C2 for 60 min at room temperature prior to adding to the tissue bath. The tetanic response to 100 Hz was measured and expressed as area/volts-sec with a) 3.46 mV sec, b) 3.47 mV sec, and c) 0.91 mV sec.
3.4. DISCUSSION

Previously in our laboratory, a naïve llama phage-display \( V_{H}H \) library was used to select \( V_{H}H \)s against \( \alpha-Cbtx \), a potent \( \alpha \)-neurotoxin of the venom of *Naja kaouthia* (Stewart *et al.*, 2007). Unfortunately, the affinities of the isolated naïve \( V_{H}H \)s were too low (low \( \mu \)M range) for therapeutic use. The aim of this current study was to isolate higher affinity \( V_{H}H \)s with neutralizing capacity to \( \alpha-Cbtx \) by constructing an immune phage-displayed \( V_{H}H \) library.

Although immunization of horses with venom is routinely performed for antivenom development, this is the first report to describe the successful immunization of a llama with crude snake venom, more specifically *N. kaouthia* venom. The llama generated a good polyclonal response against crude venom and, more specifically, against \( \alpha-Cbtx \).

A closely related study reported the immunization of a camel with the purified toxic fraction of *Androctonus australis hector* (scorpion) as the immunogen (Meddeb-Mouelhi *et al.*, 2003). However, results from this thesis indicate that it is unnecessary to purify the venom to induce a strong immune response against the toxic fraction, at least pertaining to cobra venom. Furthermore, it is also not necessary to conjugate toxins to carrier proteins, to detoxify them, or convert them to a toxoid as other researchers have done (Anderson *et al.*, 2008).

To evaluate the HCAb immune response, the serum was fractionated into convIgG and HCAb fractions by protein G chromatography as described by Hamers-Casterman *et al.* (1993). Since the llama produced a HCAb immune response against \( \alpha-Cbtx \), it was decided to construct a phage-displayed \( V_{H}H \) library using RNA extracted from peripheral blood lymphocytes harvested on day 110. Following amplification and
cloning of the \( V_{H} \) gene repertoire into the pMED1 phagemid vector, the ligated product was transformed into \( E. \ coli \) TG1 to maintain the library. Subsequently, the size of the library was estimated to be \( 5.0 \times 10^{9} \) individual clones with 84% containing a \( V_{H} \) coding sequence. Sequence analysis of random clones revealed that the library was 100% diverse with few conserved amino acid residues in the CDR regions. In particular, the CDR3 region varied considerably in terms of the amino acid composition and length (Figure 18). Furthermore, this library contained a good repertoire of the different \( V_{H} \) subfamilies, except for \( V_{H} \) subfamily 4 which is naturally more rare (Harmsen et al., 2000). In short, the \( V_{H} \) library constructed is of good quality and should contain high affinity \( V_{H} \) binders against the immunogens. Nevertheless, about 15% of the clones sequenced contained a frameshift mutation (FSM), while none of the clones had a premature amber stop codon mutation.

Enrichment of \( \alpha-\)Cbtx \( V_{H} \) binders by panning this immune library was a success. As revealed by the input/output titres (Table 4), highly stringent conditions were imposed during panning by decreasing the concentration of \( \alpha-\)Cbtx and increasing the number of washes. After three rounds of panning, polyclonal phage ELISA showed good binding signal (Figure 19) and about 60% of individual phage clones (Figure 20) showed specific binding to \( \alpha-\)Cbtx, thus indicating enrichment for \( \alpha-\)Cbtx binders.

Sequence analysis of positive binders revealed that the 3\(^{rd}\) round of panning generated several unique \( V_{H} \) sequences. Based on CDR homology and CDR3 length, the \( V_{H} \) clones were grouped into either Cluster I or Cluster II (Figure 21). Binders clustered together share high CDR sequence identity; however, little CDR homology is
shared between these two clusters. Furthermore, the α-Cbtx binders from Cluster I have a CDR3 length of 17 amino acid residues while those from Cluster II have 14 residues.

Unfortunately, analysis of the coding sequences also revealed that many clones (8/27; 30%) isolated from the 3rd round of panning contained premature amber (TAG) stop codon mutations (denoted as ‘∗’ in Figure 21). Most of these mutations (6/8; 75%) occurred within the first FR of the VH sequence, more specifically, targeting the first codon of the VH sequence (50%, 4/8). The remaining two clones (C21 and C33) had amber stop mutations introduced in FR4.

These amber codon mutants were able to survive panning because E. coli TG1, a suppressor E strain, was used (Marcus et al., 2006). This strain translates amber codon (TAG) for a glutamine residue (30% efficient) thereby resulting with phage displaying VH fused to the coat protein III. However, for soluble VH expression without protein III fusion, the phagemid-VH construct is transformed into a non-suppressor strain, such as E. coli HB2151. This strain recognizes the amber stop codon as a stop signal and antibody expression is prematurely terminated.

Since the original unselected library did not contain antibody clones with amber stop mutations (Figure 18), it is plausible that these mutations were introduced by E. coli TG1 during the process of panning. Others have also reported the occurrence of positive phage binders with premature amber stop codons in the antibody coding sequence (Shinohara and Fukuda, 2002; Yan et al., 2004; Barderas et al., 2006; Marcus et al., 2006; Wu et al., 2007; Pokorny et al., 2008). In fact, Marcus et al. (2006) have proposed that the driving force for this phenomenon is likely caused by a mechanism the E. coli takes to avoid the cytotoxic effects of the antibody. Nevertheless, this constitutes an
important bottleneck when selecting antibodies by phage display since the TAG mutations need to be reverted by site-directed mutagenesis. While this current study did not undertake site-directed mutagenesis to revert amber stop codons, it would be interesting to further study α–Cbtx binders C15, C46, C7 and C33 because they likely originate from the same $V_H$ clone.

Several clones that survived panning and were detected as positive α–Cbtx binders by monoclonal phage ELISA (Figure 20) displayed frameshift mutations (data not shown). Most of these frameshift mutations were caused by a single or double nucleotide deletion at the junction of the primer annealing site and the first codon of the $V_H$ sequence, and are therefore thought to have been artificially introduced during library construction. Since these phage clones survived three rounds of stringent panning, it seems plausible that these frameshift mutants produced functional $V_H$s fused to the coat protein III of the phage particles. In fact, some frameshift mutants have been reported to be ‘leaky’ and express low levels of functional proteins (Atkins et al., 1972). For instance, Atkins et al. (1972) reported that several lac frameshift $E. coli$ mutants produced low levels of β–galactosidase activity and that this leakiness was under ribosomal control. Similar to the results obtained in this current research, Shinohara and Fukuda (2002) obtained 15 clones with frameshift mutations that survived through 3 or 4 rounds of panning an immune phage-displayed scFv library. These authors suggested that these mutants were able to produce functional scFvs on phage particles by noncanonical translation mechanisms, such as ribosome hopping, albeit at low levels. A review on these translation mechanisms are discussed by Gesteland and Atkins (1996).
Several differences were also noted when the α-Cbtx V<sub>H</sub>H binders isolated from this immune library were aligned against those isolated from a naïve library by Stewart <i>et al.</i> (2007) (Figure 26). Interestingly, Stewart’s naïve binders all belong to V<sub>H</sub>H subfamily 1 while the ones isolated from this immunized library belong to V<sub>H</sub>H subfamily 2 (Harmsen <i>et al.</i>, 2000). As expected, the immune V<sub>H</sub>Hs share little CDR homology with the naïve ones, since they had the opportunity to go through natural affinity maturation. For instance, in the research presented here, immune V<sub>H</sub>Hs had a shorter (14 or 17 residues) CDR3 region than did the naïve clones (18 or 19 residues) produced by Stewart <i>et al.</i> (2007). This result is consistent with the literature since V<sub>H</sub>H subfamily 2 have on average a shorter CDR3 region than other subfamilies (Harmsen <i>et al.</i>, 2000).

|---- CDR1 ----| |-------- CDR2 --------| |------- CDR3 -------|

| C2 | GSSSTYAMG | VTTNGN-SPNYADSVRG | EGYSRTGDSWYDGY |
| C43 | TEDDI | TTTTTTTTTTTTTTTTTTTTTTTTTTTTT | |
| C20 | TFSNG | FSSGRRSGRTTTT | GSSTNTNHEY |
| C19 | DFTN | FSSGRRSGRTTTT | GSLSVTNYE |
| Stewart C10 | RTFS | RSPGTTT | RYPRTSWAGP | |
| Stewart C12 | RTFS | RSPGTTT | RYPRTSWAGP |
| Stewart C5 | RTFS | RSPGTTT | GTDACSGHKT |

**Figure 26.** Sequence alignment of the CDR regions of the anti-α–Cbtx V<sub>H</sub>Hs isolated from an immunized (C2, C43, C20 and C19) and naïve (Stewart C10, C12 and C5; Stewart <i>et al.</i>, 2007) libraries.

In this current study, two clones from Cluster I (C19 and C20) and two clones from Cluster I (C2 and C43) were chosen for further characterization. The selected clones were all successfully expressed in 1 L <i>E. coli</i> cultures and then purified from the periplasmic fractions using IMAC. While the expression levels (12-18 mg/L) were not as
high as those observed with other camelid V_Hs (>100 mg/L; Spinelli et al., 2001), enough was recovered for kinetic measurements and in vitro neutralization assays.

Kinetic analysis by SPR revealed that high affinity α–Cbtx binders were isolated from the immune V_H library constructed in this study. In particular, C2 had the strongest affinity for α–Cbtx with K_D value of 0.4 nM. Despite having identical CDR2 and CDR3, C43 had 60-fold less affinity (K_D = 24 nM) than C2 to α–Cbtx. It is in the CDR1 that they differ the most (5/10; 50% identity) with some FR1 substitutions as well. Their differential binding affinities to α–Cbtx therefore suggests that the CDR1 is also instrumental in binding α–Cbtx, at least for these two V_Hs. Accordingly, Vu et al. (1997) have previously suggested that, in contrast to human and mice, amino acid residues from the CDR1 of camelid V_Hs play a critical role in antigen-binding.

C19 and C20 also had strong affinities to α–Cbtx with K_D values of 25 nM and 1 nM, respectively. Although these V_Hs share high sequence identity to each other, the substitutions are dispersed in different regions of the V_H sequence, making it difficult to deduce which residue or region plays an important role in α–Cbtx recognition.

Other clones not selected for further characterization in this study warrant kinetic analysis, especially C7, C15/C46 and C33. These positive α–Cbtx binders (as revealed by monoclonal phage ELISA; Figure 20) likely originate from the same V_H clone; however, they each contain an amber stop codon mutation. Nevertheless, this mutation could be reverted by site-directed mutagenesis for one of these clones to allow kinetic measurements.

Lastly, these immune α–Cbtx binders had ~1000-fold higher affinity compared to those isolated from a naïve V_H library (2-3 μM) (Stewart et al., 2007). This was not a
surprise since the immune clones had the opportunity for natural affinity maturation using the llama’s immune system.

Marks et al. (2004) have shown that botulinum toxin (BoNT), a potent neurotoxin produced by Clostridium botulinum, is more effectively neutralized in vitro and in vivo when a combination of 2 or 3 mAbs simultaneously bind non-overlapping epitopes of BoNT (Marks, 2004). Since the CDR1 and CDR3 of VHs are thought to play a critical role in antigen recognition (Vu et al., 1997; Muyldermans, 2001), it was hypothesized that α–Cbtex clones from Cluster I (Refer to Figure 21) recognize a different immunogenic epitope than those from Cluster II. However, as revealed by SPR analysis, it appears that α–Cbtex cannot be simultaneously bound by more than one VH binder. These selected binders likely target the same or adjacent (possibly overlapping) epitopes on α–Cbtex. These findings are not surprising considering the size of VHs (14-16 kDa) relative to α–Cbtex (7.8 kDa). Therefore, using combinations of different VH binders simultaneously will likely not increase the neutralization efficacy of α–Cbtex in in vitro or in vivo assays.

An in vitro muscle twitch assay showed that 100 nM C2 neutralizes most of the paralytic effects of 50 nM α–Cbtex. Therefore, C2 eliminates α–Cbtex binding to the ACh site of the post-synaptic cleft. These preliminary results also look very promising for the other clones (C19, C20 and C43) because all the clones appear to target the same epitope. Nevertheless, in vivo neutralization experiments are needed to validate these three other VHs as therapeutic reagents.

Moreover, it is speculated that C2 neutralizes α–Cbtex by binding, hindering, or by causing conformational changes that affects residues located at the end of the 2nd loop of
α–Cbtx (Refer to Figure 5). These residues are especially important in binding to the ACh receptor and their blockade offers protection against α–Cbtx.

In summary, this study demonstrated that high affinity recombinant llama single domain antibody fragments (VHs) can be generated against α-Cbtx. Furthermore, we demonstrated that at least one of these VHs (C2) is able to neutralize the paralytic activity of α-Cbtx at neuromuscular junctions in an in vitro assay. These anti-α-Cbtx VHs may represent better reagents than whole antibodies for snake envenomation because of their small size, better tissue permeability and low potential immunogenicity.
4. CONCLUSIONS AND FURTHER DIRECTIONS

Since their discovery in the late 1800s by Calmette, little progress has been made in the development of conventional antivenoms. In fact, Gutiérrez et al. (2006) have categorized snake bite envenomations as a major neglected disease of the 21st century. Current problems associated with conventional antivenoms include i) poor neutralization of toxins, ii) adverse reactions to antivenoms (anaphylaxis, serum sickness), and iii) limited supply of antivenoms (Lalloo and Theakston, 2003). There is a need for more efficacious, safer, and widely distributed therapeutics for snake envenomation.

Since conventional antivenom antibodies [IgG, F(ab')2] ineffectively neutralize venom toxins in deep tissues, alternative antibody fragments with the capacity to be distributed to a larger volume of tissue in the human body are needed. With the advent of recombinant antibody technology, antibody engineers are able to design antibody reagents with desired properties such as improved tissue permeability. Reducing the molecular mass and removing the Fc region of antibodies will alter their pharmacokinetic profile thus increasing their volume of distribution. Camelid derived V_{H}H antibody fragments are the smallest antigen-binding domain (~16 kDa) and lack the Fc region, thus it is not surprising that they have been shown to permeate tissue well (Cortez-Retamozo et al., 2002 and 2004). V_{H}H-based antivenom may therefore be more efficacious for the neutralization of venom toxins, especially those located in deep tissues. In fact, one study showed that sera from camels and llamas immunized with Echis ocellatus (viperid) venom was able to prevent the development of local haemorrhagic lesions induced by this venom in mice experiments (Harrison et al., 2006).
The research objectives of this thesis were to develop high affinity V_Hs with neutralizing capabilities against α-Cbtx, a potent neurotoxin from the venom of N. kaouthia. Since screening a naïve library resulted in low affinity V_H binders to α-Cbtx (Stewart et al., 2007), we decided, in this current research, to construct an immune phage-displayed V_H library from a llama hyper-immunized with crude N. kaouthia venom. Following successful library construction and panning, several unique anti-α-Cbtx V_H binders were isolated, expressed and characterized. Surface plasmon resonance (SPR) analyses showed that the selected immune V_H binders (C2, C19, C20 and C43) had dissociation constants (K_D) in the low nanomolar range (0.4-25 nM). In comparison, the naïve V_H binders isolated by Stewart et al. (2007) had K_Ds in the low micromolar range (2-3 μM). SPR analyses revealed that the immune V_Hs bound to the same, overlapping or adjacent epitopes on α-Cbtx. Furthermore, an in vitro muscle twitch assay showed that V_H C2 effectively neutralizes the paralytic effects of α-Cbtx at neuromuscular junctions; the other clones are currently being tested. In the future, in vivo mice experiments should be performed to further validate the therapeutic efficacy of these anti-α-Cbtx V_Hs.

It would also be valuable to determine the efficacy of these α-Cbtx V_Hs against long-chain, and possibly short-chain, α-neurotoxins from other snake venoms. For example, it is quite probable that the α-Cbtx V_H from this study are also able to bind and neutralize homologous neurotoxins from N. naja (Indian cobra) and possibly from other snakes (Refer to Figure 4). If this turns out to be the case, these V_Hs could have potential as broad spectrum and therapeutic reagents against the neurotoxins of various snake species.
The major limitation of this work is that many other pharmacologically important venom components will also have to be neutralized to create an effective therapeutic antidote. Since the llama in this research project was immunized with crude *N. kaouthia* venom, the \( V_H \) library could also be panned against other components of the *N. kaouthia* venom (Refer to Table 2). It would be particularly valuable to select \( V_H \)s against locally acting toxins including phospholipases A\(_2\) and metalloproteinases since these cause much of the tissue damage associated with Thai cobra snake bites (Gutiérrez and Rucavado, 2000). Furthermore, methodologies could be developed to simultaneously select \( V_H \)s against crude venom, while ensuring that all venom components are represented and neutralized.

In conclusion, this study showed that high affinity \( V_H \)s with neutralizing capabilities can be generated against a pharmacologically active venom component. Although much work remains to be done to create a \( V_H \)-based antivenom, we believe that \( V_H \)s may represent a safer and more efficacious treatment for snake bite envenomation compared to conventional IgG, F(ab')\(_2\) or Fab antivenoms because of their smaller size and low inherent immunogenicity. In addition, the use of \( V_H \)s may help solve the limited supply crisis of antivenoms since they are easily expressed and purified from *E. coli*/yeast expression systems.
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APPENDIX 1

Optimization of V<sub>H</sub>H Library Construction

1<sup>st</sup> set of PCR: Amplification of V<sub>H</sub>H-C<sub>H</sub>2 and V<sub>H</sub>-C<sub>H</sub>1-C<sub>H</sub>2 regions

Using C<sub>H</sub>2 primer:

![Image of gel electrophoresis showing bands for different conditions](image1)

**Legend:**
- L: 1 Kb ladder
- 1: 0.5 ul cDNA; 0.5 mM MgCl<sub>2</sub>
- 2: 1.0 ul cDNA; 0.5 mM MgCl<sub>2</sub>
- 3: 3.0 ul cDNA; 0.5 mM MgCl<sub>2</sub>
- 4: 0.5 ul cDNA; 1.5 mM MgCl<sub>2</sub>
- 5: 1.0 ul cDNA; 1.5 mM MgCl<sub>2</sub>
- 6: 3.0 ul cDNA; 1.5 mM MgCl<sub>2</sub>
- 7: 0.5 ul cDNA; 3.0 mM MgCl<sub>2</sub>
- 8: 1.0 ul cDNA; 3.0 mM MgCl<sub>2</sub>
- 9: 3.0 ul cDNA; 3.0 mM MgCl<sub>2</sub>

Figure A1.1

Using C<sub>H</sub>2b3 primer:

![Image of gel electrophoresis showing bands for different conditions](image2)

**Legend:**
- L: 1 Kb ladder
- 1: 0.5 ul cDNA; 0.5 mM MgCl<sub>2</sub>
- 2: 1.0 ul cDNA; 0.5 mM MgCl<sub>2</sub>
- 3: 3.0 ul cDNA; 0.5 mM MgCl<sub>2</sub>
- 4: 0.5 ul cDNA; 1.5 mM MgCl<sub>2</sub>
- 5: 1.0 ul cDNA; 1.5 mM MgCl<sub>2</sub>
- * 6: 3.0 ul cDNA; 0.5 mM MgCl<sub>2</sub>
- 7: 0.5 ul cDNA; 3.0 mM MgCl<sub>2</sub>
- 8: 1.0 ul cDNA; 3.0 mM MgCl<sub>2</sub>
- 9: 3.0 ul cDNA; 3.0 mM MgCl<sub>2</sub>

Figure A1.2
APPENDIX 2

Epitope mapping by SPR Results

- **600 nM C19 followed by 2 µM C43**
  - Response Difference (RU) over time (sec)
  - Maximal response at 21 RUs

- **2 µM C43 followed by 600 nM C19**
  - Similar response pattern

- **600 nM C19 followed by 500 nM C20**
  - Maximal response at 33 RUs

- **600 nM C19 followed by 1 µM C2**
  - Maximal response at 19 RUs

- **2 µM C43 followed by 1 µM C2**
  - Maximal response at 23 RUs

- **2 µM C43 followed by 500 nM C20**
  - Maximal response at 25 RUs

- **500 nM C20 followed by 1 µM C2**
  - Maximal response at 21 RUs