Two North American Arthropods of Clinical Significance: Their Venoms and the Development of Specific Antivenoms.

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A thesis submitted to the Faculty of Medicine,

University of London,

for

the Degree of Doctor of Philosophy

2001

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ABSTRACT

Large volumes of antisera were generated against *Apis mellifera* venom with which to develop a novel, platform technology for the inexpensive production of antivenoms. The ovine sera contained high levels of specific antibodies which neutralised the myotoxic, phospholipase A_2 and *in vivo* activities of the venom.

Methods of processing the antisera to provide Fab or $F(ab')_2$ were investigated. $F(ab')_2$ was thought to be clinically advantageous and, by determining the conditions necessary for the preferential breakdown of Fc and serum components other than $F(ab')_2$, it was possible to avoid salt precipitation. Diafiltration was then used to remove most of the unwanted small fragments and anion-exchange chromatography to remove any remaining acidic impurities such as pepsin and large aggregates. The $F(ab')_2$ was ~97% pure and the yield ~ 19g per L of serum. This is the first specific therapy for mass envenoming by European or Africanised bees.

Spiders of the genus *Latrodectus* (black widows) are distributed widely and about 2,500 bites are reported annually in the USA. The neurotoxic effects of the venom were studied on the isolated phrenic nerve diaphragm preparation. Low venom concentrations (1mg/L) were stimulatory while high concentrations (10mg/L) caused nerve blockade which was potentiated by increased calcium levels.

Although effective, the Merck antivenom, which is unprocessed horse serum, causes unacceptable risks. The second purpose of this project was to prepare an improved *Latrodectus* spider antivenom using the new platform technology.

Different immunisation schedules were studied to optimise the humoral immune response. Sheep immunised with 2mg *La.hesperus* venom produced the highest levels of specific antibodies as assessed by ELISA, using the isolated nerve diaphragm preparation or *in vivo* in mice. The new process provided a pure $F(ab')_2$ antivenom retaining 78% of the original antisera ED_{50} neutralising power and was ~ twice as effective as the Merck antivenom.

CONTENTS

ABBREVIATIONS

BCA	: Bicinchoninic acid
BSA	: Bovine serum albumin
ED_{50}	: Median effective dose of antivenom
EDTA	: Ethylenediamine tetra-acetic acid
ELISA	: Enzyme linked immunosorbant assay
FPLC	: Fast protein liquid chromatography
hr	: Hour(s)
HRP	: Horseradish peroxidase
IgG	: Immunoglobulin G
iv	: Intravenous
kDa	: Kilodalton
La.	: *Latrodectus*
LD_{50}	: Median lethal dose
Lo.	: *Loxosceles*
NSS	: Normal sheep serum
PAGE	: Polyacrylamide gel electrophoresis
PBS	: Phosphate buffered saline
RT	: Room temperature
SD	: Standard deviation
SDS	: Sodium doedecyl sulphate
SEM	: Standard error mean
TEMED	: N,N,N',N'-Tetramethyl-ethylenediamine
Tris	: Tris(hydroxymethyl)aminomethane or TRIZMA®

ACKNOWLEDGEMENTS

I would like to thank Professor John Landon for giving me the opportunity to perform these studies and his guidance and support and Professor Tim Chard for kindly allowing the use of his laboratory.

I am also greatly indebted to Dr David Smith for his helpful suggestions and comments during the writing of this thesis, to; Sophia, Arif, and Richard for their help and training in some of the *in vivo* techniques, the Department of Histopathology, Charing Cross and Westminster Hospital for the electron micrographs and all my colleagues, past and present, for their help and encouragement.

Finally, many thanks to the EPSRC (GR/N23677), MicroPharm Ltd, The Welsh Office (REG 2636), and Therapeutic Antibodies Inc. (now Protherics Plc) for financing this work.

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CHAPTER 1:
INTRODUCTION

1.1 Arthropods

1.1.1 History and General Introduction

Arthropods are invertebrates with jointed limbs, a segmented body, and an exoskeleton made of chitin. This Phylum includes three-quarters of all known species of animals and is subdivided into several diverse classes such as Arachnids, Insects, Crustaceans and Myriapods (centipedes). The Order of Araneae (spiders) from the Arachnids and Order Hymenoptera from the Insects will be considered in this work.

Currently, about 30,000 species of spiders have been recognised and many more have yet to be discovered (Preston-Mafham & Preston-Mafham, 1993a). Evolutionary adaptation has allowed them to occupy nearly every terrestrial habitat, from the peaks of the highest mountain ranges to the depths of the largest caves and from damp marsh to dry desert; indeed anywhere that they can find other arthropods to feed upon. Phylogenetically the spiders are very old, with the first fossil spiders belonging to the Carboniferous period (Bettini & Brignoli, 1978).

The development of the venom apparatus and the evolution of different types of web have ensured their evolutionary success. The venom is of equal, or greater, importance to spiders than silk and natural selection must have had a pronounced influence on its toxic and pre-digestive constituents. Many spiders have developed enzymes that neutralise most of the defensive toxic substances secreted, or simply contained, by the arthropods on which they prey (Bettini & Brignoli, 1978).

All spiders are venomous, with the sole exception of the small family of Uloboridae, which has lost its poison glands (Bettini & Brignoli, 1978). However, fortunately for man, only a few species have evolved venoms that are poisonous to mammals, together with the apparatus needed to pierce the skin and allow envenoming.

1.1.2 Anatomy, Including Venom Injecting Apparatus

At the front of the cephalothorax or head are the jaws (or chelicerae) which cause the spider's deadly bite (Figure 1.1). They consist of a stout basal segment, which often

bears sharp teeth along the inner edge, and a sharp fang, which hinges onto the basal segment and is used to pierce the exoskeleton of the prey. These fangs often fit into grooves on the basal segment and the way in which the chelicerae work is used as a means of separating the more primitive (mygalomorph) from the more advanced true spiders (Labidognatha). In the mygalomorphs (e.g. Tarantulas and Funnel Web spiders), the chelicerae strike forwards and downwards whereas, in the true spiders, they are turned through 90° and work from side to side, in a pincer-type movement.

The mygalomorph fang arrangement is suitable for spiders which live mainly at ground level, since the downward thrust of the fangs is opposed by the ground on which the prey is standing. However, it is of little use on a leaf or in a web and it is in such situations that the greater span between the fangs, possible in the true spiders, confers benefit. Near the end of each fang is a tiny hole which is connected to the poison sacs and through which venom is injected into the victim (Wright *et al.*, 1973). The fangs measure between 0.32 - 0.45mm in length for both *Latrodectus* and *Loxosceles* species (Bucherl, 1971). The poison glands may be contained entirely within the basal segment of the chelicerae or may extend back into the head. Each is enveloped with several muscle layers, whose contraction expels the stored venom (Wright *et al.*, 1973).

Unlike spiders, honey bees (*Apis mellifera*) use their venom only for defence. The morphology and function of the sting from an Africanised bee is almost identical to that of a European bee. The stinger is composed of two barbed lancets supported by hard plates and powerful muscles, connected to a venom sac and to specialised glands that produce alarm odours. When a bee stings, the lancets scissor their way into the victim, and barbs anchor the sting so that it remains in the skin when the bee pulls away leaving the entire distal segment of the bee's abdomen, along with a nerve ganglion, various muscles, a venom sac, and the end of the insect's digestive tract (Visscher *et al.*, 1996). The sting continues to throb for 30-60 seconds injecting at least 90% of the venom within 20 seconds and all the venom by 60 seconds. On average each Africanised bee injects 94μg of venom compared to 147μg for the slightly larger European bee (Schumacher *et al.*, 1990; 1994). Alarm odours are released by the sting that alert other bees and mark the victim for continued attack

(Schumacher *et al.*, 1994). Honey bees are unusual among stinging insects in that the bee dies following stinging.

1.1.3 Classification of American Arthropods of Clinical Importance

Although the classification of various Arthropods has changed periodically over the years, the structured form of biological classification, developed by Linnaeus during the eighteenth century, has remained unaltered.

e.g.

KINGDOM - Animalia
PHYLUM - Arthropoda
CLASS - Arachnida
ORDER - Araneae (the spiders)
SUB-ORDER - Araneomorph
FAMILY - *Theridiidae*
GENUS - *Latrodectus*
SPECIES - *hesperus* (Western black widow)

Those Arthropods thought to be of clinical significance in North America are listed in Figure 1.2.

1.1.4 Spider Venom

The purpose of the venom is to kill or immobilise the prey and it is injected into the victim via two sharp hypodermic like fangs. Digestion of the prey is normally external with some spiders injecting the digestive enzymes directly while others crunch up the prey first to expose the soft internal tissue onto which they pour digestive fluid. The liquefied contents of the insect are then pumped up by the muscular stomach into the alimentary canal of the spider.

1.1.4.1 Venom extraction methods

1.1.4.1.1 Venom gland extract

Spiders are first killed in a closed atmosphere of ether or chloroform or by freezing and the venom glands removed by gently pulling on the chelicera with forceps, or by

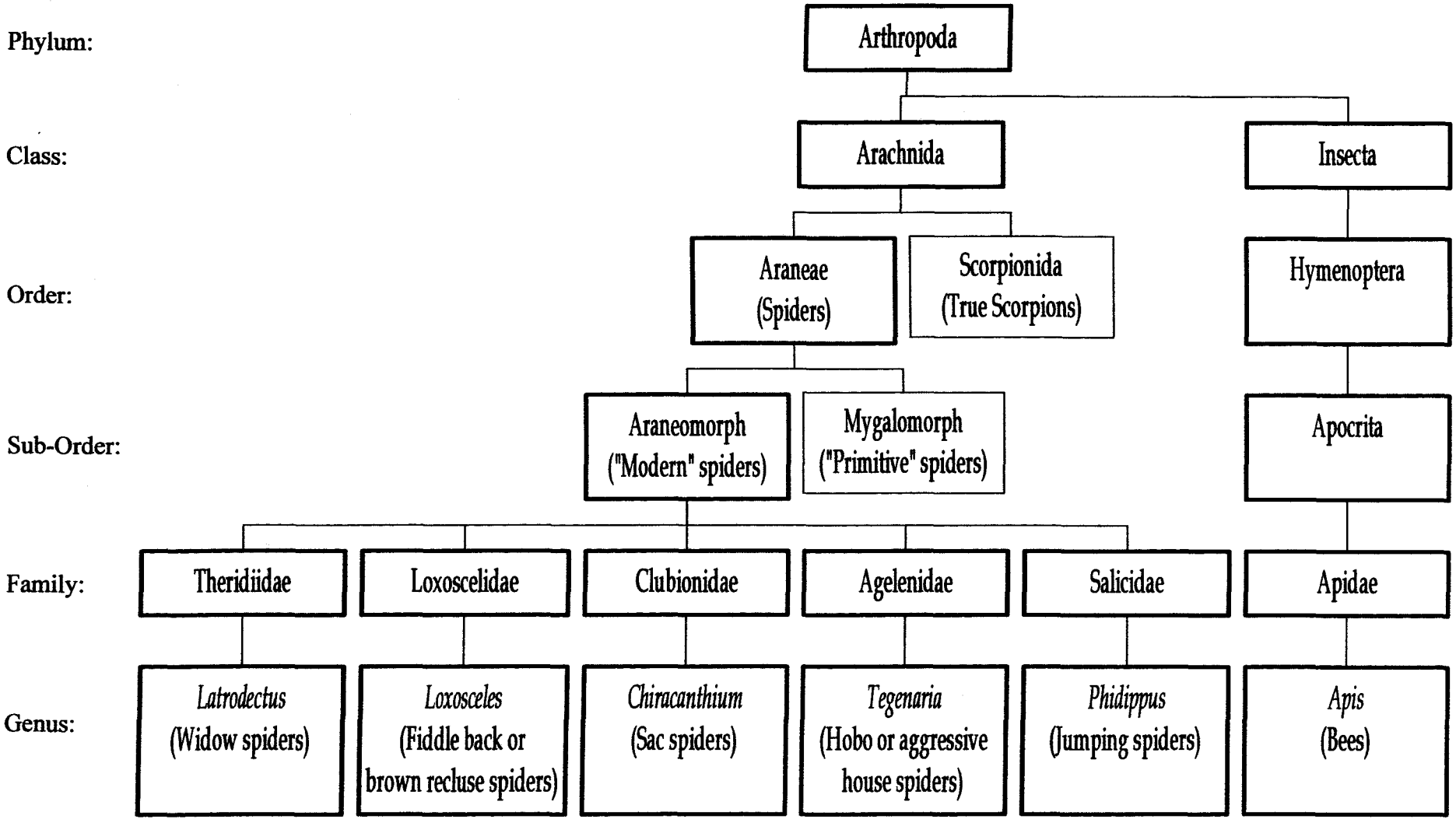


Figure 1.2. Classification of clinically important Arthropods of North America

careful dissection (Frontali & Grasso, 1964; Bettini & Brignoli, 1978). Venom glands from *Latrodectus* species measure between 1.5 - 2.1mm in length and 0.2 - 0.4mm in width (Bucherl, 1971). These may then be ground up, with or without the fangs and chelicera, using washed sand in a small volume of saline. Alternatively, they may be homogenised with saline, borate or Tris-HCl buffer followed by centrifugation to remove the precipitate (Frontali & Grasso, 1964; Frontali *et al.*, 1976; Bettini & Brignoli, 1978; Grasso & Mastrogiacomo, 1992; Krasnoperov *et al.*, 1992). The buffer used for extraction should provide the best conditions for the solubility of the venom toxins without denaturing them. This method has the advantage of providing relatively large quantities of venom protein. However, it requires large numbers of spiders and the product is contaminated with cellular material from the gland tissues (Bettini & Brignoli, 1978). Gentler methods have been used to extract the venom from the glands but, while limiting contamination, are usually associated with a greatly reduced recovery (Berger, 1973).

1.1.4.1.2 Electrical stimulation

Spiders can be anaesthetised with CO_2 to the point where they can be picked up without danger to the handler and without trauma to themselves (Meadows & Russell, 1970; Bascur *et al.*, 1980; Jack, 1996). Mild electrical stimulation (60 volts, 9 hertz, 0.1 mA) causes the slow (20-40 sec) extrusion of venom from the fangs into a micromanipulated pipette. Mean volumes of 0.29µl (80µg protein) and of 0.15µl (40µg protein) are obtained from female and male *Loxosceles reclusa* respectively (Morris & Russell, 1975). *Loxosceles laeta* yields on average 350µg and 150µg (wet weight) respectively (Bascur *et al.*, 1980). The venom produced by this method is less contaminated than that from either dissection or more vigorous electrical stimulation (Morris & Russell, 1975). A similar approach is used by our suppliers (Spider Pharm Inc. in the United States) with the addition of a small suction tube placed in the mouth to remove any contaminating secreted digestive juices or vomit caused by the stimulation (Kristensen personal communication). This method has the additional advantage of allowing several extraction's from the same spider since it is unharmed.

Many others have used crude electrical stimulation and collection from the fang pits, where the collected venom is likely to be contaminated with a large amount of

digestive fluids. This explains why large quantities (of 600 and 700μg dry weight) of material are collected from *Latrodectus* and *Loxosceles* species respectively, using this method (Bucherl, 1971).

1.1.4.1.3 Other methods

Venom collected when *Latrodectus tredecimguttatus* was induced to bite a cotton wool tampon was found, by Lebez (1953), to be contaminated with oral juices containing proteolytic and glycogenolytic enzymes and adrenaline oxidase, whereas such enzymes were not found in venom gland extracts. The first experiments carried out using *Latrodectus* venom involved getting the spider to bite the test animal directly. However, results were highly variable with some animals biting more vigorously than others (Marx, 1890; Baerg, 1922).

The future promise of almost unlimited spider toxins has been advanced by the sequencing of DNA encoding the putative α-latrotoxin precursor and the synthesis of this and other black widow spider neurotoxins in insect cells (Kiyatkin *et al.*, 1990; Kiyatkin *et al.*, 1995; Ichtchenko *et al.*, 1998; Volynski *et al.*, 1999). Spider venom production may also be possible using a similar method to that demonstrated by culturing snake venom gland cells *in vitro* (Sells *et al.*, 1989).

1.1.5 Bee Venom

1.1.5.1 Venom extraction methods

Venom can be extracted from homogenates of the sting apparatus which have been removed from ethanol-fixed, deeply frozen, recently killed, or immobilised insects, or by collecting venom ejected through the sting as a result of mechanical, electrical or chemical stimulation. Electrical stimulation is normally used as thousands of bees can be induced to sting in a relatively short time. The equipment consists of a wooden frame with wires stretched across it at 3 mm intervals. Alternate wires carry an electric charge and the circuit is completed when the bee comes into contact with two adjacent wires carrying 115V (AC) applied with 4 sec breaks at 3 sec intervals (Piek, 1986). The bees sting through a nylon sheet stretched beneath the wires and the venom is easy to collect.

1.1.6 Epidemiology

The poison control centres within the United States receive about 2,500 and 2,300 annual reports of bites by black widow and brown recluse spiders respectively.. This compares to just 900 rattlesnake bites (Figure 1.3a). However, in 1999, only 383 and 619 of the spider envenomings were classified as causing significant clinical features (viz moderate/major/death) (Figure 1.3b). These figures comprise data collected from ~ 80 - 95% of the poison control centres and probably represent a considerable underestimate of the real number (Litovitz *et al.*, 1991; 1992; 1993; 1994; 1995; 1996; 1997; 1998; 1999; 2000).

1.1.7 Clinical Manifestations of Envenoming

1.1.7.1 *Latrodectus*

Poisoning caused by the bite of the *Latrodectus* spider can run a serious and dramatic course, although death is unusual. Thus the victims suffer great pain and, although the most severe effects may start to dissipate after about 24 to 36 hours, recovery is often protracted for three or four weeks (Southcott, 1976; Banham *et al.*, 1994; White *et al.*, 1995). The mortality rate, before the introduction of antivenom therapy, was an estimated 5% (Ingram & Musgrave, 1933).

The bite itself is superficial and may be like a slight needle prick or not even perceived. From the time of the bite to the first clinical manifestations is normally short (~ 10 - 20 minutes), although it may extend to one or two hours (Maretic & Stanic, 1954). The clinical features may vary greatly which has led to some confusion in the literature (Banham *et al.*, 1994). However, most of the clinical manifestations, as described around the world, appear identical.

In America during the 1920's and 1930's the circumstantial evidence linking the severe clinical picture seen by physicians with the small, timid black widow spider caused considerable scepticism. Experimental results also demonstrated marked differences in the susceptibility of various animal species with rabbits, cats, dogs and sheep little affected while mice, guinea-pigs, chickens and horses demonstrated severe effects (Blair, 1934; Maretic & Stanic, 1954; Wiener, 1961). This scepticism was resolved conclusively by two independent experiments in which *Latrodectus mactans*

was stimulated to bite a human subject and the effects recorded in full (Baerg, 1922; Blair, 1934). In particular, intense pain and localised sweating at the site of the bite were noted as important early diagnostic features (Wiener & Fraser, 1956; Wiener, 1961). The signs and symptoms of *Latrodectus* envenoming are considered further in Chapter 5.

1.1.7.2 *Loxosceles*

The initial bite by a *Loxosceles* spider is relatively painless so that patients often remain unaware until they experience the first symptom, a local burning-stinging sensation (Atkins *et al.*, 1957; Atkins *et al.*, 1958; Schenone & Suarez, 1978; Rees *et al.*, 1987). The venom may then bring about either of two clinical variants of Loxoscelism, namely the cutaneous or the systemic (viscerocutaneous) form. The systemic form is much less common in North America than the cutaneous form and is associated with the rare instances of death associated with *Lo. reclusa* envenomation (Nance, 1961; Madrigal *et al.*, 1972; Novak *et al.*, 1979; Litovitz *et al.*, 1999). More deaths appear to be related to the South American species *Lo. laeta*, with approximately 13% of the cases of Loxoscelism in Chile resulting in the systemic form (Schenone & Suarez, 1978; Schenone *et al.*, 1989), possibly due to the large amounts of venom available (see earlier).

1.1.7.2.1 Cutaneous form of Loxoscelism

The cutaneous form may result in local skin necrosis or local oedema without necrosis, with the patient seeking medical help from 12 - 36 hours after the bite (White *et al.*, 1995). Both lesions are associated with pain after 2 - 8 hours, which varies from mild to severe (Atkins *et al.*, 1957; Atkins *et al.*, 1958; Schenone & Suarez, 1978; Rees *et al.*, 1987; Masters, 1998). There may be two small puncta and transient erythema, with itching, swelling and mild to severe tenderness. This is followed by the development of a bleb or blister after ~ 12 - 24 hours, surrounded by a erythematous halo (Atkins *et al.*, 1957; Schenone & Suarez, 1978). The bleb or blister is dark in colour, varies in size from 3 - 35cm, and is gradually replaced by a characteristic blackish eschar (scab), which was earlier called the 'gangrenous spot of Chile' (Macchiavello, 1947). The eschar sloughs within 2 - 5 weeks, either

superficially (67%) or leaving a necrotic ulcer (33%). The latter is frequently slow to heal and may extend to, but not involve, the underlying muscle layers (Schenone & Suarez, 1978). Secondary lesions with their subsequent eschar and necrotic ulcer may appear below the primary lesion, perhaps due to gravitational spread (Schenone & Suarez, 1978). The worst lesions and scarring are often found over fatty areas, such as the thighs, abdomen or buttocks (Wasserman & Siegel, 1977). There may also be persistent damage to underlying structures such as nerves (Gross *et al.*, 1990).

1.1.7.2.2 Systemic form of Loxoscelism

In addition to the local manifestations, which are identical to those observed in the cutaneous form, patients with systemic Loxoscelism have haemolytic anaemia, haemoglobinuria, haematuria, jaundice, fever and sensorial involvement, all of which generally appear within the first 24 hours. The temperature may reach 42°C and the patient may become prostrate, with laboured breathing, and pass dark coloured urine. The occurrence of this syndrome has no relationship to age, sex, season, site of bite, or size of the local lesion (Schenone & Prats, 1961; Schenone & Suarez, 1978). Systemic cases have been documented, although not frequently, in the United States and can cause a fatal "total haemolysis" just 2 days after a bite (Dillaha *et al.*, 1964; Minton & Olson, 1964; Taylor & Denny, 1966; Litovitz *et al.*, 1999).

1.1.7.3 Other spiders

The bites of *Tegenaria agrestis* (hobo or aggressive house spider), *Phidippus* (jumping spiders) and *Cheiracanthium* spp. (yellow sac spiders) have occasionally been implicated with causing clinical features in North America (Minton, 1972; Vest, 1987; Vest *et al.*, 1996; Russell, 1996).

Envenoming by *Phidippus* species is reported to cause a sharp pinprick and, sometimes, the development of a painful, tender area immediately around the bite. The symptoms and signs usually abate within 48 hours.

Envenoming by *Cheiracanthium* species is associated with a sharp pain, which increases during the first 30 to 45 minutes (Russell, 1996) and, since they are tenacious biters, they must sometimes be physically removed which explains why large numbers of spider bites by this species are positively identified. Restlessness and

a dull pain over the injured part, often accentuated by movement, are described (Russell, 1996) and a reddened weal with a hyperaemic border may develop together with small petechiae or purple to red spots under the epidermis caused by extravasation of blood near the centre of the weal. The injured area is tender to pressure and the skin temperature over the lesion often elevated, although the body temperature is usually normal. Inflammation of the lymph glands may develop. Vital signs are normal, as are blood findings. Abdominal cramps develop in some patients bitten on the lower extremity, and there may be diarrhoea but usually no vomiting. Pruritus (itching) is common.

1.1.7.4 *Apis mellifera*

The typical response to a single sting by a honey bee is intense, immediate pain with a burning sensation. This usually lasts for a few minutes after which the pain subsides. A white weal with a central red spot often forms soon after the sting, then rapidly fades to be replaced by a red flare and some local swelling which, by this time, is usually warm or hot to the touch. These symptoms subside and generally after a day or two all that remains is an itchy spot at the sting site (Schmidt, 1986).

Over 50 of these stings simultaneously can cause systemic effects due to the relatively large dose of venom delivered and about 500 are probably necessary to cause death by this direct toxicity, although one death in Arizona was due to only ~ 125 stings (Koszalka, 1949; Michener, 1975; Bousquet *et al.*, 1982; 1984; Mejia *et al.*, 1986; Franca *et al.*, 1994; Kolecki, 1999). Circulating venom levels of up to 3.8mg/l have been recorded and clinical features can include rhabdomyolysis, nausea and vomiting, intravascular haemolysis, disseminated intravascular coagulation, respiratory distress, hepatic dysfunction, hypertension, myocardial damage, shock, renal tubular necrosis and / or renal failure (Humblet *et al.*, 1982; Mejia *et al.*, 1986; Franca *et al.*, 1994; Kolecki, 1999). Typical features normally include evidence of a generalised rhabdomyolysis often resulting in myoglobinaemia and myoglobinuria with elevated serum levels of muscle enzymes (Franca *et al.*, 1994). Patients often develop renal failure and acute renal tubular necrosis with pigment and erythrocytes in the tubules and collecting ducts (Humblet *et al.*, 1982; Franca *et al.*, 1994). Intravascular

haemolysis is a major feature of most reported cases of multiple bee stings and some patients become comatose and die 1-3 days later (Franca *et al.*, 1994).

1.2 *Latrodectus*

1.2.1 Classification

The classification of different species of *Latrodectus* has changed considerably over the years depending mainly on the features studied (Kaston, 1970; Bucherl, 1971; Bettini & Brignoli, 1978). They are currently classified into the following species in the United States; *Latrodectus hesperus, La. mactans, La. variolus, La. geometricus and La. bishopi* (Kaston, 1970). Other species found around the world include; *La. tredecimguttatus, La. hasselti, La. pallidus, La. indistinctus, La. menavodi, La. katipo, La. curacaviensis, La. corrallinus, La. quartus, La. mirabilis, La. variegatus, La. carteri, La. cretaceus, La. sagittifer, La. diaguita, La. revivensis, La. hysterix, La. cinctus, La. concinnus, La. menavoidi, La. obscurior, La. luzonicus, and La. atritus.*

1.2.2 Distribution

Spiders of the genus *Latrodectus* are widely distributed across the World (Figure 1.4) and range as far south as New Zealand through to Mongolia in the northern hemisphere.

As noted above, at least five species of *Latrodectus* occur within the United States (US). All are quite large, subglobose theridiid spiders in which the lateral eyes are widely separated, a condition virtually exclusive to this genus. Very large females sometimes grow to over half an inch in length. Only *La. hesperus*, *La. mactans* and *La. variolus* merit the name 'black widows' in as much as the adults of all three are mostly jet black and marked with crimson spots. However, when the spiderlings leave the egg sac they are whitish and progress through a series of stages in which black gradually replaces most of the white, yellow, and red spots and bands. In adult females the dorsal markings on the abdomen are frequently lost, leaving it almost all black. However, the red hourglass marking is rarely lost on the venter. The males retain much of the bright colour pattern of the immature stages and are much smaller (Kaston, 1970; Gertsch, 1979).

The three 'black widows' are very similar and often difficult to separate. The southern

black widow, *Latrodectus mactans*, ranges north to New England and frequently lives where its irregular web can be placed under ground detritus near buildings. The cephalothorax and legs are usually all black while the abdomen is shiny black with a red spot just above the spinnerets and, sometimes, red spots or a band extending forward. The red hourglass marking on the venter is usually present and entire. The males are black with red and white spotting on the abdomen.

The northern black widow, *Latrodectus variolus*, ranges widely from the eastern US south into western Florida and eastern Texas. In Florida it occupies moist areas and places its webs in the branches of trees. However its habitats in other parts are more varied. Females are much like *mactans* but typically have a central row of red spots on the abdomen while the ventral hourglass is divided into two transverse bands. Males are somewhat larger in this sub-species and often have legs marked with paler rings.

The western black widow, *Latrodectus hesperus* (Figure 1.5), ranges widely over Oklahoma, Kansas, and middle Texas throughout the south-western states. It often builds its web near the ground, in animal burrows, under bridges and, frequently, on shrubs above the ground or even in trees. Females have a black cephalothorax and legs with the upper abdomen typically all black. Occasionally, some paler spots of the immature pattern are present. The ventral hourglass is often complete, but somewhat variable and occasionally divided. The males are typically much paler than those of the other species, with bands and spots on the cephalothorax and abdomen subdued to give a general grey appearance.

The brown widow, *Latrodectus geometricus*, is widely distributed around the world in the tropics and is the dominant species over most of Brazil and the eastern coastal portions of South America. Although it now ranges widely as the result of commerce, its occurrence in North America is sporadic and limited to southern Florida where it lives mainly in or near buildings. The red widow, *Latrodectus bishopi*, is found only in sand-pine scrub in central and south-eastern Florida. The carapace and legs are bright orange or reddish and the abdomen is usually brilliantly spotted with red or yellow marks on the dorsum which are retained by the adult female.

1.2.3 Venom Composition

The major toxic fractions of the venom are variations of a protein, latrotoxin. The

Figure 1.5 Adult female black widow spider (*Latrodectus hesperus*).

majority of these components are not toxic to mammals but are potent insect toxins (e.g. α, β and ε-latroinsectotoxins. Krasnoperov *et al.*, 1992). Indeed the only toxin said to be active in mammals is α-latrotoxin. The proportions of these toxins may partially explain the large differences in susceptibility of different animal species to the lethal effects of the whole venom (Table 1.1). Other components of the venom include the 'spreading factor' hyaluronidase, bradykinin-potentiating peptides and 5-hydroxytryptamine (Pansa *et al.*, 1972; Bettini & Maroli, 1978; Akchunov *et al.*, 1992).

1.2.4 Pathophysiology

1.2.4.1 α-latrotoxin

α-latrotoxin is toxic to vertebrates and thought to be responsible for the signs and symptoms of human envenoming (Frontalli *et al.*, 1976). This large toxin (131 kDa) constitutes ~3% of the venom protein and has been purified by a combination of gel filtration and ion-exchange chromatography or by immuno-affinity chromatography (Frontalli *et al.*, 1976; Grasso, 1976; Dalimov *et al.*, 1988; Krasnoperov *et al.*, 1992; Grishin, 1998; Volynski *et al.*, 1999). It has been shown that preparations previously considered homogeneous also contain a low molecular weight (8 kDa) component called latrodectin which expresses no toxicity in either mammals or insects (Grishin *et al.*, 1993; Pescatori & Grasso, 1994; Grishin, 1998). The recent production of a functional α-latrotoxin molecule from cultured insect cells infected with a recombinant baculovirus encoding for the toxin which is free of latrodectin further implies that the low molecular weight contaminant has no significant functional role (Ichtchenko *et al.*, 1998; Volynski *et al.*, 1999).

Nanomolar (nM) concentrations of α-latrotoxin can produce a massive release of neurotransmitters from synapses of both the central and peripheral nervous systems (Frontali *et al.*, 1976; Tzeng & Siekevitz, 1978; Hurlbut & Ceccarelli, 1979; Grasso *et al.*, 1982; Nicholls *et al.*, 1982; Scheer *et al.*, 1984). It has no selectivity for specific types of synapses but seems to affect them all (Hurlbut & Ceccarelli, 1979; Scheer *et al.*, 1984). This stimulation by the toxin evokes exocytosis of neurotransmitter-containing small synaptic vesicles exclusively in frogs (Matteoli *et al.*, 1988), whereas in mammals the neuropeptide-containing large dense core vesicles

Test Animal	LD_{50} (mg/kg)
Frog	145
Blackbird	5.9
Canary	4.7
Cockroach	2.7
Chick	2.1
Mouse	0.9
Housefly	0.6
Pigeon	0.36
Guinea pig	0.075

Table 1.1 Toxicity of *Latrodectus tredecimguttatus* venom on different species (Bettini & Brignoli, 1978).

are affected equally (Einhorn & Hamilton, 1974; Janicki & Habermann, 1983; Waterman & Maggi, 1995). More importantly from the clinical perspective, sensory nerve endings which contain large dense core vesicles have also been found to be sensitive to the venom or to α-latrotoxin both *in vivo* (Queiroz & Duchen, 1982) and *in vitro* (Waterman & Maggi, 1995). The direct (IM) injection of sub-lethal amounts of venom (from *Latrodectus tredecimguttatus* and from *La. hasselti*) was shown to result in several morphological changes in murine sensory nerve terminals. Within 30 minutes some sensory nerve endings became swollen with a progressive degeneration of annulospiral endings over time and, after 24 hours, every spindle was devoid of sensory nerve terminals. Degenerated nerve endings were then taken up into the sarcoplasm of intrafusal muscle fibres and the regeneration of sensory nerve axons was almost complete after one week. Similar features have also been described for motor terminals (Queiroz & Duchen, 1982).

1.2.4.2 α-latrotoxin receptors

α-latrotoxin has two structurally and pharmacologically distinct classes of high affinity (10^{-9}-10^{-10}M) receptors. The calcium-dependent receptor (neurexin Iα) is a large (160-220 kDa) cell surface membrane protein existing as multiple isoforms that will bind α-latrotoxin only in the presence of Ca^{2+} (Petrenko *et al.*, 1990; 1993; Grishin, 1998). It has one transmembrane segment and structurally resembles cell adhesion proteins (Ushkaryov *et al.*, 1992). A second high affinity receptor is the calcium-independent receptor (CIRL or latrophilin) (Krasnoperov *et al.*, 1996; Davletov *et al.*, 1996). CIRL is thought to be the more important because the toxin-receptor interaction can stimulate neurotransmitter release from neurones in a Ca^{2+}-free media (Misler & Falke, 1987; Capogna *et al.*, 1996). CIRL belongs to a family of closely related orphan G protein, protein-coupled receptors homologous to the secretin receptor family (Krasnoperov *et al.*, 1997; Lelianova *et al.*, 1997). In this family of three closely related homologous proteins, CIRL-1 is a brain-enriched high affinity α-latrotoxin-receptor, whereas CIRL-2 is a ubiquitously expressed low affinity receptor of the toxin (Ichtchenko *et al.*, 1999).

α-latrotoxin acts extracellularly by binding to these endogenous membrane receptor families. The formation of receptor-toxin complexes is followed by cation influx

through α-latrotoxin-induced channels and by, as yet, only partially identified signalling mechanisms that eventually result in massive spontaneous exocytosis (Figures 1.6 and 1.7).

There is ample evidence that α-latrotoxin receptors are crucial for the effects of α-latrotoxin and two different hypotheses have been proposed (Rosenthal & Meldolesi, 1989; Geppert *et al.*, 1998). According to the first, α-latrotoxin needs the receptor just to target the membrane at the right location. The toxin then makes pores in the plasmalemma, as it does in artificial lipid bilayers (Finkelstein *et al.*, 1976), and causes influx of Ca^{2+} into the cytoplasm, a process known to lead to exocytosis (Grasso *et al.*, 1980; Nicholls *et al.*, 1982). The second hypothesis argues that the toxin causes substantial neurotransmitter release even in the absence of Ca^{2+}, provided other divalent cations are present (Rosenthal *et al.*, 1990). For example, Mg^{2+} (which is unable to elicit a response when applied directly) can apparently replace Ca^{2+} in supporting the toxin's action (Misler & Hurlburt, 1979). This indicates that ion fluxes through the latrotoxin-induced channels may not be the main stimulus in the toxin-evoked release.

It has been shown recently that α-latrotoxin can stimulate insulin secretion from pancreatic β-cells in the absence of any ion fluxes (Lang *et al.*, 1998). Moreover, even when Ca^{2+} is present in the medium, it does not need to enter the α-latrotoxin-sensitive cells to stimulate secretion (Capogna *et al.*, 1996; Michelena *et al.*, 1997; Davletov *et al.*, 1998). These findings suggest that the toxin possesses a signalling function instead of, or in addition to, its channel-forming capability (Rahman *et al.*, 1999). Therefore, the receptor, apart from providing specifically localised binding sites, is likely to actively participate in the transduction into the cell of the signal elicited by α-latrotoxin (Rahman *et al.*, 1999).

Evidence for this interaction has been demonstrated and indicates that CIRL is a G-protein coupled receptor (Rahman *et al.*, 1999). The CIRL-G protein complex is very stable in the presence of GDP (guanosine diphosphate) but dissociates when incubated with GTP (guanosine triphosphate), suggesting a functional interaction. Also, using a α-latrotoxin-evoked norepinephrine (noradrenaline) secretion model from rat brain synaptosomes, it was found that the aminosteroid U73122, which inhibits the coupling of G proteins to PLC, blocks the Ca^{2+}- dependent action of the toxin.

Cleavage of phosphatidylinositol-4,5-bisphosphate (PIP_2) by PLC produces IP3, a second messenger that regulates Ca^{2+} release from intracellular stores. PLC activity towards PIP_2 is maximal at micromolar Ca^{2+} concentrations (Kozawa *et al.*, 1987). Therefore, this enzyme may constitute the high affinity Ca^{2+} sensor for exocytosis (Rahman *et al.*, 1999). Thapsigargin, which depletes intracellular Ca^{2+} stores, also decreases potently the effect of α-latrotoxin in the presence of extracellular Ca^{2+} (Rahman *et al.*, 1999).

Conversely in the absence of Ca^{2+}, the toxin-CIRL interaction induces pores in the presynaptic membrane which are non-selective and permeable to small fluorescent dyes, neurotransmitters and cations (Hurlbut *et al.*, 1994; Davletov *et al.*, 1998; Rahman *et al.*, 1999).

Although a second functional neurexin receptor has recently been identified (β-neurexin) there is no evidence to-date of the transduction into the cell of a signal elicited by α latrotoxin binding to this class of receptors (neurexin Iα, β-neurexin) (Sugita *et al.*, 1999).

Certain cell types (rat adrenal-medulla-tumour cell line, PC12) contain endogenous α latrotoxin receptors which are insufficient for a complete response using low concentrations of α latrotoxin. Induction of more receptors (via transfection), either as neurexins or CIRL-1, dramatically increases their α latrotoxin response, implying that the receptor density drives the α latrotoxin response (Sugita *et al.*, 1999). Similarly, in neurexin 1α knockout mice (that do not express neurexin 1α), the α latrotoxin response is decreased in the presence of Ca^{2+}, even though the other neurexins and CIRL-1 are still present (Geppert *et al.*, 1998). This finding also suggests that the receptor density is limiting, which may help explain some of the specific characteristic symptoms of *Latrodectus* envenoming (such as pain) due perhaps to differences in receptor densities of different types of neurones. Possible variations such as these may also help explain why certain mammalian species are more susceptible to the lethal effects of the venom (Table 1.1).

1.2.5 Treatment of Envenoming

Treatment of latrodectism is principally directed at relieving the severe pain and reversing the neurotoxic action of the venom.

A variety of therapies have been advocated, but no large scale controlled studies have been performed to determine the optimal treatment (Miller, 1992). As a result, therapy in the United States has evolved largely from anecdotal experience with relatively few patients. Recommended therapies have included hot baths, the administration of calcium gluconate, muscle relaxants, benzodiazipines, narcotic analgesics and antivenom (Blair, 1934; Barron, 1960; Maretic, 1983; Timms & Gibbons, 1986; Clark *et al.*, 1992; Miller, 1992; Zukowski, 1993).

Low blood calcium levels are known to be associated with some similar symptoms including muscle aches, seizures and spasms as well as tetany (general stiffening and spasms of the muscles) and are treated with calcium injections or supplements. Intravenous calcium gluconate has, therefore, long been recommended for pain relief after envenoming (Maretic, 1983; Timms & Gibbons, 1986). Investigators have had inconsistent results in this respect (Russell, 1962; Key, 1981; Clark *et al.*, 1992). For instance Key (1981) found calcium gluconate to be effective in only six of 13 patients and, since the symptoms found in severe *Latrodectus* envenoming are known to "wax and wane", many patients will demonstrate this clinical pattern and will appear to improve regardless of treatment if they are observed for only a limited period after envenoming (Maretic, 1983; Clark *et al.*, 1992).

Muscle relaxants such as methocarbamol (Robaxin) and diazepam (Valium) have been used in patients with significant muscle spasm (Russell, 1962; Timms & Gibbons, 1986) but one investigator found it to be effective in only one of 10 patients (Key, 1981). Several narcotic analgesics have also been used for pain relief with a combination of parenteral opioids and benzodiazepines producing the greatest improvement (Clark *et al.*, 1992). However, hospital admission for observation and pain control with large repeated doses may be required (Clark *et al.*, 1992; Miller, 1992).

Antivenom use in the United States is limited usually to patients with signs of severe envenoming or with a high risk of developing complications e.g. age under 16 or over 60 years, respiratory difficulty, marked hypertension, pregnancy, or distress that does not respond to other measures (Miller, 1992; Russell, 1996). This limited use reflects the high incidence of life-threatening reactions that may be associated with the systemic administration of the available product (Clark *et al.*, 1992; Miller, 1992;

Zukowski, 1993). Nevertheless, the response to this antivenom is often dramatic, with full and lasting relief of symptoms without the need for hospital admission, unless reactions to the antivenom occur (Timms & Gibbons, 1986; Clark *et al.*, 1992; Miller, 1992; Sutherland, 1992; Zukowski, 1993). The use of antivenom has also been reported, both in the U.S. and Australia, to be highly effective, even when delayed several days (90 and ≥120 days respectively) after the bite (Sutherland, 1992; Banham *et al.*, 1994; O'Malley *et al.*, 1999).

1.3 Bees

1.3.1 Classification and Distribution

The order Hymenoptera includes bees, wasps, hornets and ants. However, only mass attacks by honey bees are capable of injecting toxic quantities of venom and are, therefore, of clinical significance in the United States. Honey bees (*Apis mellifera*) originate from Africa and Europe where they have evolved different characteristics to survive in their respective tropical and temperate habitats (Winston, 1994). Most of the bees in the Americas were imported from Europe and, until the mid-1950s, those in South America were also of European origin. While European bees are good honey producers in temperate climates, they are less so in the tropics. African honey bees, in contrast, had a good reputation for tropical honey production, and were also known to exhibit intense defensive behaviour (Winston, 1994).

In 1956, Brazilian scientists imported some African queens, from South Africa and Tanzania, to Sao Paulo, Brazil. The objective was to cross the African and European bees to produce a gentle and productive tropical honey bee. However, before these hybridisations could be accomplished, 26 swarms escaped and formed the nucleus of a feral population (Winston, 1994). The bees spread at a rate of 300-500 km per year, and now occupy most of South and Central America and the southern states of the US. These Africanised bees are much more aggressive than their European counterparts and are capable of sudden large scale attacks induced by only minimal disturbances (Collins *et al.*, 1982). Within the southern United States an increasing number of mass bee attacks have been reported with the occasional fatality since the arrival of the Africanised bee (Kolecki, 1999). In Mexico there have been more than 190 such

deaths between 1988 and 1993, with future estimates of 60 deaths per year (Guzman-Novoa & Page, 1994).

1.3.2 Venom Composition

Bee venom contains many biologically active components such as melittin, phospholipase A_2, apamin, mast cell degranulating peptide, hyaluronidase, histamine, and dopamine (Habermann, 1972).

1.3.2.1 Melittin

Melittin is the major component, comprising 40 - 50 % of the dry weight (Habermann, 1972). It is a highly basic 26 amino acid peptide (2,847 Da) with strongly hydrophobic and hydrophilic regions and no disulphide bridges.

1.3.2.2 Phospholipase A_2

Bee venom contains 10-12% phospholipase A_2 (PLA_2) which forms the major allergen of the venom and has a molecular weight of 15,800Da with four disulphide bridges (Shipolini *et al.*, 1971; 1974a; 1974b; Schmidt, 1986).

1.3.2.3 Hyaluronidase

Hyaluronidase forms a minor constituent (2-3%) of the venom and is a glycoprotein with molecular weight of 41,000Da (Kemeny *et al.*, 1984).

1.3.2.4 Apamin

Apamin, a minor (~2%) basic peptide component of the venom contains 18 amino acids (2,027Da) with 2 disulphide bridges. It is a centrally acting neurotoxin that causes uncoordinated movements of the skeletal musculature increasing to spasms, jerks and convulsions when injected in mice (Habermann, 1984).

1.3.2.5 Mast cell degranulating peptide

Mast cell degranulating peptide (Peptide 401) is also a minor component (1-2%) of the venom consisting of 22 amino acids (2,593Da) with 2 disulphide bridges. This highly basic peptide has an isoelectric point above pH 10.5 and causes the degranulation of mast cells without their lysis (Banks & Shipolini, 1986).

1.3.3 Pathophysiology

The principal effects of bee venom are thought to result from its main components melittin and PLA_2. Melittin is known to damage cell membrane systems, causing the direct lysis of erythrocytes and effects on leukocytes, thrombocytes and other pharmacological systems (Habermann, 1972; Hider *et al.*, 1983; Fletcher & Jiang, 1993). It also causes the depolarisation and contraction of skeletal and heart muscle cells and *in vivo* myonecrosis (Habermann, 1972; Ownby *et al.*, 1997).

Bee venom PLA_2 hydrolyses the 2-acyl bonds of phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols and phosphatidylserines, disrupting membrane integrity and releasing lysophospholipids and fatty acids which themselves may further damage the membrane. Unlike melittin, the purified enzyme has no direct effect on washed erythrocytes but, when combined with melittin, there is a synergistic effect, and erythrocytes are lysed under conditions in which neither alone would cause lysis (Vogt *et al.*, 1970; Habermann, 1971).

It seems likely that most of the toxic effects seen after a mass bee attack are due to the general membrane damaging effects of the melittin and PLA_2 which cause the myotoxic features (Azevedo-Marques *et al.*, 1992; Ownby *et al.*, 1997).

1.4 Antibodies

Protein antigens encountering the immune system are processed by antigen-presenting cells (e.g. macrophages, dendritic cells, Langerhans' cells) which then express fragments of the antigen on their surface together with MHC (Major Histocompatibility Complex) II molecules. Specific T-helper cells recognise the antigen fragment presented with the MHC II molecule via their specific surface

receptors and facilitate, via the release of cytokines, B-cells which have also recognised the antigen (either free or via antigen presenting cells) with their own specific surface receptors (a specific idiotype of immunoglobulin). The specific B-cell clones are then stimulated to proliferate and divide into antibody-forming cells (plasma cells) which secrete large quantities of the specific antibodies. A few B-cell clones differentiate into memory cells which are utilised in any secondary exposure to the antigen and are responsible for the rapid production of large quantities of antibody seen in the secondary response.

1.4.1 Summary of Antibody Structure

Antibody molecules all have a basic four polypeptide chain structure, consisting of two identical light and two identical heavy chains, stabilised and cross-linked by intra-chain disulphide bonds. There are five major types of immunoglobulin heavy chains (γ, μ, α, δ, ε) and the type determines the class of the antibody (e.g. IgG, IgM, IgA, IgD and IgE, respectively). IgG and IgA are further divided into subclasses. For example, in man there are four IgG subclasses compared to two in sheep. Light chains are of two main types (κ or λ) and either type may associate with any of the heavy chains.

Antibody domains (globular regions) consist of 3 or 4 polypeptide loops stabilised by a β-pleated sheet and an intra-chain disulphide bond. Light chains have two domains and heavy chains four or five. The hinge region resides between the Fc and Fab and contains the inter-heavy chain disulphide bonds and confers segmental flexibility on the antibody molecule. The highest degree of amino acid variability between different antibody molecules of the same class is found concentrated within the N-terminal domains of the light and heavy chain; hence called the variable (V) region. The V regions of one light and one heavy chain form an antigen binding site. The remaining domains are relatively invariant, within any particular class of antibody, and so are called the constant (C) region.

IgG is the major serum immunoglobulin and constitutes the main antibody type in the secondary immune response to most antigens. In humans it accounts for 70-75% of the total immunoglobulin pool and has a molecular weight of ~146,000 and a sedimentation coeficient of 7S. The IgG class is distributed between the intra- and

extravascular pools and is the exclusive anti-toxin class. Sheep IgG is unusual in that it fails to bind staphylococcal protein A.

1.4.2 Summary of Antibody Function

Antibodies are bifunctional molecules. One function is to bind to specific epitopes on the surface of micro-organisms or of macromolecules and the second is to interact with effector systems to facilitate the destruction of the microbe or its removal. Some antibody functions can be mediated just by binding to the antigen (e.g. toxin neutralisation or preventing viral penetration of cells). However, most antibody functions require binding to its Fc region by various cells and / or the first component of the classical complement system. Thus the binding interaction between the Fab of the antibody and the antigen causes a conformational change in the Fc region which allows the Fc to interact with cells of the immune system, phagocytes and C1q of the classical complement pathway. The different antibody classes and subclasses interact with different cells, and so have slightly different functions (see Table 1.2).

The complement system is a complex group of serum proteins which mediate inflammatory reactions. Having bound to antigen, IgM, IgG1 and IgG3 can activate the complement cascade while IgG2 appears to be less effective and IgG4, IgA, IgD and IgE are ineffective in this respect.

The immunoglobulins display a complex pattern of interactions with various cell types which display Fc receptors (FcR). Different immunoglobulins (IgG, IgM, IgE, and IgA) bind to different Fc receptors: FcγR, FcμR, FcεR, FcαR, respectively, with FcγR being further subdivided into FcγRI, FcγRII, and $Fc\gamma R_{1o}$. The FcγRI is expressed on monocytes and macrophages but not on lymphocytes; the FcγRII is expressed on monocytes, granulocytes, platelets, and B lymphocytes but not on T lymphocytes; the $Fc\gamma R_{1o}$ is expressed on a variety of non-lymphoid cells and a subset of T lymphocytes (the so-called Tγ cells) but not on B lymphocytes.

1.5 Immunotherapy

1.5.1 History

The protective effect of immunising an animal with small amounts of venom was first described by Sewall, in 1887, who repeatedly injected pygmy rattlesnake venom into

	IgG	IgA	IgM	IgD	IgE
Complement fixation:					
Classical	++	-	+++	-	-
Alternative	-	±	-	-	-
Cross placenta	++	-	-	-	-
Fix to homologous mast cells and basophils	-	-	-	-	++
Binding macrophages and polymorphs	++	±	-	-	±

Table 1.2 Biological properties of major immunoglobulin classes in the human.

a pigeon and showed its subsequent ability to survive the injection of six lethal doses of the venom - an example of active immunisation. Roux and Yersin (1888) demonstrated the passive transfer of immunity by protecting non-immune animals against diphtheria following the transfer of blood from an animal immunised with diphthera toxin. These findings were confirmed by Von Behring and Kitasato (1890) against diphtheria and tetanus toxins as well as by Phisalix and Bertrand (1894) and Calmette (1894) against the European viper (*Vipera aspis*) and Vietnamese cobra venoms respectively. The first commercial antivenom was developed by Calmette to treat envenoming by the Indian cobra.

The first antivenoms consisted of crude hyperimmune horse serum and some such products still exist today. However, most current antivenoms consist of purified antibodies or antibody fragments. Thus the dangers associated with the systemic administration of unrefined equine serum were quickly recognised. Most patients developed serum sickness after about 8 days while a few, who had been exposed previously to horse proteins, experienced acute and sometimes fatal early anaphylaxis. The incidence of deleterious effects was reduced by the use of an ammonium sulphate precipitated immunoglobulin fraction of the serum as summarised by Landon *et al.*, (1995). Another major advance followed the work of Pope (1938; 1939a; 1939b), in which pepsin digestion was introduced to prepare $F(ab')_2$ before the final salt precipitation step. This improved both the purity and the activity of the final product. These processes remained unchanged until the recent incorporation of ion-exchange (Grandgeorge *et al.*, 1996) and affinity chromatography (Karlson-Stiber *et al.*, 1997) steps.

1.5.2 Side Effects

Adverse reactions occur commonly in patients who are receiving foreign proteins and even homologous proteins when given intravenously. Such reactions are usually mild and easily controlled by decreasing the rate of administration of the solution (Kirkpatrick, 1991). They may be due to contaminants in the solution (such as pyrogens) or to immunological mechanisms and it is usual to divide them into either early (anaphylactic or anaphylactoid) or late (serum sickness) responses.

1.5.2.1 Early

Anaphylaxis is an immunological term to describe a type I hypersensitivity reaction mediated by specific IgE. Briefly an antivenom component interacts with pre-formed specific IgE fixed to mast cells and basophils, triggering the release of histamine and other mediators. These reactions develop rapidly and may be associated with anything from just a mild urticarial reaction to severe and life-threatening anaphylactic shock.

The exact cause of immediate anaphylactoid (anaphylaxis-like) reactions remains elusive. However, numerous studies have been performed to elucidate the cause of human intravenous IgG reactions. From this it appears likely that reactions to human IgG may be due to the release of inflammatory mediators caused by the activation of phagocytic cells predominantly in the liver, through the interaction of IgG dimers or aggregates with Fc receptors (Bleeker *et al.*, 1987; 1989; 2000; Lobatto *et al.*, 1987; Jenei *et al.*, 1991; Sylvestre *et al.*, 1996). Aggregates may also activate the complement system which may contribute to the reaction (Waldesbuhl *et al.*, 1970). Commercial antivenoms have also been shown to have a complement activating effect *in vitro* that varies substantially between manufacturers, and probably reflects different degrees of purity ranging from whole antiserum to pure $F(ab')_2$ (Sutherland, 1977).

Pyrogen (or endotoxin) contamination may also result in side effects although, with modern production facilities and testing, this is becoming a thing of the past.

1.5.2.2 Late

Serum sickness is a type III hypersensitivity reaction where specific antibodies (IgG) are produced by the recipient after about a week and combine with the foreign protein to form small immune complexes in the circulation. Normally, immune complexes are removed from the circulation via the reticulo-endothelial (mononuclear phagocyte) system, particularly in the liver (by Kupffer cells), spleen, and lungs. However, if they are formed in large quantities, they are also deposited in various other tissues. The deposited immune complexes then bind and activate complement and the C3a and C5a fragments so generated bind to basophils in the blood and cause their degranulation. Immune complexes may also interact directly with basophils and platelets (via their immunoglobulin Fc regions) and cause their degranulation. Some

of the released mediators, in particular histamine and 5-hydroxytryptamine, cause retraction of endothelial cells and so increase the permeability of the blood vessels leading to the deposition of still more immune complexes. The activated platelets aggregate and initiate the formation of small clots on the collagen of the exposed basement membrane beneath the endothelial cells. Other mediators attract neutrophils which then attempt to phagocytose the deposited complexes. However, since the tissue-bound complexes cannot be easily engulfed, the macrophages release their lysosomal contents causing local damage (Klein, 1990). This is associated with an abrupt fall in total complement levels and the clinical signs of serum sickness (e.g. fever, glomerulonephritis, pain in the joints, erythema, oedema and urticaria).

1.5.2.3 Merck Antivenom

The only spider antivenom available in the United States is specific for the black widow spider (Antivenin *Latrodectus mactans*, Merck & Co., Inc.) and consists of unrefined horse serum. As a result, its administration carries a high risk of causing hypersensitivity reactions such as anaphylaxis and / or serum sickness (Kobernick, 1984; Timms & Gibbons, 1986; Clark *et al.,* 1992; Zukowski, 1993). These reactions can be severe and even fatal and pose a severe therapeutic dilemma (Clark *et al.,* 1992). Thus although the antivenom is highly effective, the mortality rate from black widow spider bites is low (<5%) with the majority of deaths in children and the elderly (Miller, 1992; Zukowski, 1993). As a result this antivenom is recommended only for patients with severe envenoming that fall into one of the following categories; age under 16 or over 60 years; patients experiencing respiratory difficulty or marked hypertension; pregnant woman and those with severe distress that does not respond to other measures (Miller, 1992; Russell, 1996).

By comparison, highly purified ovine antibody fragments directed specifically against digoxin have an excellent safety record and an incidence of adverse reactions of less than 1% (Kirkpatrick, 1991). This clearly demonstrates that a safe antibody based product is needed and could be developed to treat spider envenoming in North America.

1.5.3 Purification of Polyclonal Antibodies

Although originally crude antisera were used for therapy, most antivenoms are now purified by a process involving one or more steps to reduce the incidence of adverse reactions. After removal of the red and other cells by centrifugation, one approach is to first digest the antiserum with pepsin followed by thermocoagulation and removal of the precipitated material by centrifugation or filtration. Finally the majority of remaining contaminants are removed when the antibody fragments are precipitated with ammonium sulphate (Pope, 1939a; 1939b; Harms, 1948 (Figure 1.8)). Alternatively the γ-globulins are precipitated first with either ammonium or sodium sulphate, to yield a γ-globulin rich fraction which may be used as it is or then enzymatically cleaved with pepsin (or papain) to produce smaller active fragments (Figure 1.9).

Extra steps such as anion-exchange and affinity chromatography have been incorporated recently into certain manufacturing processes to remove contaminating proteins and increase the potency of the final product (Grandgeorge *et al.*, 1996; Raw *et al.*, 1996; Karlson-Stiber *et al.*, 1997; Chippaux & Goyffon, 1998).

1.5.3.1 Fractionation of immunoglobulins

Mineral salts such as ammonium and sodium sulphate are widely used for the purification of immunoglobulins, causing their precipitation as a result of 'dehydration' of the protein by the salt. Polyethylene glycol (PEG) also selectively precipitates proteins in proportion to their molecular size and concentration. Conversely caprylic acid precipitates most of the plasma proteins except the immunoglobulins (Steinbuch & Audran, 1969; McKinney & Parkinson, 1987). The precipitation of unwanted proteins has a major advantage for large scale production, when continuous flow centrifuges are used. Thus these machines are not designed to retain the precipitate and, as a result, large losses are often found with ammonium and sodium sulphate precipitates (Dos Santos *et al.*, 1989).

1.5.3.2 Enzymatic Cleavage

1.5.3.2.1 Pepsin

Pepsin A (EC 3.4.23.1) is the predominant aspartic endopeptidase found in the gastric

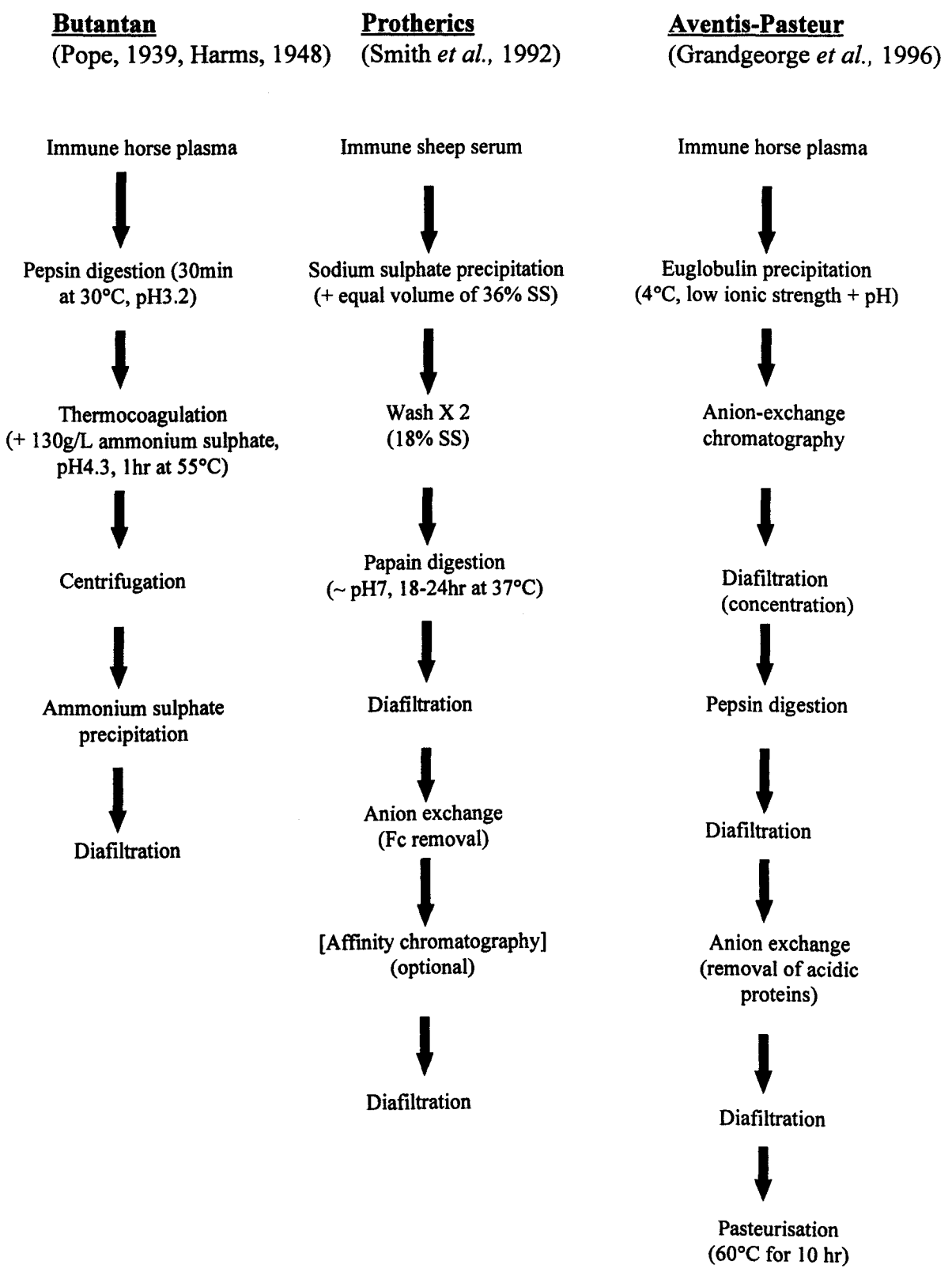


Figure 1.8 Some commercial antivenom production processes.

juice of vertebrates. Commercial grade preparations can also contain gastricsin (pepsin C, EC 3.4.23.3) and a minor component called pig gelatinase (pepsin B, EC 3.4.23.2) which have more restricted specificities and either a pronounced or little activity against haemoglobin respectively. Un-phosphorylated pepsin A is also known as pepsin D.

Pepsin A is produced in the form of an inactive precursor (zymogen) called pepsinogen with a MW of 40,000Da, by the parietal (chief) cells of the stomach. A reduction of the pH below 5 causes a conformational change in the molecule and an autocatalytic process with the cleavage of a 44aa highly basic pro-segment to form the active pepsin with a MW of 35,000Da and isoelectric point of 2.2 which is capable of hydrolysing peptide bonds (Jones *et al.,* 1995; Jonsson, 1972).

The aspartic proteinases have a typical bilobal structure composed predominantly of β-sheets separated by a deep cleft that serves as an extended binding site (Figure 1.10). This may accommodate at least seven amino acid residues. When all the binding sites are occupied, a conformational change occurs which, in an unknown way, enhances catalytic activity. In pepsin the aspartic acid residues found at positions 32 and 215 lie in close proximity in this cleft and are involved in the catalytic process. Two antiparallel β-strands form a flexible loop, L71-G82, which is located at the entrance to the active site and commonly known as the flap. The flap projects over the cleft forming a channel into which substrate binds. The role of a threonine residue on this flap, at position 77, has also been shown to provide an essential hydrogen bond that contributes to proper substrate alignment and, indirectly, to a catalytically favourable geometry of the transition state (Sielecki *et al.,* 1990; Okoniewska *et al.,* 1999). The enzyme is considered rather non-specific, in that no clear cut rules for its specificities have been found.

Although the enzyme shows a preference for hydrophobic (e.g. Ala, Val, Leu, Ile) or aromatic (e.g. Phe, Tyr, Trp) amino acids at the P1 and P'1 positions, the residues found at up to 3 or 4 amino acids from the cleavage point also play an important role in the enzyme's specificity (Figure 1.11). The conformational shape of the substrate (e.g. haemoglobin or albumin) also plays a significant role, as demonstrated by Schlamowitz and Peterson (1959).

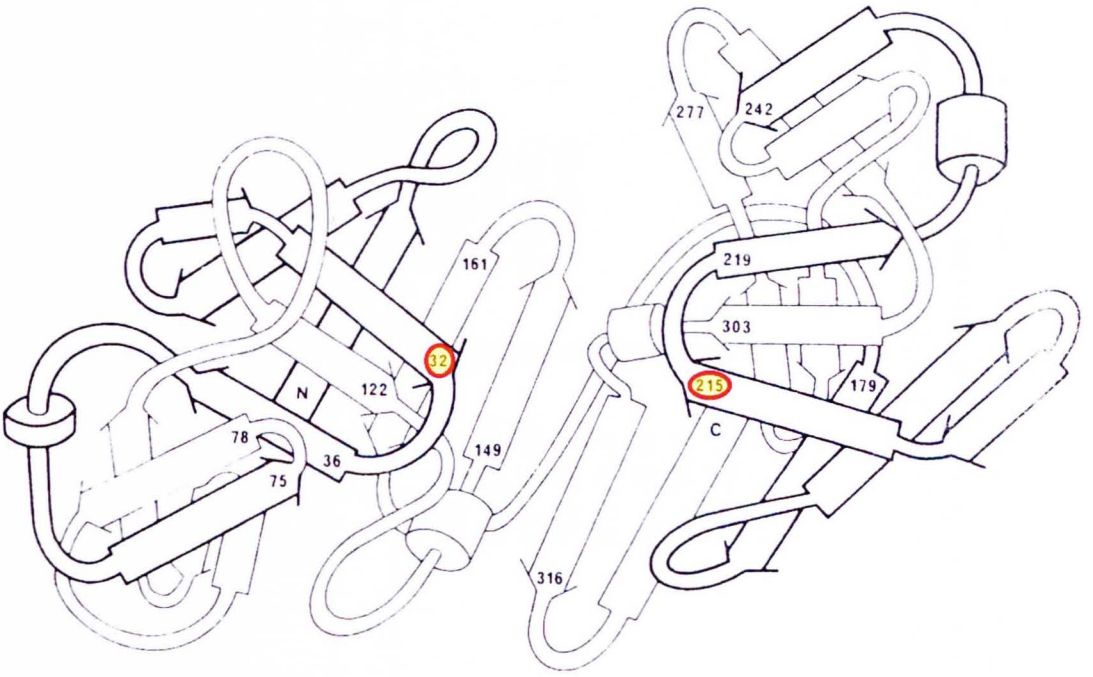


Figure 1.10 Schematic illustration of the folding of the peptide chain of the aspartic proteinases (Foltmann, 1981). The arrows indicate parallel or anti-parallel β-structures, the cylinders indicate α-helices, N-terminus and C-terminus are marked by N and C respectively. The model is opened along the cleft, in the real model Asp 32 and Asp 215 are located close to each other.

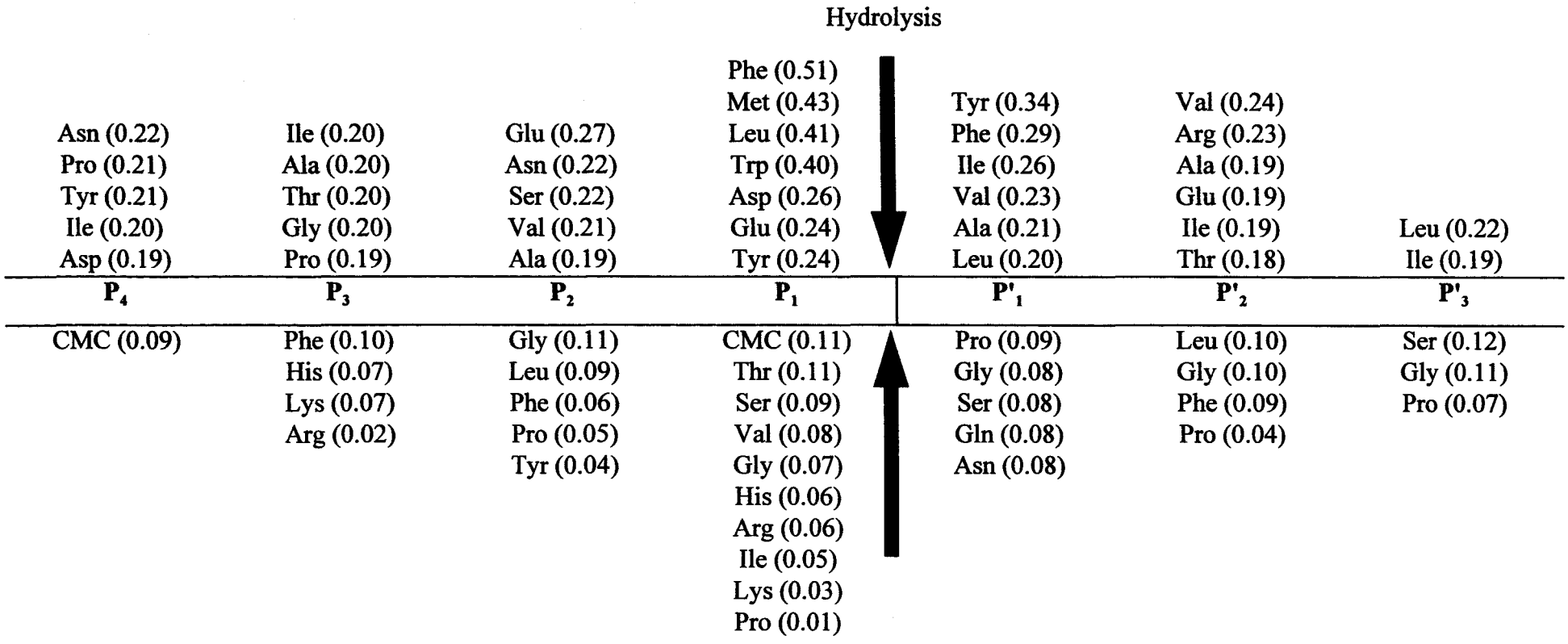


Figure 1.11 Subsite preferences and dislikes for pig pepsin (Powers *et al.,* 1977). The sample used for this calculation consisted of peptides comprising 6910 bonds of which 1020 were cleaved by pig pepsin. The amino acid residues on the top occupy positions relative to the cleavage point, with probabilities greater than the mean value (0.148). Likewise those listed at the bottom have cleavage probabilities less than the mean. CMC (carboxy-methyl-cysteine).

1.5.3.2.2 Papain

Papain (papaya peptidase I, EC 3.4.22.2) is a cysteine endopeptidase which hydrolyses proteins with a broad specificity for peptide bonds and a preference for a residue bearing a large hydrophobic side chain at the P2 position. However, it does not accept Val at P1'. Papain is a major protein constituent of the latex and the melon-like green fruit of the small softwood tree *Carica papaya*. It has a molecular weight of 23,700 Da and an isoelectric point of 8.75. Papain was the first recognised member of this class of proteolytic enzymes that need a free sulfhydryl group for activity. Other enzymes belonging to this group are chymopapain (also isolated from papaya latex), ficin from the fig tree, and bromelain from pineapples. The enzymes from animal tissue, called cathepsins, belong to this group, as well as clostridio-peptidase B and streptococcal proteinase from bacteria. These enzymes usually need an activator to release the blocked SH group. The most usual activators are cysteine, cyanide, or glutathione (Drenth *et al.*, 1971).

1.5.3.2.3 Trypsin

Trypsin (EC 3.4.21.4) is a serine endopeptidase which preferentially cleaves substrates containing Arg or Lys. It is produced as an inactive precursor called trypsinogen in the pancreas and cleaved initially by a specific enteropeptidase secreted in the duodenum to produce a small amount of active trypsin which can then activate more trypsinogen.

1.5.3.3 Enzymatic digestion of immunoglobulins

Treatment of immunoglobulins with proteolytic enzymes found practical use over 50 years ago when Pope (1939) employed pepsin to remove the Fc of horse anti-tetanus and anti-diphtheria antibodies. Proteolytic enzymes were subsequently used to determine the structure of antibodies, by Porter (1959). He described the partial digestion of rabbit gamma globulin by papain to yield three fragments, two antibody binding fragments (Fab) and a slightly larger fragment (Fc) that had much of the antigenic specificity of the original molecule. Other studies using increasing concentrations of the enzyme demonstrated the further digestion of part of the rabbit gamma globulin molecule into small dialysable fragments representing 42% of the

total protein, but with only a 25% decrease in specific binding capacity of the remaining fragments (Nisonoff & Woernley, 1959).

Enzymes used for the digestion of antibodies today include pepsin, trypsin, bromelain and ficin (Mariani *et al.*, 1991; Zou *et al.*, 1995). However, only pepsin and papain are used to digest polyclonal antibodies in the commercial production of antivenoms.

Despite the generally over-simplified view of antibody digestion, there are subtle differences in the susceptibility of different classes and sub-classes of antibody to digestion by various enzymes (Turner *et al.*, 1970). The antibodies from different species can also demonstrate a considerable variation in their relative susceptibility to enzymes. For example, the digestion of rabbit antibodies by pepsin at pH 4.5-5.0 (Nisonoff *et al.*, 1960), even after 48hr, produces an intact $F(ab')_2$ fragment, without appreciable loss of antibody combining activity. However, at pH 2.5, digestion proceeds via the formation of an intermediate Fab' fragment until all parts of the molecule are reduced to small peptides without any binding activity (Haber & Stone, 1967). Davies *et al.*, (1978) have shown that sheep IgG, by comparison, is only slowly digested at pH 4.7 and even after 48hr at 37°C some residual intact IgG remains. Despite this partial digestion, both subclasses of ovine IgG (IgG1 and IgG2) were susceptible to proteolytic cleavage producing $F(ab')_2$ and small fragments of Fc (Davies *et al.*, 1978).

1.5.4 Antivenom

1.5.4.1 The ideal antivenom

The ideal antivenom should be rapidly distributed to the tissues where the venom may be present, including its site of introduction, and must be retained sufficiently long to continue to neutralise any venom that is being released slowly from the bite site (Cardoso *et al.*, 1990). It should have a high avidity for the toxic components of the venom and, therefore, be effective at preventing morbidity or mortality. It should also not produce any side effects. Its stability should reflect the likely storage conditions in the field and it should be of a cost affordable by the people or country that it is destined for.

1.5.4.2 Types and availability of antivenom

All commercial antivenoms are produced by immunising the recipient animal(s) with the specific venom or venoms and collecting the antisera. This may then be refined to IgG or $F(ab')_2$ fragments to reduce the amount of foreign protein given per treatment and, thereby, the incidence of side effects. Normally horses are used due to their large size although other species are also used occasionally. Table 1.3 lists the commercially available spider antivenoms.

Product Name / Type	Venoms Covered	Manufacturer
Antivenin (*Latrodectus mactans*), whole serum (equine)	*Latrodectus mactans*	Merck & Co., Inc., USA
Red-backed spider antivenom, $F(ab')_2$ (equine)	*Latrodectus hasselti*	Commonwealth Serum Laboratories (CSL), Australia
Spider antivenom, $F(ab')_2$ (equine)	*Latrodectus indistinctus*	The South African Institute for Medical Research, South Africa
Anti-*Latrodectus mactans tredecimguttatus* serum, $F(ab')_2$ (equine)	*Latrodectus tredecimguttatus, (Latrodectus hasselti, Latrodectus indistinctus)*	Institute of Immunology, Croatia
Soro-antiloxoscelico, whole serum (equine)	*Loxosceles reclusa*	Instituto Butantan, Brazil
Suero-antiarachnidico, $F(ab')_2$ (equine)	*Loxosceles sp.*	Instituto Nacional de Salud, Peru
Soro-antiarachnidico polyvalente, whole serum (equine)	*Loxosceles sp., Phoneutria sp., Tityus bahiensis, Tityus serrulatus*	Instituto Butantan, Brazil
Funnel-web spider antivenom, IgG (lapine)	*Atrax robustus, Atrax sp. Hadronyche sp.*	Commonwealth Serum Laboratories (CSL), Australia

Table 1.3 Commercially available spider antivenoms (Theakston & Warrell, 1991; Meier, 1995).

CHAPTER 2:
MATERIALS AND GENERAL METHODS

2.1 Materials

2.1.1 Antivenoms and Immunochemicals

Black widow spider antivenom (*Latrodectus mactans*) was purchased from Merck & Co., Inc. (West Point, USA). Normal sheep and horse serum and donkey anti-sheep IgG serum were obtained from MicroPharm Ltd (West Wales, UK).

Peroxidase-conjugated affinity purified rabbit anti-sheep $F(ab')_2$ was purchased from Jackson Immuno Research Laboratories Inc. (Luton, UK).

2.1.2 Venoms

Frozen spider venoms from *La. hesperus, La. mactans, Loxosceles reclusa* and *Lo. deserta* were purchased from Spider Pharm Inc. (Feasterville, USA). These venoms were obtained by the electrical stimulation of CO_2 anaesthetised spiders. Suitable precautions were taken to prevent venom contamination with gastric secretions by inserting a suction tube into the spider's mouth while collecting venom from the fang tips using a capillary pipette. *La. tredecimguttatus* freeze dried venom gland extracts and purified venom components were purchased from Latoxan (Rosans, France) and *La. hasselti* venom from Venom Supplies Pty. Ltd. (Tanunda, South Australia). Alpha latrotoxin and other venom components were purified according to the method of Krasnoperov *et al.*, (1992). Freeze-dried *Apis mellifera mellifera* venom and its purified venom components were obtained from Latoxan (Rosans, France) and Africanised bee venom was obtained from Southwest Venoms (Tucson, USA).

2.1.3 General Chemicals and Materials

CNBr-activated Sepharose 4B and Q Sepharose Fast Flow were purchased from Pharmacia (Uppsala, Sweden); Ultra Pure Protogel (30% acrylamide / 0.8% bis-acrylamide w/v, solution) from National Diagnostics Ltd (Atlanta, USA); flat-bottom polystyrene Nunc Microwell plates from Life Technologies Ltd (Paisley, UK); and ultrafilters and filters from Sartorius (Goettingen, Germany).

All other general chemicals and solvents were of AnalaR or similar grade and supplied by BDH / Merck (Poole, UK) or Sigma (Poole, UK) unless otherwise stated.

2.2 List of General Instruments

ELISA plate washer; Flow Laboratories (Lugano, Switzerland).

ELISA plate reader; Titertek Multiscan PLUS (MKII), Flow Laboratories (Lugano, Switzerland).

Multipipette; Life Sciences International (Basingstoke, UK).

Incubator; Genlab Ltd (Widnes, UK).

Water bath; Grant SUB14 (Cambridge, UK).

Centrifuge; Mistral 3000 (Crawley, England).

Spectrophotometer; Jenway 6105 U.V./VIS (Felsted, England) .

Electrophoresis system; Mini-protein II dual slab cell, Biorad (California, USA).

FPLC; comprising two P-500 pumps, LCC-501 PLUS control unit, HR10 flow cell, motor valve MV-7, mixer, UV-1 optical/control units, REC 2, Pharmacia (Uppsala, Sweden).

Fraction collector; FRAC-100, Pharmacia (Uppsala, Sweden).

Micro balance; BP110S, Sartorius (Goettingen, Germany).

PH meter; Jenway 3200 (Felsted, England).

Nerve muscle equipment; Maclab 4e, Quad Bridge Amp, Isometric force transducers, Performa 6300 Power PC, ADInstruments (Hastings, UK). Two channel dual impedance stimulator, 60mL tissue baths, tissue holder electrodes, Harvard Apparatus Ltd (Edenbridge, UK).

2.3 General Methods

2.3.1 Protein Concentration

2.3.1.1 Optical density (OD) at 280nm

A spectrophotometer set at 280nm was first blanked or zeroed against the appropriate buffer in a 1cm path length silica (quartz) cuvette and then the sample at an appropriate dilution measured (ideally reading at ~ 1.0). Extinction coefficients (1g/L) of 1.5 for ovine IgG (Curd *et al.,* 1971) and 1.4 for ovine Fab (Allen, 1996) were used throughout.

2.3.1.2 Bicinchoninic acid (BCA) assay

The macromolecular structure of a protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for the colour formation with the BCA assay.

Proteins in an alkaline medium reduce copper II to copper I (the biuret reaction) which then reacts with BCA to form an intense purple colour at 562nm. BCA forms alkaline metal salts which are soluble in water due to the polar carboxylic acid groups. The purple reaction product, formed by the interaction of two molecules of BCA with one cuprous ion, is also water-soluble enabling the spectrophotometric measurement of an aqueous protein solution. Protein to protein variation is said to be less with BCA than with OD at 280nm resulting in a more accurate quantitation of impure solutions or of those with an unknown extinction coefficient.

Wells of a microtitre plate were filled with 200μL of BCA working reagent which is a 50:1 mix of BCA reagent A (containing sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate in 0.2M sodium hydroxide) and B (containing 4% cupric sulphate, Pierce Chemical Company) and 10μL protein samples added to each well. Both a standard curve (20-2,000μg/mL), using bovine serum albumin (BSA) standard, and unknown samples were assayed in duplicate on the same plate which was mixed by shaking for 30 seconds, then incubated for 1hr at 37°C and the absorbance measured at 562nm. The protein concentration of unknowns was read off from the standard curve (Figure 2.1).

2.3.2 Antisera Production

Antisera were raised by immunising half-breed Welsh ewes every 28 days with immunogen dissolved in phosphate buffered saline (1.3mL/sheep) and mixed extensively with Brij 35 (0.2mL/sheep) and adjuvant (3.25mL/sheep) to form a stable water-in-oil emulsion. Freund's complete adjuvant was used for the primary immunisation, and Freund's incomplete adjuvant for all reimmunisations. The sheep were immunised in six sites, namely intramuscularly in each forelimb and hindlimb and subcutaneously on either side of the neck.

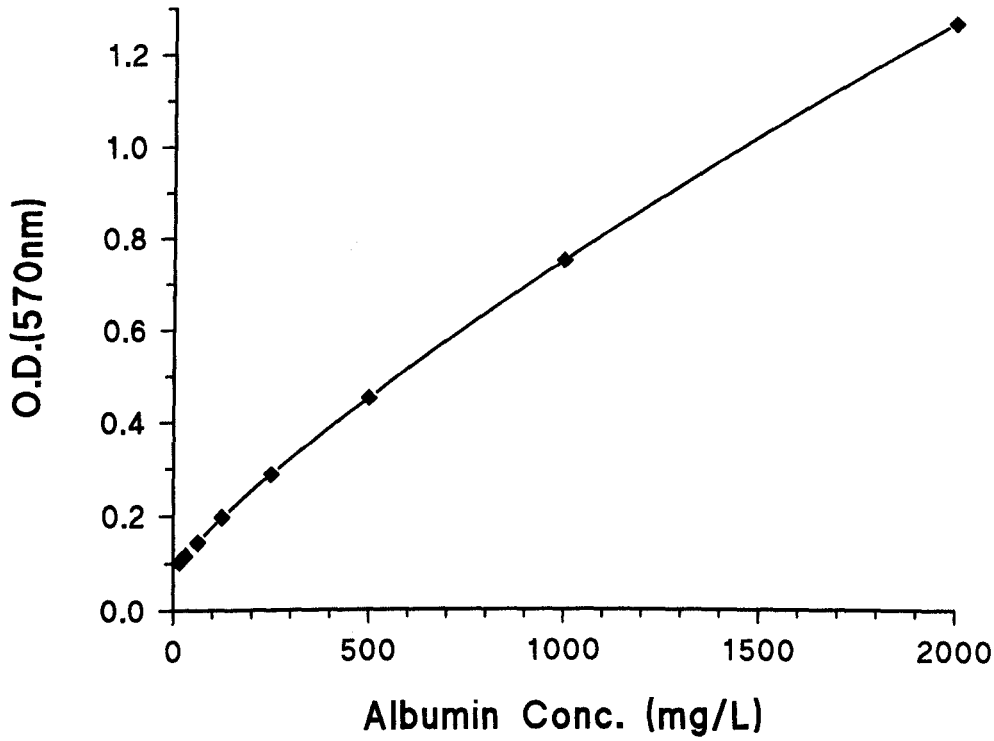


Figure 2.1 BCA protein assay standard curve

Ten mL blood samples were collected from each sheep via the external jugular vein two weeks after each immunisation and, once adequate antibody levels had been achieved, 10mL / kg body weight of blood was collected at the same intervals. The blood was then rolled slowly for 2hr at room temperature to enhance clotting, centrifuged and the serum aspirated and stored frozen (-20°C) until used.

2.3.3 Fast Protein Liquid Chromatography (FPLC)

Gel filtration columns consisted of a cross linked agarose medium (Superose 12 or Superose 6 HR 10/30, volume 25mL). Superose 12 and 6 columns had a range of pore sizes suitable for the separation of proteins with molecular weights between 1 and 300 kDa and between 5 and 5,000 kDa respectively. Samples were diluted in running buffer (Tris, 0.05M, pH8.0, NaCl 0.2M) and centrifuged or filtered to remove any insoluble material. A 50μL sample loop attached to a MV-7 motor valve was used to apply the sample to the column and the appropriate sensitivity levels set for measuring the eluted proteins (UV-1 at 0.1AU, chart recorder REC2 at 10mV) at 280nm through a HR10 flow cell. A constant flow rate of 0.5mL/min was provided by a P-500 pump and samples were run for a minimum of 1hr. Peak detection was set at 5% above the baseline and the integrator on the LCC-501 PLUS control unit used to calculate both the peak retention times and the percentage area under the peak.

Known molecular weight standards (aprotinin 6,500Da, cytochrome C 12,400Da, carbonic anhydrase 29kDa, bovine serum albumin 66kDa, alcohol dehydrogenase 150kDa, β-amylase 200kDa, apoferritin 443kDa, thyroglobulin 669kDa) and blue dextran (2,000kDa, used to calculate the void volume) were run under identical conditions and their retention times used to plot a molecular weight calibration curve with the elution volume (Ve) expressed in terms of the void volume (Vo) which was used to estimate unknown molecular weights (Figure 2.2).

2.3.4 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate is an anionic detergent which binds strongly to proteins, causing their denaturation. In the presence of excess SDS, about 1.4g of the detergent binds to each gram of protein, giving the protein a constant negative charge per unit mass. Consequently all protein-SDS complexes move towards the anode during

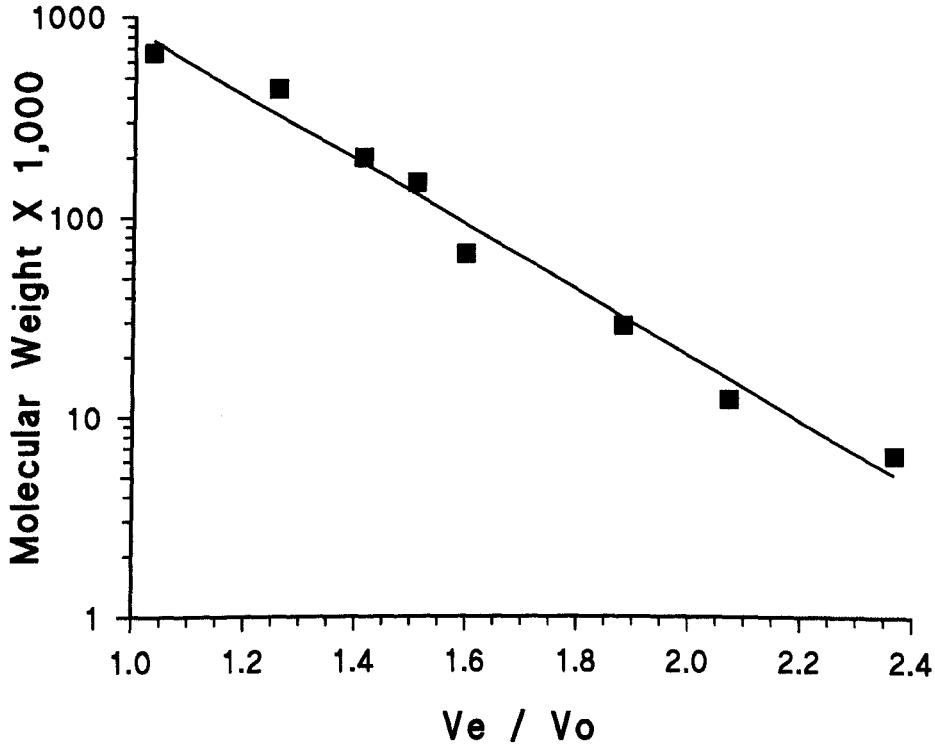


Figure 2.2 Superose 12 size exclusion chromatography standard curve

electrophoresis and, owing to the molecular sieving properties of the gel, the mobility of each is proportional to its molecular weight.

The stacking gel serves to concentrate the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilising differences in ionic strength and pH between the electrophoresis buffer and the stacking gel and involves a phenomenon known as isotachophoresis. The stacking gel has a very large pore size, which allows the proteins to move freely and concentrate, or stack, under the effect of the electric field. The band sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have a lower electrophoretic mobility than the protein -SDS complexes which, in turn, have a lower mobility than the chloride ions of the loading buffer and the stacking gel.

SDS-PAGE gels were prepared to characterise the various components of the venom or antivenom using the buffer system described by Laemmli (1970). They were prepared between two glass plates which had been thoroughly cleaned using distilled water and acetone. The plates were separated using Teflon spacers with the base of the gel sealed using a rubber seal onto which the glass plates were pressed and the running gel (Table 2.1) poured between the plates. The gel was then covered by an overlay solution (acetone) which ensured even setting at the acrylamide/acetone interface. Once set, the overlay solution was washed off with distilled water and the stacking gel (composition also shown in Table 2.1) poured and a Teflon comb inserted at the top to form the wells. Once set, the comb was removed and the sample wells and upper and lower buffer chambers filled with running buffer (Tris 25mM, glycine 0.192M, SDS 0.1% w/v, pH 8.3) before use.

The samples were diluted at least 1:4 with sample buffer containing 62.5mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol and 0.005% (w/v) bromophenol blue for non-reducing conditions. Reducing buffer was identical but also contained 5% 2-mercaptoethanol and the samples were heated at 95°C for 4 min. The molecular weights of unknown proteins were calculated by running the MW standards myosin (205kDa), β-galactosidase (116kDa), phosphorylase b (97kDa), fructose-6-phosphate kinase (84kDa), albumin (66kDa), glutamic dehydrogenase (55kDa), ovalbumin (45kDa), and glyceraldehyde-3-phosphate dehydrogenase (36kDa) on the same gel

	Separating Gel		Stacking Gel
	12%	7.5%	
Water	3.35mL	4.85mL	6.1mL
1.5M Tris-HCl, pH 8.8	2.5mL	2.5mL	
0.5M Tris-HCl, pH 6.8			2.5mL
10% (w/v) SDS stock	100µL	100µL	100µL
Acrylamide/Bis (30 / 0.8% w/v)	4.0mL	2.5mL	1.3mL
10% ammonium persulfate	50µL	50µL	50µL
TEMED	5µL	5µL	10µL

Table 2.1 Constituents of SDS-PAGE gels.

and plotting a calibration curve of the R_F (distance protein has migrated from origin / distance from origin to tracking dye) against MW (Figure 2.3).

Gels were run at a constant voltage of 150V and then visualised by staining (Coomassie brilliant blue G 0.05% w/v in destain) for 1 hr before being destained (water/acetic acid/methanol mixture 5:1:4), prior to drying.

2.3.5 Diafiltration.

Small volumes of material were diafiltered or concentrated using 2mL centrifuge concentrator units with polyethersulfone membranes (Vivaspin) according to the manufacturer's instructions in a swing-out bucket type centrifuge and repeatedly washed as required. Larger volumes were diafiltered using Sartocon-Micro cross flow ultrafiltration units with either 30 or 50kDa MWCO polyethersulfone membranes (Sartorius, Goettingen, Germany). Fluid was pumped around the ultrafiltration unit as shown in Figure 2.4 and membranes primed with at least 50mL buffer before being used. Material to be ultrafiltered was initially concentrated to ~ 100mL and regularly topped up with fresh buffer as the volume decreased until it had been washed with 10 volumes or 1L of buffer. Ideally fresh buffer should continually be added at the same rate as the filtrate waste is produced in order to increase the efficiency of the diafiltration. Any residual material left trapped in the tubing and ultrafiltration unit at the end was flushed out with a small volume of buffer and added to the product. The membrane was regenerated between use by thoroughly washing the system with distilled water and then with 1M NaOH for 2 hr.

2.3.6 Enzyme Linked Immunosorbant Assay (ELISA)

Serum antibody titres directed specifically against purified alpha latrotoxin or whole venom, were determined by ELISA according to the method of Sjostrom *et al.*, (1996). Polystyrene 96-well micro-titre plates were coated with a solution of venom (2μg/mL in sodium carbonate/bicarbonate buffer 0.1M, pH 9.6) or α latrotoxin (500ng/mL) by incubating 100μL per well for 2 hours at 37°C. Coated plates were then washed three times with 300μL of ELISA washing buffer per well (NaCl 137mM, Na_2HPO_4 8.1mM, KCl 2.68mM, $NaH_2PO_4.2H_2O$ 1.28mM, Thimerosal 0.247mM, Tween 0.1%), blocked with ELISA washing buffer for a further 2 hours at

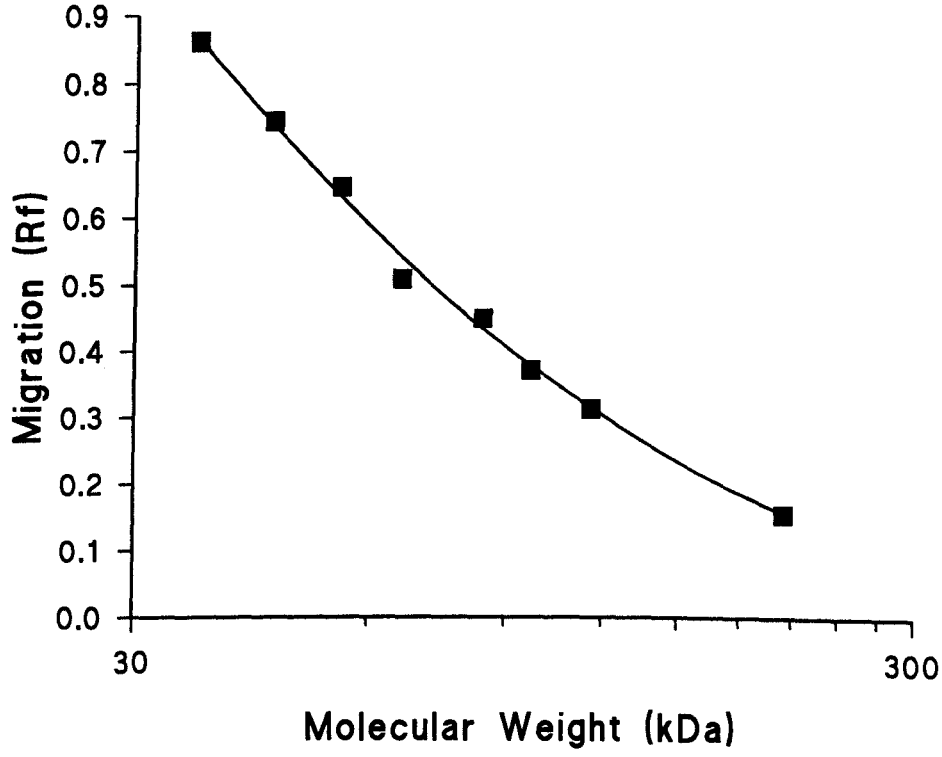


Figure 2.3 An SDS-PAGE (7.5%) calibration curve

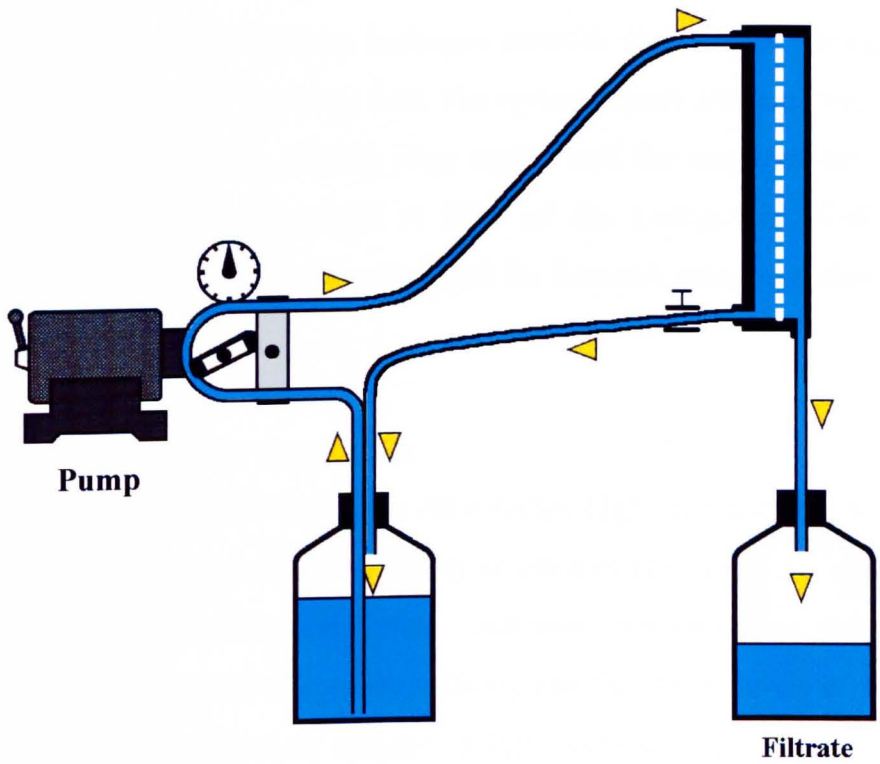


Figure 2.4 Diafiltration using a cross flow ultrafiltration unit.

37°C and finally washed to remove any unbound antigen. The plates were then incubated with 100μL of doubling dilutions of the immune serum diluted with ELISA washing buffer. A reference antiserum (from a pool of sera with an established titre) and normal sheep serum (NSS) were included on each plate as controls. All dilution curves were performed in duplicate. Plates were subsequently incubated at 37°C for 1 hr, washed three times and then incubated with 100μL of donkey anti-sheep immunoglobulin (1:500 dilution in ELISA washing buffer) to which had been conjugated horse radish peroxidase (HRP). Colour development was achieved at room temperature using 100μL of o-phenylenediamine dihydrochloride (OPD, 0.1mg/mL in citrate buffer, 0.07M, pH 5.0) with hydrogen peroxide (0.02%) and stopped after 15 minutes with 50μL of 3M sulphuric acid. The optical density (492nm) was read using a Titertek Multiscan PLUS (MKII) plate reader and the mean of the duplicates calculated. Titres were determined at 50% of the background NSS corrected absorbance (Figure 2.5) and values corrected for between assay variation using the reference antiserum.

2.3.7 Small Scale Affinity Purification.

Cyanogen-bromide activated Sepharose 4B columns (1g) were prepared according to the manufacturer's instructions using venom or toxin as the ligand at a concentration of 5mg/g of dry gel. Loading and eluting conditions were optimised and conditions providing the highest levels of specific antibody and the lowest levels of nonspecific binding were chosen. Antiserum samples (500μL) were diluted in 0.9% saline / 2% acetonitrile (6mL), mixed end-over-end overnight with the gel at 4°C, washed (saline / 2% acetonitrile), and eluted (100mM glycine, pH 2.5). Specific antibody concentrations were determined based on their optical density at 280nm, using an extinction coefficient of 1.5 for ovine IgG. Non-specific binding was determined by running NSS and levels of specific antibody were corrected by subtracting the value due to non-specific binding.

2.3.8 Venom Phospholipase Assay.

Venom phospholipase A_2 (EC 3.1.1.4) activity was assessed by measuring free fatty acid release from a phospholipid substrate (phosphatidyl-choline, egg yolk type) using

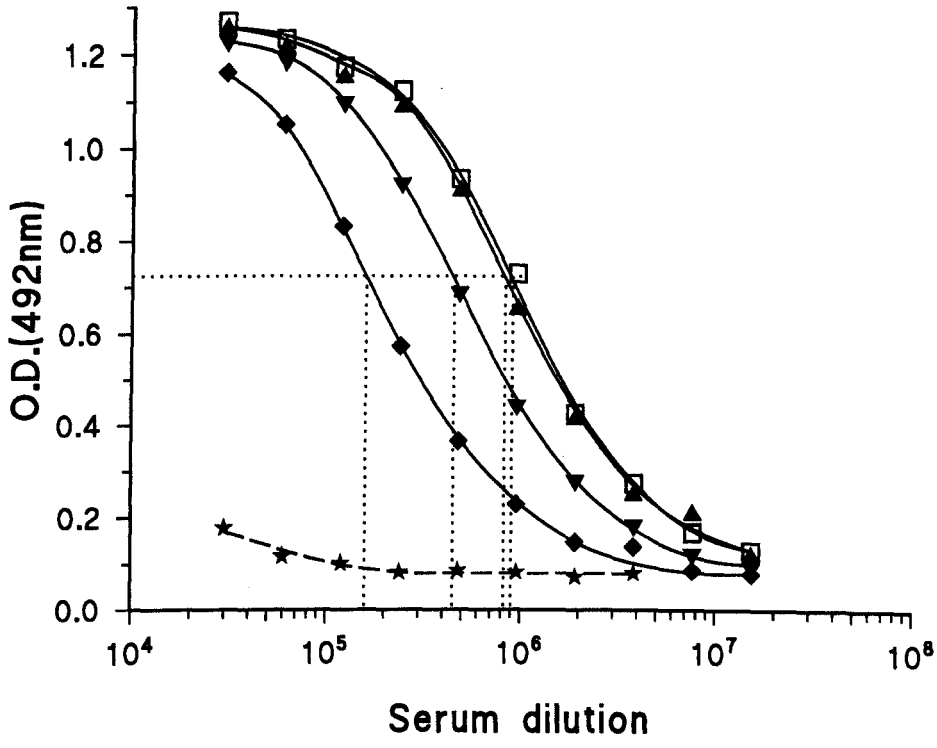


Figure 2.5 *La.hesperus* venom specific antiserum dilution curves by ELISA. Antiserum samples were; standard (□), week30 (2mg) *La.hesperus* (▲), week58 (0.1mg) *La.hesperus* (▼), week58 (0.1mg) *La.hasselti* (◆), NSS (★).

cresol red as a pH indicator (deAraujo & Radvanyi, 1987).

To 1mL of the freshly prepared lipid suspension (1.2g phosphatidyl-choline dissolved in 1mL methanol made up to 10mL with assay buffer), was added 25mL of assay buffer (100mM NaCl, 100mM KCl, 10mM $CaCl_2$, pH 8.6) followed by 0.3mL of Triton-X-100. The solution was mixed thoroughly until it became clear and the pH adjusted to 8.5. One mL of the indicator solution (10mg cresol red in 10mL of assay buffer) was added and the resultant solution made up to a final volume of 30mL with assay buffer, producing a red coloured substrate solution. This solution was protected from light as the indicator solution is light sensitive. Next, 100µL of assay buffer was added to 2.8mL of the substrate solution in a plastic cuvette and the optical density (573nm) measured. To this was added 100µL of venom solution (10µg/mL in assay buffer) and a stop-watch started. To a second cuvette containing 2.8mL of substrate solution and 100µL of assay buffer was added a further 100µL of assay buffer as a control, which was run concurrently with the assay cuvette. Readings were taken every minute for 25 minutes and expressed as percentage hydrolysis of the substrate, taking the second cuvette as a zero standard (0% hydrolysis).

Effects of various concentrations of venom were measured and the optimum concentration was used to assess antivenom neutralisation. Venom (10mg/L) was premixed with antivenom for 30 min at 37°C prior to substrate addition and the optical density at 573nm measured after 10 min.

2.3.9 Nerve-Diaphragm Assay

The two phrenic nerves are made up from branches of the 3rd, 4th and 5th cervical (neck) nerve roots and descend through the neck and thorax to innovate the diaphragm.

Left phrenic nerve-hemidiaphragm preparations were isolated from 20-35g male, out-bred white mice (Kitchen, 1984; Bulbring, 1946).

Mice were killed by cervical dislocation (avoiding over stretching the mice as this may damage the diaphragm and break the nerves) and placed under a lamp/extractor hood on an ice block wrapped with a paper towel. Krebs buffer (NaCl 118mM, $NaHCO_3$ 25mM, $NaH_2PO_4.H_2O$ 1.0mM, KCl 4.8mM, $CaCl_2.2H_2O$ 1.9mM, $MgSO_4.7H_2O$ 1.2mM, D-glucose 11.1mM) was placed on a separate ice block and

gassed with 95% O_2 / 5% CO_2. The skin covering the upper part of the abdomen and thorax was removed to reveal the muscle layers covering the chest which were removed in turn to expose the rib cage. An incision was made along the midline half way between the xiphisternum and the neck and a quick examination performed to ensure that the phrenic nerves were not adhering to the chest wall. The cavity and diaphragm was then irrigated with cold gassed Krebs solution at regular intervals. Lateral cuts were made on either side of the midline incision, parallel to the ribs, and the overlying section of rib cage carefully removed to leave just one or two ribs attached to the diaphragm on the animal's left side. The left phrenic nerve was carefully trimmed of connective tissue / fat and a short length of thread tied around it as far from the diaphragm as possible and the nerve cut leaving the thread attached to the nerve and diaphragm. The wall of the abdomen was then cut to free the rib cage. Holding the xiphisternum with forceps the diaphragm was cut straight down through the xiphisternum to the spine, taking care not to cut or stretch the phrenic nerve. The diaphragm was then cut laterally from its attachment to the body wall so freeing the preparation from the animal, placed in a petri dish containing gassed Krebs and trimmed to a suitable size to fit in the tissue holder. Using a needle, a piece of thread was attached to the tip of the tendon at the apex of the diaphragm preparation for attachment to the transducer.

The recording equipment was calibrated by applying a 5g calibration weight (Ohaus) to each transducer. The rib was attached to the mounting bar of the tissue holder (Harvard Apparatus Ltd) and the nerve pulled gently across the sliding jaw-hook electrode and secured in place. The mounted preparation was then transferred to an organ bath and connected to the transducer (see Figure 2.6).

Each tissue preparation was bathed in either 40 or 60mL Krebs buffer maintained at 32°C, and supplied with 95% O_2 + 5% CO_2. Indirect stimulation (via the nerve) was applied at supramaximal voltage (~3V, 0.2Hz, 0.2ms) and muscle contractions recorded using an isometric transducer linked to a bridge amplifier and MacLab chart recorder (200samples/sec, DC high pass, 200Hz low pass, 1mV range). Indirect stimulation was continued for at least 30min until a consistent twitch response was produced after which venom was added (t_0), and washed out once after 30min (t_{30}) and again after a further 30min (t_{60}). The decrease in contractions was calculated as a

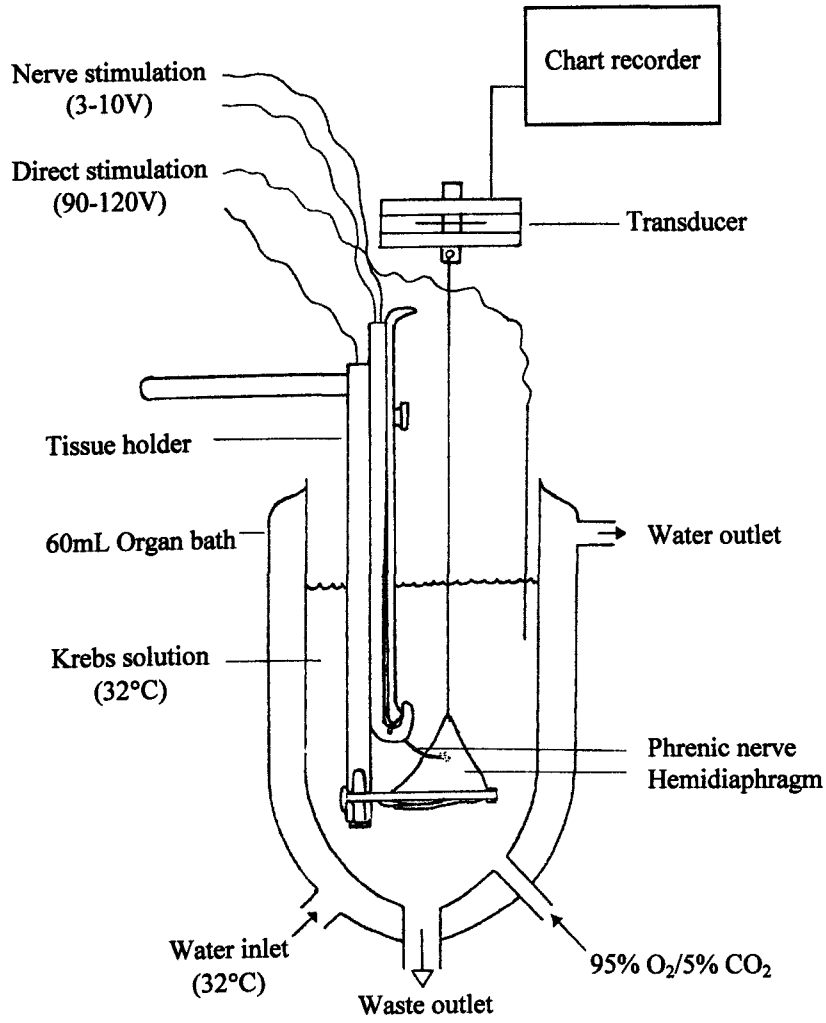


Figure 2.6 Mounted phrenic nerve hemidiaphragm preparation.

percentage of the contractions just before venom addition. Only tissue preparations with muscle contractions producing greater than 1g of tension were used. The myotoxic effects of the venom were assessed by applying a short burst of direct (muscle) stimulation (0.2Hz, 1ms, ~100V) before venom addition and at 30min intervals thereafter.

The ability of antivenom to neutralise the effects of the venom was assessed by premixing a fixed venom concentration with antivenom and incubating at 37°C for 30 min before addition to the hemidiaphragm preparation (t_0) followed by an identical dose cycle as outlined above.

2.3.10 Venom Lethality in Mice and Antivenom Efficacy

Where possible all solutions were sterile filtered (0.2μm) prior to use. The LD_{50} of the venom (the amount that causes the death of 50% of the animals) was assessed by intravenous (i.v.) injection of various doses of venom in 0.2mL of physiological saline into the tail vein of 18-20g male out-bred white (TFW) mice (Theakston & Reid, 1983). Animals were placed under a heat lamp to dilate the tail vein and, thereby, aid injection. Five mice were used for each venom dose and the LD_{50} was calculated from the number of deaths within 24hr by probit analysis. Animals were regularly checked during the 24hr period and dead animals removed. Any animals in severe distress and likely to die before the full 24hr period were humanely killed according to Home Office guidelines.

To assess antivenom potency, various amounts of antivenom were mixed with two or five times the LD_{50} of venom; the mixture was then incubated at 37°C for 30 min and injected intravenously into mice. Five mice were used at each antivenom dose. The ED_{50} or median effective dose of the antivenom (the amount that prevents 50% of the mice from dying) was calculated from the number of deaths within 24 hr after injection of the venom/antivenom mixture using probit analysis (Laing *et al.*, 1992).

CHAPTER 3:
PRODUCTION OF ANTISERA FROM WHICH TO MANUFACTURE ANTIVENOMS
- USING AFRICANISED BEE VENOM AS AN EXAMPLE

3.1 Introduction

The development of a platform technology with which to manufacture a range of antivenoms involves two distinct stages

(1) Production of a suitable pool of antisera, processing using a conventional method and its assessment.

(2) Development of a new improved method of manufacturing antivenom from the antisera pool.

This chapter deals with (1) and chapter 4 with (2).

3.1.1 Production and Assessment of Antiserum

Before beginning to produce a new pool of antisera there must be adequate supplies of high quality venom - for immunisation and for use in assessment techniques. An appropriate species must be selected that produces adequate volumes of antisera such as the horse, goat or sheep, and their immunisation schedule optimised to produce the maximum amount of specific antibodies. Appropriate assessment techniques must also be selected and should include a bioassay to measure the neutralisation of toxicity.

Because of problems in obtaining large quantities of high quality spider venom and its high cost, it was decided to develop a new platform technology using antisera raised against an easily available venom. This chapter describes the production of large quantities of antisera to bee venom for use in the next stage. Bee venom was chosen as a model because it is easily collected in large quantities and is, therefore, widely available at low cost. In addition, there are concerns in the US about increasing

numbers of mass bee attacks for which there is no specific antivenom available. Thus a safe, effective treatment is required urgently as only normal supportive intensive care treatment of the clinical features as they occur is currently available (Franca *et al.*, 1994; Schumacher & Egen, 1995; Schumacher *et al.*, 1996). Treatments include haemofiltration or haemodialysis, exchange transfusions or plasmaphoresis, antihistamines, corticosteroids, calcium gluconate and bicarbonate with mannitol (Franca *et al.*, 1994; Schumacher & Egen, 1995).

The pre-clinical development and assessment of a model antiserum specific for *A. mellifera* venom is described here that was subsequently used in the next chapter to develop a new procedure for processing antivenoms.

3.2 Methods

3.2.1 Venom

Freeze dried *Apis mellifera mellifera* venom and its purified venom components were obtained from Latoxan (Rosans, France) and Africanised bee venom was obtained from Southwest Venoms (Tucson, USA).

3.2.2 Venom Analysis by Electrophoresis

A conventional SDS-PAGE technique was performed as described in Chapter 2.

Propionic acid - urea polyacrylamide (22%) gel electrophoresis was performed according to the method of Chettibi and Lawrence (1989). Gels were prepared by mixing 15 mL of Protogel (30% acrylamide, 0.8% bis-acrylamide, w/v) with 0.4ml propionic acid and 7.2g urea. Once dissolved, 133µL of freshly prepared 10% ammonium persulphate and 16µL of TEMED were added, quickly mixed and poured to form two mini gels. Running buffer consisted of 2% acetic acid in distilled water and sample buffer contained 30% sucrose, 2% acetic acid, and 0.01% neutral red dye. Samples were dissolved in distilled water, diluted with an equal volume of sample buffer and run at 10mA/gel with the anode connected to the top of the gel. Gels were stained with 0.1% Coomassie blue in methanol : water : acetic acid (50:50:7) and destained in methanol : water : acetic acid (5:100:7).

3.2.3 Venom Phospholipase A_2 Assay

Venom phospholipase A_2 (EC 3.1.1.4) activity was assessed by measuring free fatty acid release from a phospholipid substrate (phosphatidylcholine, egg yolk type) using Cresol red as a pH indicator (deAraujo & Radvanyi, 1987), as described in Chapter 2.

3.2.4 Nerve-Diaphragm Assay

Mouse left phrenic nerve-hemidiaphragm preparations were isolated, set up and run as described in Chapter 2.

3.2.5 Electron Microscopy

Following a period of stimulation of 3.5 hr in the above assay, control and venom treated (20mg/L) diaphragm preparations were fixed and mounted using standard methods for transmission electron microscopy. Tissue was bathed in formaldehyde overnight and a series of increasing ethanol concentrations used to totally dehydrate the sample, followed by staining with osmium tetroxide, baking in resin and post sectioning staining with uranium. Numerous sections were made from each preparation and photographs taken which were representative of the entire tissue.

3.2.6 *In vivo* Toxicity

Mouse LD_{50} was assessed by intravenous (i.v.) injection of the venom as described in Chapter 2 and calculated from the number of deaths within 24 hr.

3.2.7 Antisera Production

Antisera were raised by immunising half-breed Welsh ewes every 28 days (see Table 3.1) with *A. m. mellifera* venom dissolved in phosphate buffered saline as described in Chapter 2.

3.2.8 Sodium Sulphate Precipitation

Serum (at room temperature) was pooled, filtered through a glass wool plug and mixed with an equal volume of sodium sulphate (36% in water at 37°C) for 15 min at room temperature to precipitate the immunoglobulins. The mixture was then

		IMMUNOGEN DOSE (mg) PER SHEEP		
IMMUNISATION / SAMPLE / BLEED	**WEEKS POST PRIMARY IMMUNISATION**	**LOW DOSE GROUP**	**MEDIUM DOSE GROUP**	**HIGH DOSE GROUP**
Primary Immunisation	0	0.25	0.5	0.5
Re-Immunisation	4	0.25	1.0	1.0
Sample	6			
Re-Immunisation	8	0.25	1.0	2.0
Sample	10			
Re-Immunisation	12	0.25	1.0	4.0
Sample	14			
Re-Immunisation	16	0.25	1.0	4.0
Sample	18			
Re-Immunisation	20	0.25	1.0	4.0
Sample	22			
Re-Immunisation	24	0.25	1.0	4.0
Sample	26			
Re-Immunisation	28	0.25	1.0	4.0
Bleed + Aliquot	30			
Re-Immunisation	32	0.25	1.0	4.0
Bleed + Aliquot	34			
Re-Immunisation	36	0.25	1.0	4.0
Bleed + Aliquot	38			
Re-Immunisation	40	0.25	1.0	4.0

Table 3.1 *Apis m.mellifera* venom immunisation schedule.

centrifuged at 2,750g for 1 hr at ~ 25°C, the supernatant containing the albumin and other small molecular weight serum proteins discarded and the precipitate washed by resuspending with 18% sodium sulphate and re-centrifuging. This washing was repeated and the final precipitate was redissolved in saline to give a final volume approximately equivalent to that of the original serum. The resultant solution was dialysed against at least 20 volumes of saline at 4°C for 48hr, the volume of the dialysed material recorded and any remaining precipitate removed by centrifugation or filtration. Optical density (280nm) was measured to determine the IgG concentration using an extinction coefficient of 1.5 (1g/L solution, path length 1cm).

3.2.9 Papain Digestion

An established papain digestion method was used (Smith *et al.,* 1992) in which IgG (typically between 15-40g/L) was adjusted to pH 7.0 using either hydrochloric acid or sodium hydroxide and 0.8mg /ml EDTA added, mixed, and allowed to equilibrate in a water bath at 37°C for 30 min. Papain (16% of the total IgG weight, Merck 30,000 grade) was added, mixed gently and, once dissolved, activated by the addition of L-cysteine HCl (1.6mg/mL) with gentle mixing for 1 hr at 37°C. After standing at this temperature for a further 23 hr, the reaction was stopped by the addition of iodoacetamide (2.8mg/mL) and mixed for 15 min at 37°C. The digest was then dialysed against at least 20 volumes of saline at 4°C for 48hr, and centrifuged at 2,750g for 1 hr to pellet the precipitate. The Fab supernatant was decanted off, filtered and the concentration determined by optical density at 280nm using an extinction coefficient of 1.4 for ovine Fab. Final Fab concentrations were typically between 20-30 g/L. The Fab was then stored at 4°C or at -20°C for long term storage.

3.2.10 Enzyme Linked Immunosorbant Assay (ELISA)

Serum antibody titres directed specifically against purified melittin or whole venom were determined by ELISA according to the method of Sjostrom *et al.,* (1996) (see Chapter 2). Titres were determined at 50% of maximum absorbance after correction for the background NSS absorbance (Figure 3.1) and values corrected for between assay variation using the reference antiserum.

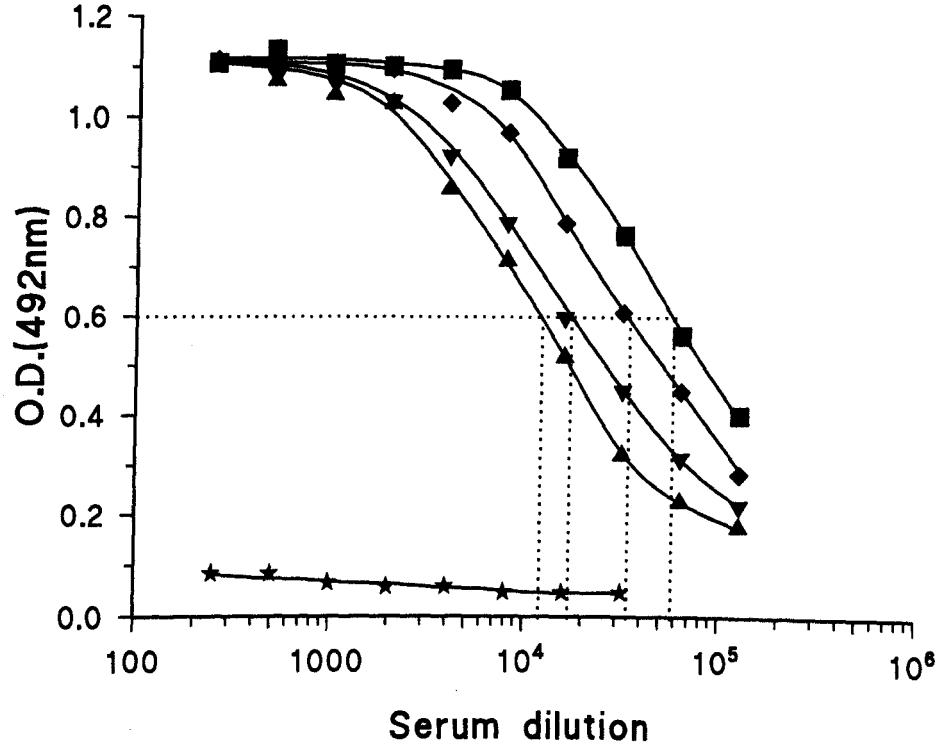


Figure 3.1 Melittin specific antiserum dilution curves by ELISA. Antiserum samples were: standard (◆), week 38 high (■), medium (▲) and low (▼) immunisation dose groups. NSS (★).

3.2.11 Small Scale Affinity Purification

Venom or melittin were covalently conjugated to cyanogen-bromide activated Sepharose 4B gel according to the manufacturer's instructions. Briefly 1g of CNBr-activated Sepharose 4B was re-swollen on a sintered glass filter for 15 min and washed with 1mM HCl, followed by 5ml of coupling buffer (0.1M sodium hydrogen carbonate, 0.5M sodium chloride, pH 8.3). The washed gel was quickly placed in a chromatography column containing 5mg of the protein to be coupled, dissolved in 6mL of coupling buffer, and mixed end over end at 4°C overnight. Unbound material was collected and its OD (280nm) used to assess the amount bound by difference. Any remaining active groups were blocked with 6ml of blocking reagent (1M ethanolamine, pH 8.0) and mixed overnight at 4°C. The blocking agent was drained off and the column washed with 12mL coupling buffer followed by 12mL acetate buffer (0.1M sodium acetate, 0.5M sodium chloride, pH4.0) followed by coupling buffer and the wash step repeated once more.

Loading and eluting conditions were optimised and conditions providing the highest levels of specific antibody and the lowest levels of nonspecific binding were chosen. Non-specific binding was found to be reduced by the inclusion of 2% acetonitrile in the wash buffer without affecting specific binding. Antiserum samples (500μL) were diluted in 0.9% saline / 2% acetonitrile (6mL), mixed end-over-end overnight with the gel at 4°C, washed (saline / 2% acetonitrile), and the columns eluted with 100mM glycine, pH 2.5. Specific antibody concentrations were determined based on their optical density at 280nm, using an extinction coefficient of 1.5 for ovine IgG. Non-specific binding was determined by running NSS and levels of specific antibody were corrected by subtracting the value due to non-specific binding.

3.2.12 Venom Phospholipase A_2 Neutralisation.

Venom (10mg/L) was premixed with antivenom for 30 min at 37°C prior to substrate addition and the optical density at 573nm measured after 10 min.

3.2.13 Neutralisation of *In Vivo* Toxicity.

Mouse ED_{50} was assessed by intravenous (i.v.) injection as described in chapter 2 using a Fab-based antivenom prepared from a pool of antisera raised using the high immunisation dose against five times the LD_{50}.

3.3 Results

3.3.1 Venom Analysis

A conventional SDS-PAGE analysis (Figure 3.2) clearly separated the phospholipase A_2 (~19,000Da) and melittin (2,847Da) components of the venom, but could not separate the melittin from the other low molecular weight components such as mast cell degranulating peptide (2,593Da) and apamin (2,027Da).

Using an acid urea gel system which separates proteins by a combination of their native charge at low pH and size, the venom was clearly separated into its individual components and these were identified by running the purified major components: melittin, phospholipase A_2, mast cell degranulating peptide (at the electrophoresis front) and apamin (Figure 3.3). No differences between the European or Africanised bee venoms could be detected.

3.3.2 PLA_2 Activity

A. m. mellifera venom concentrations of 10mg/L produced a rapid decrease in OD representing ~ 90 % hydrolysis of the phosphatidylcholine substrate after 10 min compared to a slower rate of hydrolysis produced by 7 mg/L (Figure 3.4). Minimal hydrolysis was produced by 0.7mg/L and no enzymatic activity could be detected in 10mg/L of melittin, indicating that there was no major enzymatic contamination of this material. A snake (*Crotalus scutulatus scutulatus*) venom known to have high levels of PLA_2 activity was run as a comparison and shown to have slightly less activity than the bee venom.

3.3.3 Toxicity as Shown Using a Nerve-Diaphragm Assay

After their addition to the phrenic nerve diaphragm preparation an initial tonic contraction was produced both by the whole venom and by the purified melittin, followed by a progressive decrease in muscle contractions.

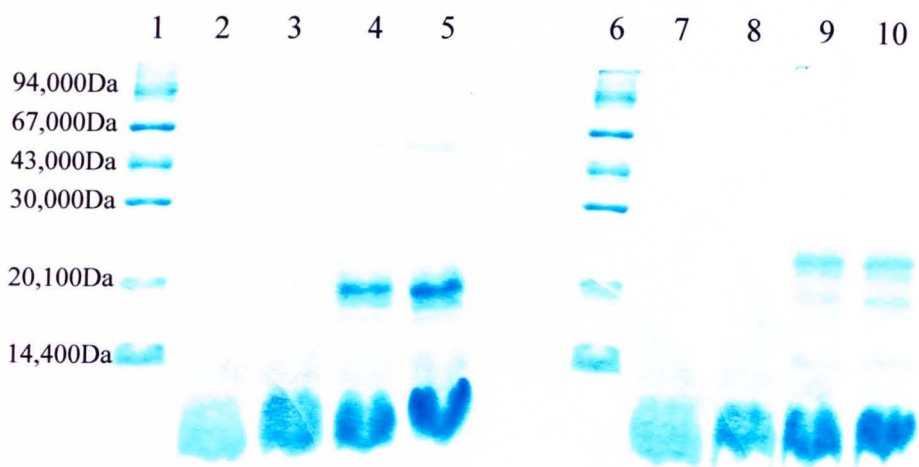


Figure 3.2 SDS-PAGE (15%) analysis of *A. m. mellifera* venom and melittin. Molecular weight markers (1, 6), 28μg melittin-reduced (2, 3), 14μg venom-reduced (4, 5), 28μg melittin non-reduced (7, 8), 14μg venom non-reduced (9, 10).

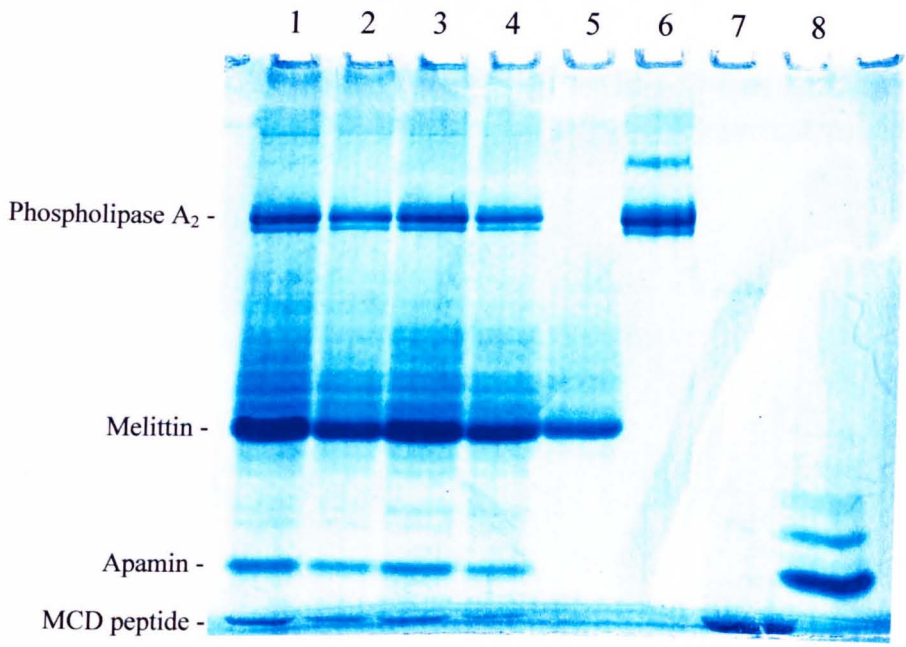


Figure 3.3 Venom composition by acid-urea polyacrylamide gel electrophoresis. Whole bee venom (30μg or 15μg) from either European *A. m. mellifera* (lanes 1 and 2 respectively) or Africanised bees (lanes 3 and 4) and 5μg of each of the purified components; melittin (lane 5), phospholipase A_2 (lane 6), mast cell degranulating (MCD) peptide (lane 7) and apamin (lane 8).

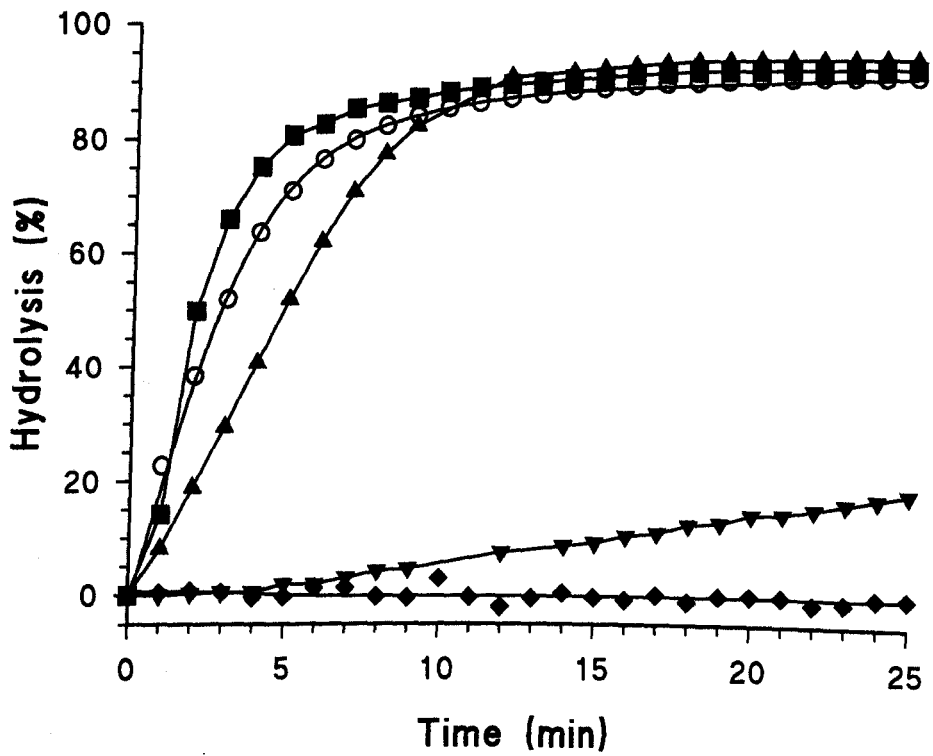


Figure 3.4 Phospholipase A_2 activity measured by the hydrolysis of phosphatidylcholine. *A. m. mellifera* venom 10 (■), 7 (▲), and 0.7 (▼) mg/L. Melittin 10mg/L (◆). *Crotalus scutulatus scutulatus* venom 10mg/L (O).

3.3.3.1 Venom

The decrease in muscle contraction showed a similar time course whether the preparation was stimulated indirectly via the nerve or directly via the muscle with a range of venom concentrations (2-20 mg/L, see Figure 3.5 and 3.6). This indicates a direct myotoxic effect and no specific neurotoxic activity could be demonstrated. The progression of the toxicity appeared unaffected by two washes of the tissue with Krebs buffer and, using a venom concentration of 20mg/L, muscle contractions had virtually stopped before the end of the experiment.

3.3.3.2 Melittin

Melittin also had an inhibitory effect on muscle contractions that followed a similar time course whether stimulated via the nerve or muscle, indicating a direct myotoxic effect (Figure 3.7). A 5mg/L concentration of melittin only produced up to 65.6% ±2.9 (SEM, n=4) inhibition of direct stimulation compared to 91.4% ±3.1 (SEM, n=4) by an equal concentration of whole venom.

3.3.4 Electron Microscopy

Electron microscopy of muscle fibres from a control diaphragm showed a normal characteristic banding pattern (Figure 3.8a), due to the presence of parallel myofibrils interspersed with mitochondria, sarcoplasmic reticulum and triads, indicating that the tissue was unaffected by the procedure. By contrast, muscle fibres from the venom treated (20mg/L) diaphragm showed complete disorganisation of the normal architecture with clumping of myofibrils and disruption of the sarcoplasmic reticulum and triads (Figure 3.8b). These features were found across all the tissue sections analysed. This venom concentration also produced a parallel loss of functional activity of the tissue.

3.3.5 *In vivo* Toxicity

The *A. m. mellifera* venom had an LD_{50} of 3.1 mg/kg (59.7µg/mouse, 95% confidence limits from probit analysis 53.2 - 81.6) and mice receiving high venom concentrations often suffered immediate convulsions leading to death within minutes.

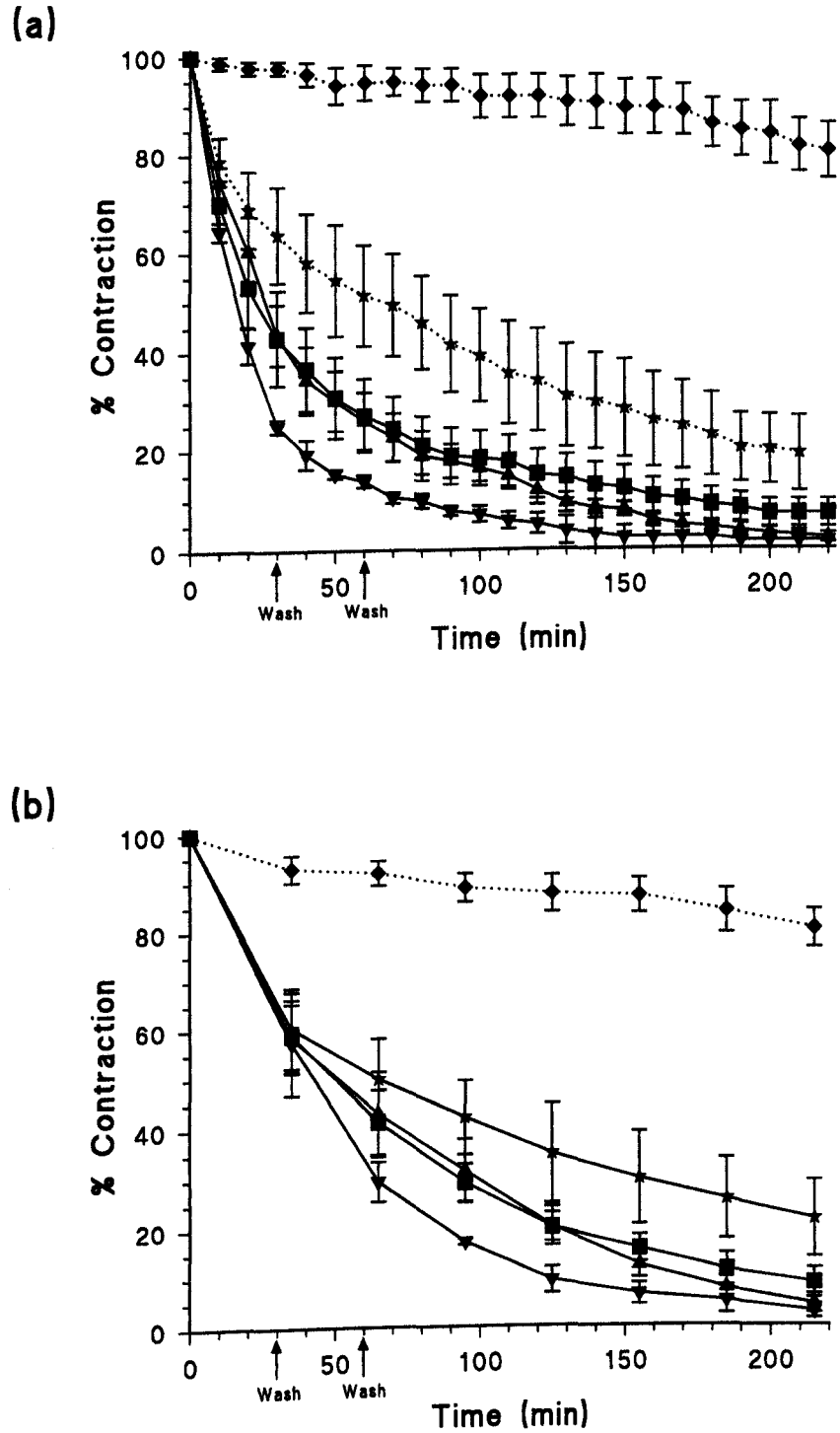


Figure 3.5 Effects of *A. mellifera* venom on the phrenic nerve diaphragm preparation. Nerve (a), and direct (b) stimulation, using venom concentrations of 0 (◆), 2 (★), 5 (■), 10 (▲), and 20 mg/L (▼), ± SEM (n=3 or 4).

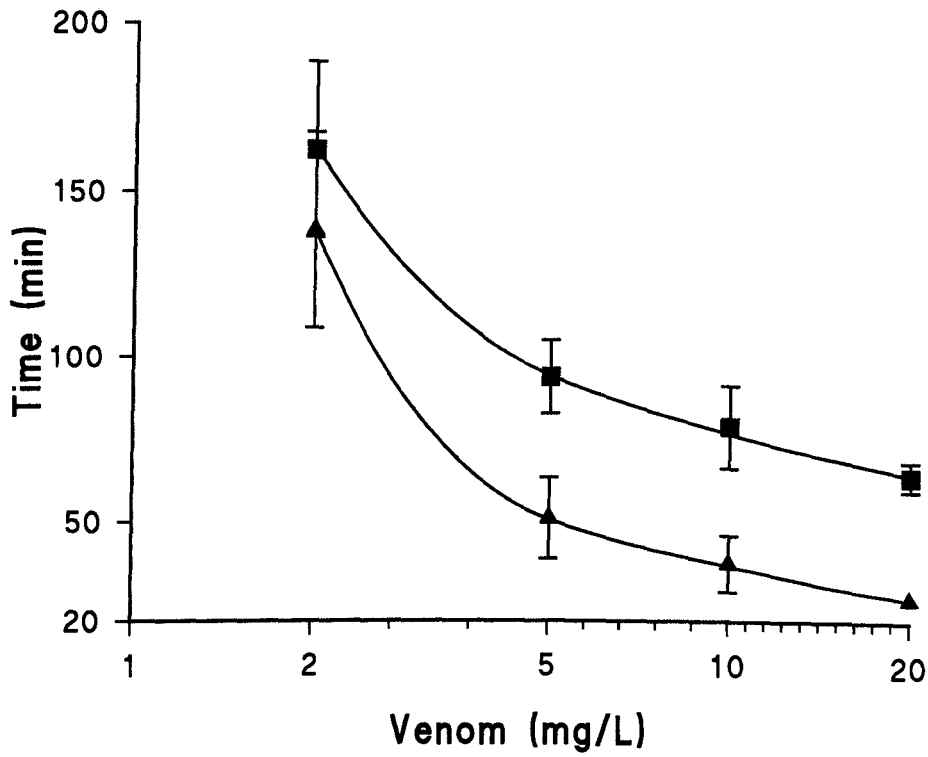
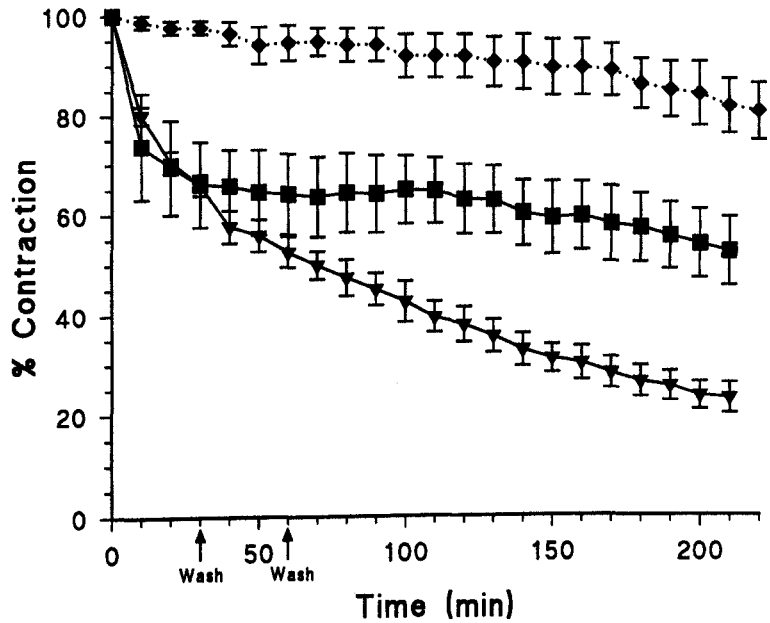


Figure 3.6 Time taken for *A. mellifera* venom to cause 70% inhibition of nerve muscle contractions. Direct (■), and nerve (▲) stimulation, ± SEM (n=4-7).

(a)



(b)

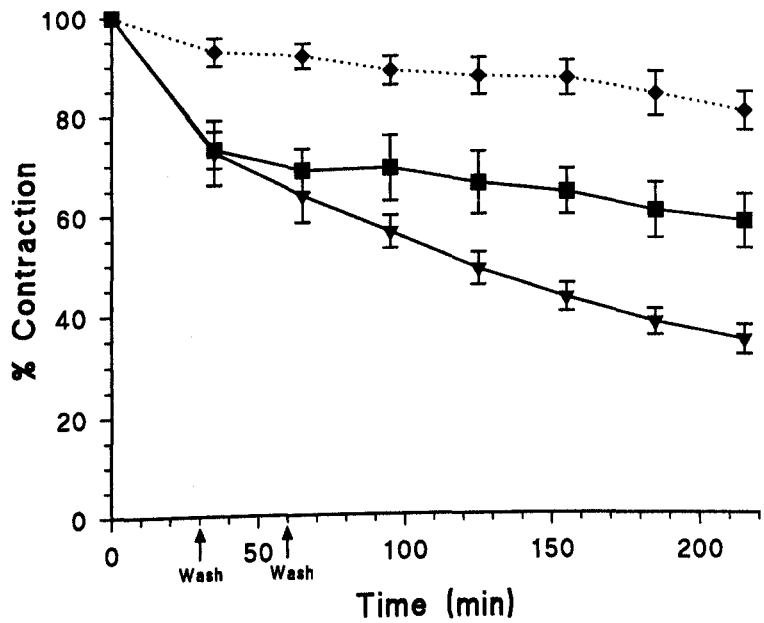
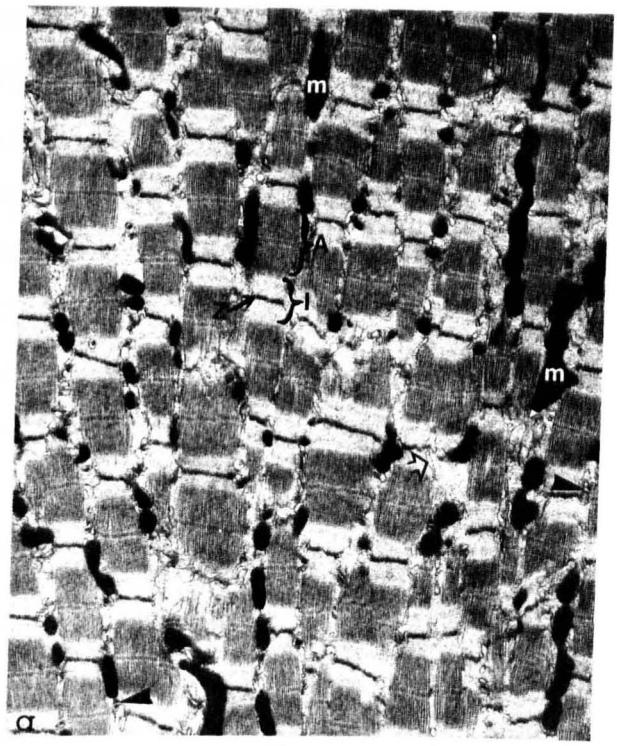


Figure 3.7 Effects of melittin on the phrenic nerve diaphragm preparation. Nerve (a), and direct (b) stimulation, using melittin concentrations of 0 (◆), 3 (■) and 5mg/L (▼), ± SEM (n=4).

(a)



(b)

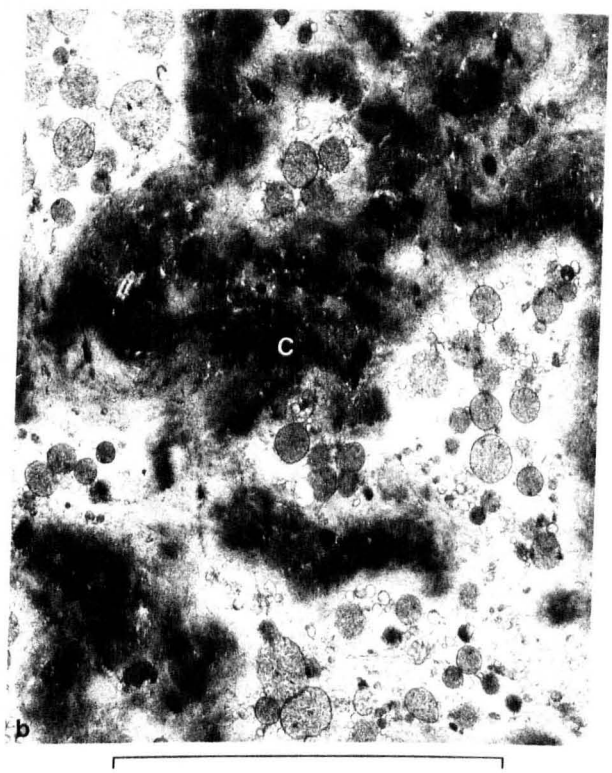


Figure 3.8 Electron micrograph of control (a) and venom treated (b) diaphragm. The former shows parallel myofibrils and characteristic banding patterns (labelled A, I, and Z) interspersed with mitochondria (m), sarcoplasmic reticulum (empty arrow) and triads (arrowheads). Venom treatment resulted in areas of complete disruption with clumped myofibrils (C). Scale bar = 6.6μm.

3.3.6 Specific Antibody Levels

3.3.6.1 ELISA

ELISA titres of up to 1:300,000 and 1:75,000 were produced against whole venom and melittin respectively, when immunising with the largest dose schedule (viz up to 4mg/sheep every 28 days). The highest dose consistently produced higher antibody titres than the medium and low dose groups (Figure 3.9). Antibody titres appeared directly related to the dose of immunogen except for those against melittin, when the medium dose group displayed similar or lower titres than the low dose group.

3.3.6.2 Small scale affinity chromatography

A typical elution profile from an *A. m. mellifera* venom small scale affinity chromatography column is shown in Figure 3.10, with most of the material eluted in the 4th and 5th fractions. Consistent with the ELISA titres, the high immunisation dose produced the highest levels of specific antibodies with up to 8.8 and 4.5 g/L (after subtracting the non-specific binding) against whole venom and melittin respectively (Figure 3.11). Conversely the lowest specific antibody levels were produced using the lowest immunisation dose schedule (viz up to 0.25mg/sheep every 28days). A slow progressive increase in the antibody response to melittin was noted without any sign of reaching a plateau after more than a year.

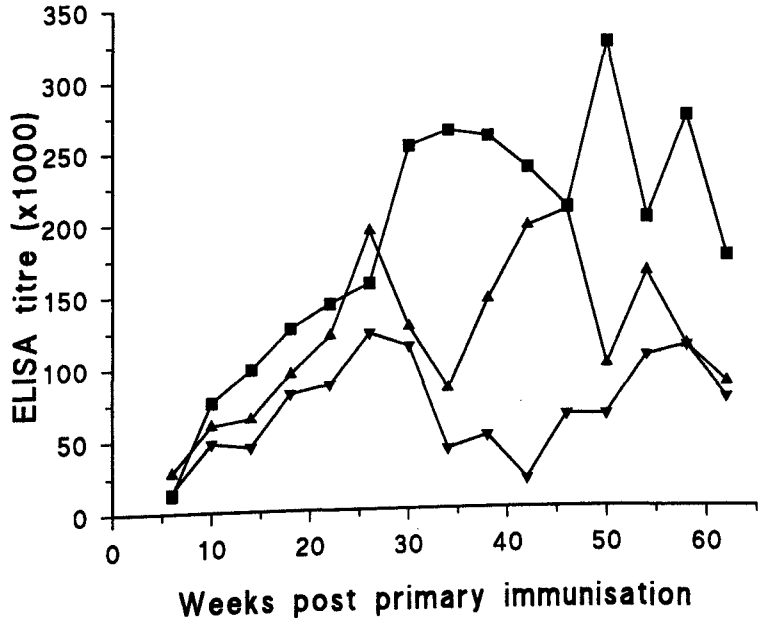
3.3.7 SDS-PAGE Analysis of IgG and Fab

The fractionated IgG showed just one major band and contained only minor traces of albumin (Figure 3.12). Fab prepared using conventional papain digestion had no visible intact IgG and principally consisted of a band of ~46,000Da.

3.3.8 Neutralisation of Venom Phospholipase A_2 Activity

Neutralisation of phospholipase A_2 activity was most pronounced using the high and medium immunisation dose group antivenoms, with 400mg/L of these IgG or Fab preparations able to fully neutralise 10mg/L of venom phospholipase A_2 activity (Figure 3.13). From this data (50% neutralisation) it was calculated that ~14mg of IgG or 16mg of Fab antivenom would be required to neutralise the PLA_2 activity of 1mg of venom.

(a)



(b)

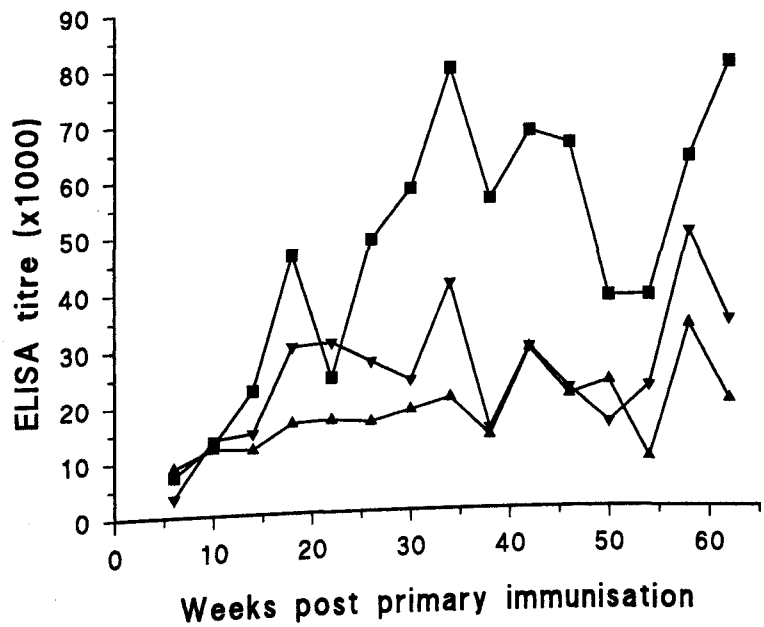


Figure 3.9 Specific antibody levels determined by ELISA, directed against *A.m.mellifera* venom (a) and against melittin (b). High (■), medium (▲) and low (▼) immunisation dose groups.

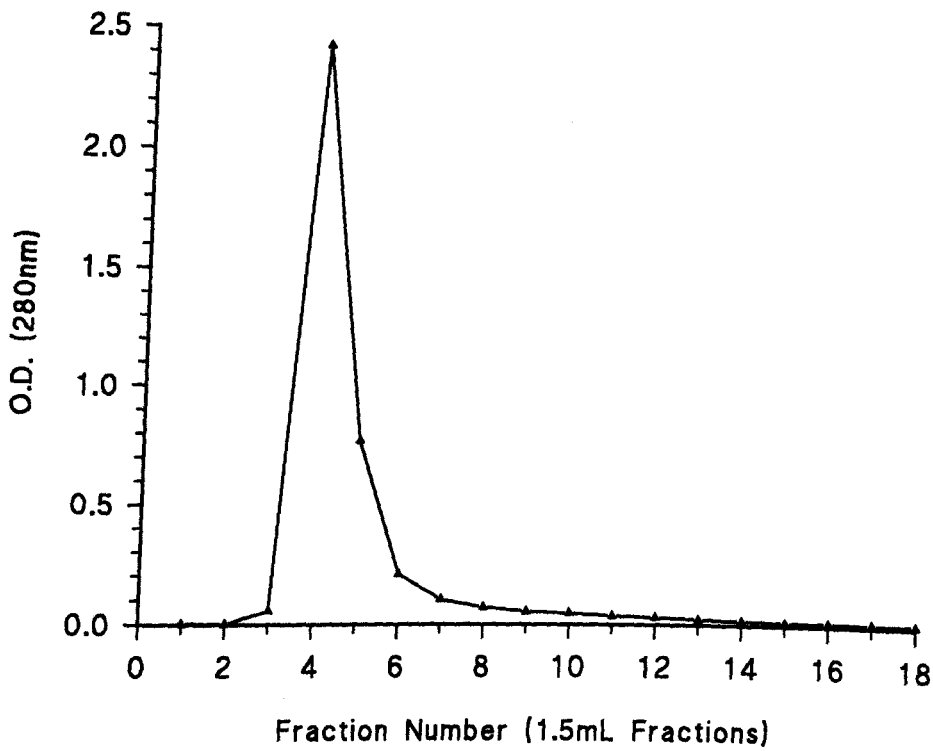
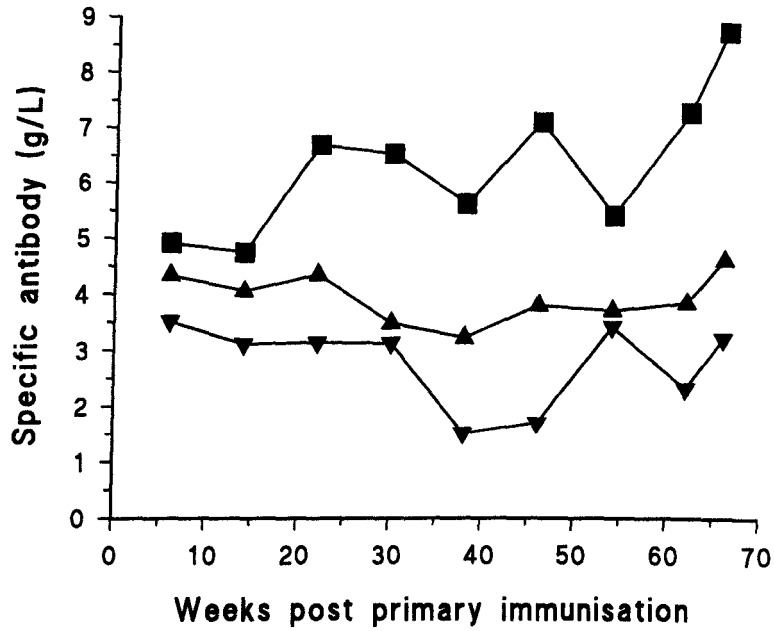


Figure 3.10 Typical *A. mellifera* affinity chromatography elution profile

(a)



(b)

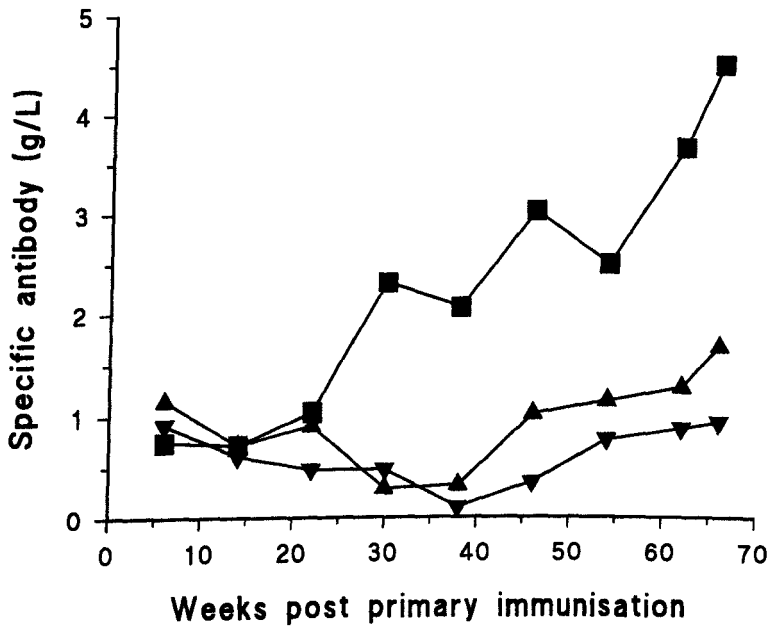


Figure 3.11 Specific antibody levels, determined by small scale affinity chromatography, directed against whole *A. m. mellifera* venom (a) and against melittin (b). High (■), medium (▲) and low (▼) immunisation dose groups.

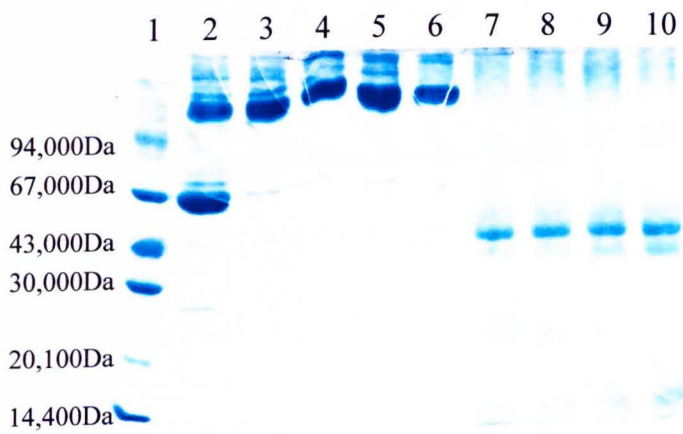


Figure 3.12 SDS-PAGE (10%) analysis of IgG and Fab purities. Molecular weight markers (1), normal sheep serum (2), week 30 low dose IgG (3) or Fab (7), week 30 medium dose IgG (4) or Fab (8), week 30 high dose IgG (5) or Fab (9), normal sheep serum IgG (6) or Fab (10).

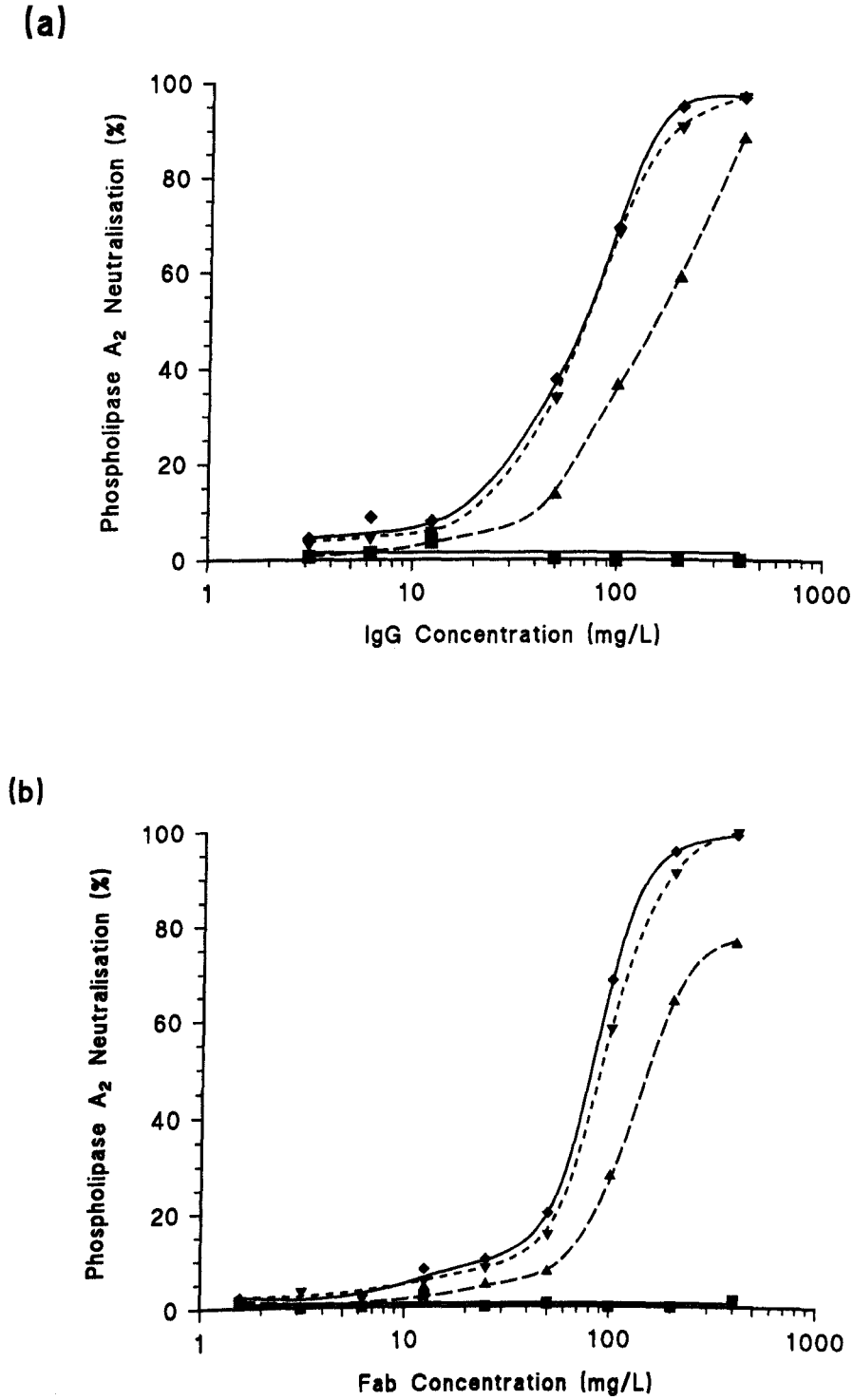


Figure 3.13 Neutralisation of *A. mellifera* venom phospholipase A_2 activity using anti-*A. mellifera* IgG (a) or Fab (b), prepared from serum produced by the high (◆), medium (▼) and low (▲) immunisation dose groups or normal sheep serum (■).

3.3.9 Venom Toxicity Neutralisation as Shown Using a Nerve-Diaphragm Assay

Increasing total Fab antivenom concentrations had a progressive neutralising effect but even a high concentration (800mg/L) could not completely neutralise the venom's toxicity (Figure 3.14).

3.3.10 Melittin Toxicity Neutralisation as Shown Using a Nerve Diaphragm Assay

Increasing total Fab antivenom concentrations had a progressive neutralising effect but even a high concentration (1600mg/L) could not completely neutralise melittin's toxicity (Figure 3.15). The tonic contraction was, however, fully neutralised with these high antivenom concentrations (Figure 3.16).

3.3.11 *In vivo* Toxicity Neutralisation

The ED_{50} for the antivenom preparation (total Fab) was 258 mg/kg (4.9mg/mouse, 95% confidence limits from probit analysis = 4.3 - 5.3) against five times the LD_{50}. From this data it can be calculated that ~20.5mg of antivenom is required to neutralise each mg of venom.

3.4 Discussion

Bee venom was chosen as a model because it is easily collected in large quantities and is, therefore, widely available at a low cost. Also, the frequency of mass bee attacks has dramatically increased in the Americas following the introduction and spread of the aggressive Africanised 'killer' bee. As yet no specific therapy is available for these toxic effects (Franca *et al.*, 1994; Schumacher *et al.*, 1996), which led us to develop an ovine antisera which could be refined to produce a potential new antivenom treatment.

Venom from European and Africanised bees appeared identical when resolved by electrophoresis systems utilising differences in both the charge and size of the components. Based on this finding and their similar toxic properties and cross reactivity (Schumacher *et al.*, 1990; 1996; 1989; Nelson *et al.*, 1990), it is probable that an antivenom raised against the European bee venom will be equally effective at neutralising the venom of the Africanised bee.

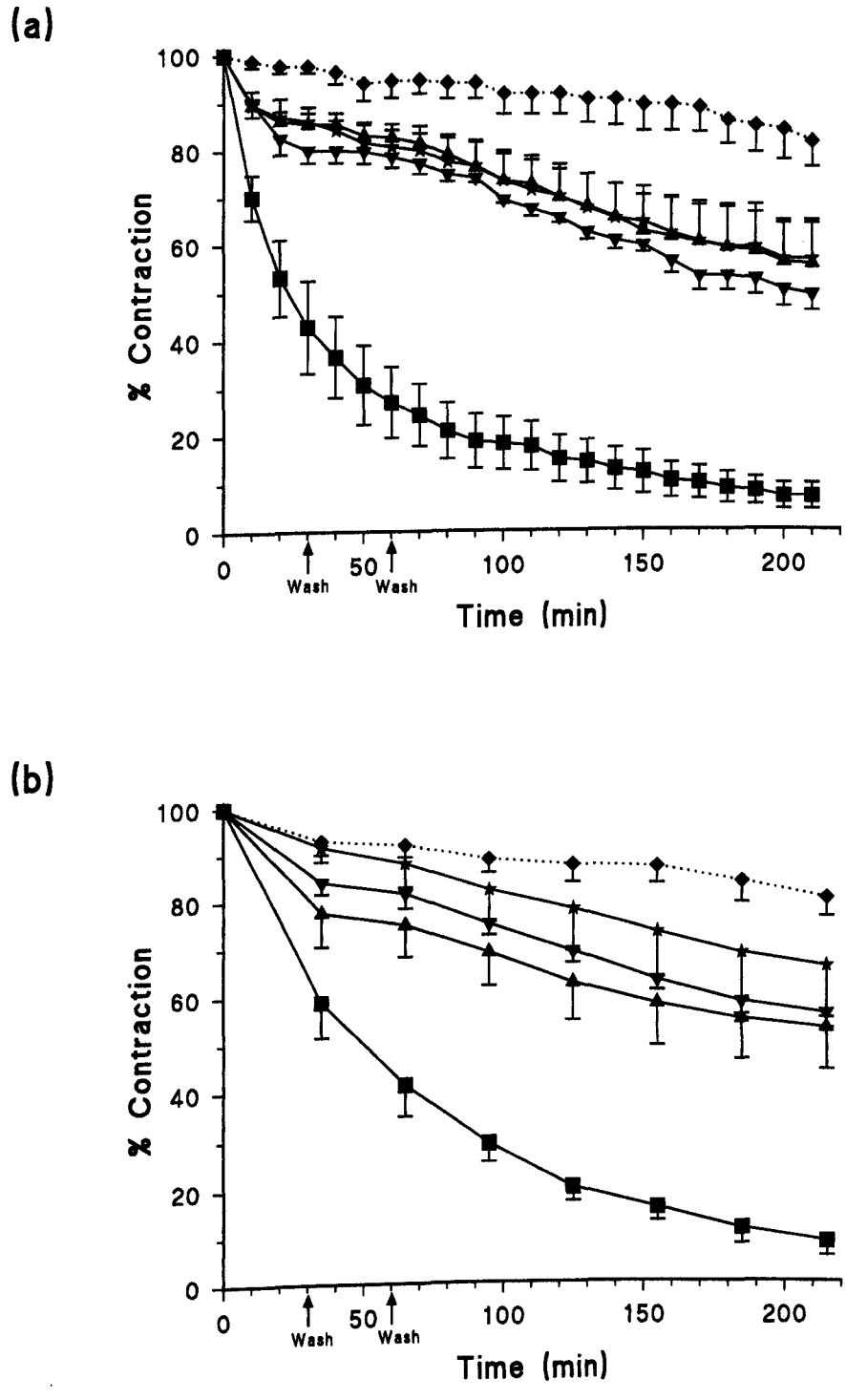


Figure 3.14 Neutralisation of venom toxicity as evidenced using nerve (a) or directly (b) stimulated diaphragm preparations, bathed in 5mg/L venom only (■), or plus 200mg/L (▲), 400mg/L (▼) and 800mg/L (★) of Fab antivenom. Control (◆). ±SEM (n=4).

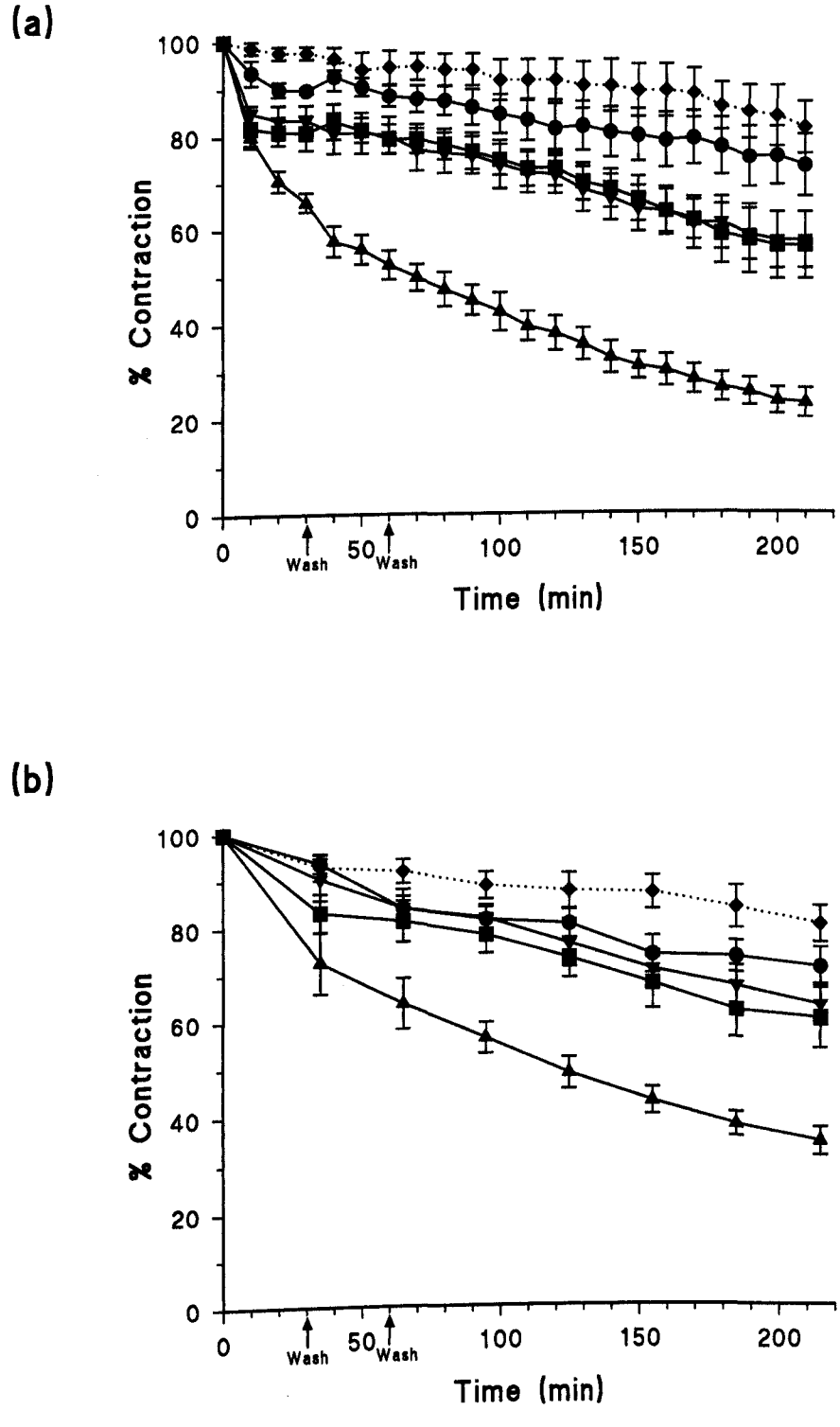


Figure 3.15 Neutralisation of melittin toxicity as evidenced using nerve (a) or directly (b) stimulated diaphragm preparations, bathed in 5mg/L melittin only (▲), or plus 200mg/L (▼), 800mg/L (■), 1600mg/L (●) of Fab antivenom. Control (◆). ±SEM (n=3-4).

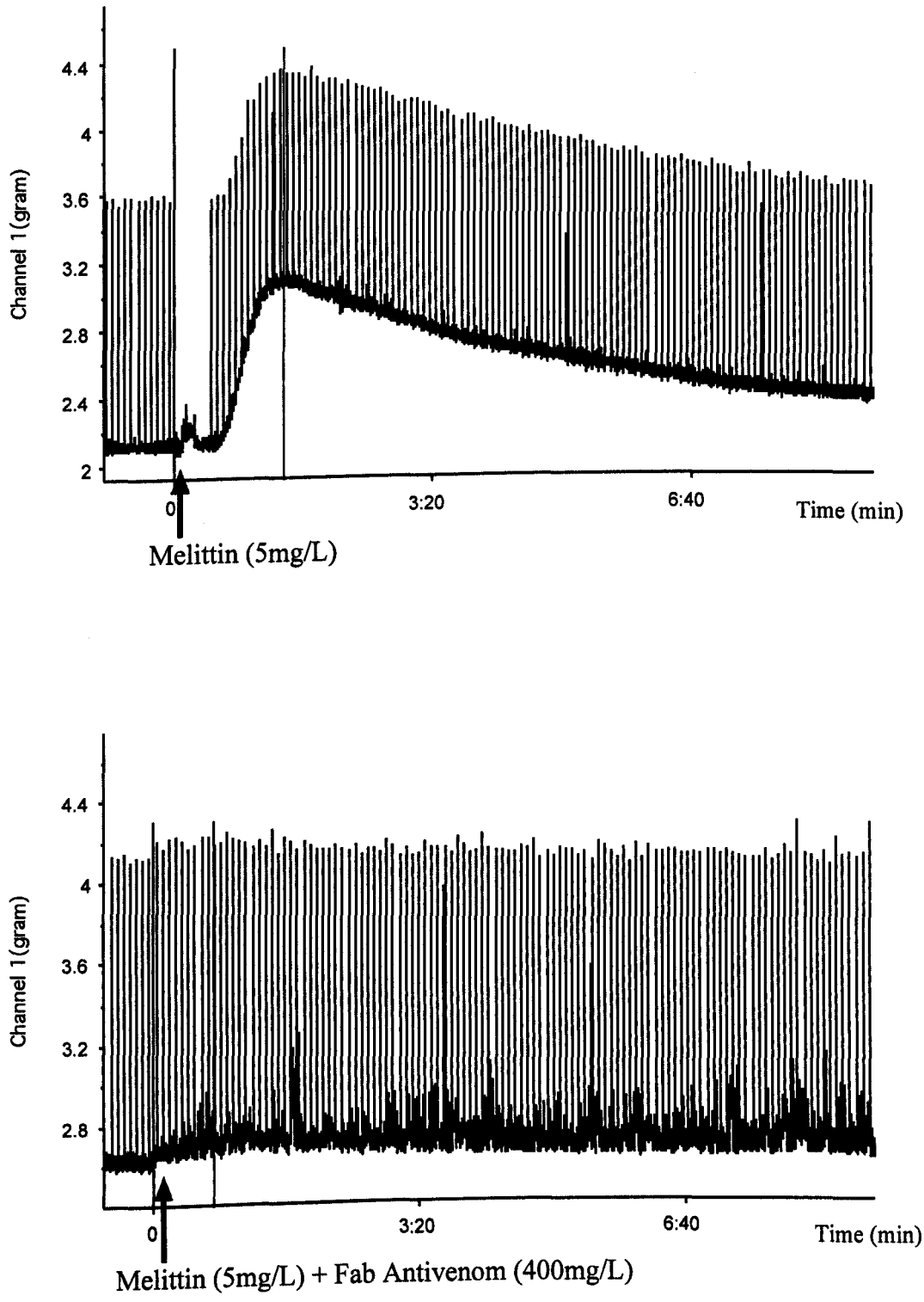


Figure 3.16 The tonic contraction induced by melittin and its neutralisation.

The LD_{50} of *A. m. mellifera* venom in this study was similar to that previously reported for both European and Africanised bee venoms (Schumacher *et al.*, 1989; 1990; 1996). The dramatic manifestations and rapid lethal effects produced by high venom concentrations in mice are probably due to apamin which acts on the central nervous system (Habermann, 1972; 1984; Vincent *et al.*, 1975; Spoerri *et al.*, 1975; van-Rietschoten *et al.*, 1975).

A powerful PLA_2 activity was found in the venom with a potency similar to that found in snake venoms.

The effects of the venom on the phrenic nerve diaphragm preparation were of a predominantly myotoxic type, as evidenced by both ultrastructural and functional changes. It produced an immediate tonic contraction, similar to the cardiotoxins (Lin *et al.*, 1975), which appears to be produced by the melittin. Although melittin produced a myotoxic effect on this isolated tissue preparation, an equal concentration of whole venom was significantly more toxic, suggesting a synergistic interaction between melittin and PLA_2.

In our laboratory total IgG levels of 28g/L were found in pooled antisera from sheep and others have reported levels in normal sheep serum greater than 26g/L (Strain *et al.*, 1984). These high levels of IgG may explain why sheep often produce relatively high levels of specific antibodies (e.g. 7 - 15g/L) to an immunogen (Smith *et al.*, 1992). The size of sheep also helps ensure their suitability for scaling up to produce antivenoms commercially, as each sheep will yield ~ 4L of antiserum per year (Meyer *et al.*, 1997; Karlson-Stiber *et al.*, 1997). Although horses are much larger and will produce ~ 8 times this volume, they are considerably more expensive to buy and maintain. Sheep were, therefore, used as the species of choice in this study. Levels of venom-specific antibodies were found to be consistently greater using the highest immunogen dose (4mg/sheep).

The present results indicate that early in the immunisation schedule most of the specific antibodies were directed against venom components other than melittin. Phospholipase A_2 is one of the most immunogenic components of bee venom and may contribute to the generalised toxicity of envenomation by a synergistic interaction with melittin (Schumacher & Egen, 1995; Vogt *et al.*, 1970; Mollay & Kreil, 1973; Ownby *et al.*, 1997; Schumacher *et al.*, 1996; King *et al.*, 1976). The antivenoms

raised by immunising with 1 or 4mg of venom fully neutralised this important enzymatic activity. Melittin is also important, with its insertion into cell membranes being thought to cause disruption of the surrounding phospholipid bilayer which allows phospholipase A_2 to gain access to its phospholipid substrate (Banks & Shipolini, 1986). However, melittin is said to be poorly immunogenic probably because of its small size (26 amino acids with a molecular weight of 2,800 Da) and strongly hydrophobic regions (Schumacher & Egen, 1995; Schumacher *et al.*, 1996; King *et al.*, 1976). It forms the major venom component and, fortunately, up to 4.5 g / L of specific antibodies to this peptide could be generated in the high dose group of sheep provided the period of immunisation was sufficiently prolonged. This led to the selection of the 4mg per sheep dose as the optimum immunisation schedule.

A conventional procedure for producing Fab-based antivenoms using a sodium sulphate precipitation step followed by papain digestion was performed and the resultant preparations tested. Similar proportions of PLA_2 neutralisation were found using either IgG or Fab preparations derived using the various immunisation schedules, although the IgG appeared to be slightly more active on a weight for weight basis than the Fab, indicating a loss of some of the activity as a result of the digestion procedure.

Antivenom was capable of neutralising most of the toxicity on the phrenic nerve diaphragm preparation produced by venom or melittin, as well as the initial tonic contraction. However, it was incapable of producing 100% neutralisation at the doses tested, which may be due to the large proportion of melittin found in the venom, together with its low molecular weight and, therefore, high molar concentration (5mg/L = 1.76μM).

The antivenom was capable of neutralising the venom *in vivo* when assessed using the standard mouse ED_{50} test and it was calculated that ~20.5mg of antivenom was required to neutralise each mg of venom. The ED_{50} results indicate, indirectly, that the antivenom is also effective against apamin.

The vast majority of bee sting reactions involve just a single sting and are severe only in the 0.15 - 4.0% of people with true systemic hypersensitivity to hymenoptera venoms (Schmidt, 1986). These 'allergic' type individuals are at a high risk of developing life threatening IgE-mediated or anaphylactoid reactions, which must be

treated conventionally (e.g. antihistamines, steroids, alpha/beta agonists, bronchodilators and adrenaline) (Ewan, 1998). In the US some 40 deaths per year result from such a reaction which usually occurs within 1hr (Schmidt, 1986). Such subjects will not benefit from the availability of an antivenom whose use will be limited to those stung by 50 or more bees and who often develop features of systemic toxicity many hours latter, with the majority of deaths after 22 hr or more (Franca *et al.*, 1994; Kolecki, 1999). This suggests that the use of antivenom may still be beneficial even if delayed for several hours. This is supported by the benefits that may arise by receiving antivenom several days after snake envenoming (Meyer *et al.*, 1997; Warrell, 1995).

Clinical studies are now essential to assess the effectiveness of an antivenom produced from this material to neutralise the high systemic levels of venom found after mass bee attacks and, thereby, alleviate the clinical manifestations and improve the prognosis. Unlike with snake bites where the amount of venom injected may vary greatly (e.g. 0-1.5g) and is unknown (Bdolah, 1979), the ability to assess the amount of bee venom injected after a mass attack by counting the number of stings should, theoretically, be of help in evaluating whether (a) antivenom is required (e.g. >50 stings), and (b) the quantity of antivenom required. Again clinical trials will be necessary to assess this.

In conclusion, this study has demonstrated that a specific *A. mellifera* antivenom can be raised in sheep that will neutralise the toxic components of the venom, and protect mice. Large quantities of antisera were successfully produced for use in the development of a new improved antivenom production process, as described in the next chapter.

CHAPTER 4:

A SIMPLE NEW PROCESS FOR PURIFYING ANTIBODY FRAGMENTS ($F(ab')_2$) IN HIGH YIELD FROM SERUM.

4.1 Introduction

For more than 50 years proteolytic enzymes have been used in the refinement of antitoxins, and were a key factor in the subsequent elucidation of immunoglobulin structure (Imray, 1902; Parfentjev, 1936; 1938; Pope, 1939; Porter, 1959). Today, enzyme cleaved antibodies are widely used throughout the world for the treatment of, for example, victims of snake envenoming.

The commercial production of polyclonal antibody fragments for therapeutic use often involves numerous steps such as ammonium or sodium sulphate precipitation, enzyme digestion, diafiltration, thermocoagulation and, more recently, ion-exchange and / or affinity chromatography (Harms, 1948; Schultze *et al.*, 1965; Kirkpatrick, 1991; Grandgeorge *et al.*, 1996; Karlson-Stiber *et al.*, 1997). Such procedures can result in an effective and safe, but expensive product (Kirkpatrick, 1991; Karlson-Stiber *et al.*, 1997; Theakston & Warrell, 2000). The latter is unfortunate, especially with regard to antivenoms, for which the greatest demands are from the poorest regions of the world. Thus, in many regions antivenoms are either not available or are relatively impure and cause a high (10-76%) incidence of side effects (Karlson-Stiber & Persson, 1994; Wilde *et al.*, 1996; Meyer *et al.*, 1997; Moran *et al.*, 1998; Theakston & Warrell, 2000). These side effects are generally attributed to contaminants including serum proteins, Fc or aggregates (Porter, 1957; Hristova, 1968; 1970; Waldesbuhl *et al.*, 1970; Sutherland, 1977; Malasit *et al.*, 1986).

The aspartic proteinase pepsin, purified from the gastric mucosa of pigs, is used extensively by commercial producers of antibody-based therapeutic products. Its use has to be optimised since, for example, it will hydrolyse rabbit IgG at pH 2.5 to small peptides without any binding activity while, at a pH of 4.5-5.0, the IgG is digested to $F(ab')_2$ and small fragments of Fc (Nisonoff *et al.*, 1960; Haber & Stone, 1967). Davies and his colleagues (1978) found that the production of $F(ab')_2$ by digesting

sheep IgG at pH 4.7 required a high pepsin concentration, was slow and left some residual intact IgG even after 48 h at 37°C.

The plant enzyme papain has been used to produce some recent antitoxins. Papain cleaves an antibody molecule at ~ neutral pH to give two molecules of Fab and one of Fc, each with a similar molecular weight. Fc contaminants then have to be removed using further steps such as ion-exchange chromatography (Meyer *et al.,* 1997; Karlson-Stiber *et al.,* 1997; Ariaratnam *et al.,* 1999). Unfortunately such ion-exchange methods are limited by the wide isoelectric range of polyclonal antibody fragments, with a resultant trade-off between purity and yield (Hristova, 1968; 1970; Saetang *et al.*, 1997). Papain, unlike pepsin, is also a known allergen in man (Quarre *et al.*, 1995; Baur *et al.*, 1995).

The initial salt precipitation of the globulin fraction from serum is time consuming, difficult to perform on a large scale under sterile conditions, and may be associated with large losses and / or aggregate formation (Schultze *et al.*, 1965; Friesen, 1987). Various direct serum digestion methods have been devised. For example Parfentjev (1936; 1938), managed to directly digest equine antisera and remove the unwanted components by a combination of diafiltration, ammonium sulphate precipitation, dialysis and tri-calcium phosphate absorption. Another commercial method which also avoids initial salt precipitation is based on those of Pope (1939) and Harms (1948) in which brief (30min, 30°C, pH 3.2) pepsin digestion is followed by a critical heat denaturation step (1 hr at 55°C, pH 4.3, 13% ammonium sulphate, 0.12% Tricresol) to precipitate non-antitoxic protein. However, this still requires a later salt precipitation step, results in 40-50% losses of antitoxin activity, and gives a product of variable purity (Saetang *et al.*, 1997). Similar losses have also been described using a process utilising diafiltration and ion-exchange chromatography, although the product was highly pure (92-98% by electrophoresis (Benanchi *et al.*, 1988)). A simpler method was described by Poulsen and Hjort (1980) in which they attempted to digest human serum directly to $F(ab')_2$, and dialysable fragments. However, a large proportion of unwanted material was still retained with the $F(ab')_2$.

To my knowledge pepsin digestion of ovine serum with the complete degradation and removal of essentially all unwanted serum proteins without a salt precipitation step yet with a high yield of $F(ab')_2$ has not been described.

The following studies were performed to investigate the enzyme digestion of both ovine whole antiserum and its IgG fraction as part of the development of a simple method to produce pure $F(ab')_2$ at high yield and low cost suitable for both bench scale work and large scale production processing.

4.2 Methods

4.2.1 Antisera and IgG Preparation

Antisera used in these studies were raised against venom from the European honey bee (*Apis mellifera mellifera*) and IgG fractions were prepared as described in Chapter 3 (Jones *et al.*, 1999). Protein concentration of serum was determined using the BCA assay (Pierce, Rockford, USA; Smith *et al.*, 1985), with a bovine serum albumin standard as described in Chapter 2.

4.2.2 The Effects of Trypsin

IgG (30mg/mL in saline) or serum were diluted with an equal volume of digestion buffer (100mM Tris, 20mM $CaCl_2$, pH 8.0), placed in a water bath at 37°C for 30 min and type IX trypsin (15,200 Units/mg protein) dissolved in digestion buffer at a concentration of 50mg/mL was added to give an enzyme to substrate ratio of 1:50, mixed and incubated at 37°C. Samples (1.5mL) were removed at set time intervals and the digestion stopped by adding 15µL phenylmethylsulphonylfluoride (PMSF) dissolved in isopropanol (100mM; James, 1978).

4.2.3 The Effects of pH on Pepsin Digestion

Aliquots (15 mL) of either the IgG fraction (30mg/mL) or whole antisera (diluted with an equal volume of distilled water) were adjusted to one of a range of pH (3.0, 3.25, 3.5, 4.0, 4.5, 5.0) at room temperature using 0.4 M HCl and then warmed in a water bath at 37°C for 30 min. Porcine pepsin A, 1:60,000 grade (Sigma, Poole, UK, 2,260U/mg at 50mg/mL in distilled water) was added while mixing at an enzyme to substrate ratio of 1:50 w/w (45.2 U/mg substrate) and incubated at 37°C. Samples (1.5 mL) were removed at set intervals (0.5, 1, 2, 4, 8, 24, 48, 72 hr) and enzymatic

cleavage stopped by adding a small volume (20 - 125μL) of 0.5 M Na_2HPO_4, in 0.9% saline to raise the pH to ~6.0, sub-aliquoted and stored at -20°C until required.

4.2.4 The Effect of Post Digestion pH on Soluble Aggregate Formation

The antisera and IgG fractions were each adjusted to pH 3.5 and digested with pepsin for 24 hr, as described above, then immediately sub-aliquoted and stored at -20°C without neutralisation. When required, samples were thawed, diluted with the appropriate pH buffer (pH 4.0, 5.0, 6.0, 7.0, 8.0) and centrifuged or filtered to remove any precipitate before running on a Superose 12 column equilibrated with the same buffer.

4.2.5 Pepsin Enzymatic Activity

Pepsin enzymatic activity was measured using a method based on that described by Ryle (1970). Stock bovine haemoglobin (2.5%) substrate was dissolved in distilled water by thorough mixing and filtered through a filter paper on a Buchner funnel. This solution could be stored for a maximum of 2-3 days at 4°C. Acidified haemoglobin was prepared by mixing 4 parts with 1 part of HCl (0.3M) and 1mL aliquots were dispensed into test tubes. These were briefly warmed to 37°C in a water bath and 0.2mL of enzyme (or sample) diluted in Clark and Lubs solution (50mM KCl, pH 2.0) added, mixed and incubated for 10 min at 37°C. Trichloroacetic acid (TCA, 5mL of a 4% solution) was added, and the tubes were mixed and incubated for a further 5 min before being centrifuged for 10 min at 3,500 RPM at RT. The OD (280nm) of the supernatant was then measured using distilled water as a blank. Controls were performed for all samples by adding the TCA (5mL) to the acidified haemoglobin substrate (1mL) before the enzyme or sample (0.2mL), after which the tubes were mixed, incubated at 37°C for 5 min, centrifuged and the OD of the supernatant measured.

4.2.6 Pepsin Purity

Pepsin purity was assessed by SDS-PAGE (as described elsewhere) and by ion-exchange chromatography. The proportion of active material was assessed by passing 100 mg of pepsin through a Mono Q HR anion exchange column equilibrated with

20mM piperazine, 10mM NaCl, pH5.0 and eluted with 20mM piperazine, 1M NaCl, pH4.75, collecting the fractions and determining their concentrations by OD at 280nm using an extinction coefficient for a 1 mg/mL solution (1cm path) of 1.47 (Knowles *et al.*, 1969). These were then adjusted to pH 2.4 and assayed for pepsin activity.

4.2.7 Pepsin Inactivation

Pepsin (50 or 100 mg/mL respectively) was diluted 10 - fold with the appropriate pH inactivation saline buffer (50mM Tris, pH 9.0; 50mM Tris, pH 8.0; 100mM phosphate, pH 7.0; 100mM citrate, pH 6.0; 100mM citrate, pH 5.0) and incubated at 37°C for 2 hr before either diluting 1 in 50 with 50 mM KCl, pH 2.4 (Clark and Lubs solution) buffer and measuring enzyme activity as described above or diluting 1 in 10 with pH 3.5 citrate buffered saline, centrifuging and measuring the OD at 280nm to determine the amount of precipitated material.

4.2.8 The Effect of High pH on Pepsin Digested Material

The effect of high pH on pepsin digested material was assessed by raising the pH to 7.5, 8.0 or 9.0 with 0.5 M Tris base and incubating for 2 or 24hr at 37°C before adjusting the pH to 4.0 with 0.4 M HCl, centrifuging or filtering and running on a Superose 12 column in pH 4.0 citrate buffered saline.

4.2.9 Diafiltration

Low molecular weight digestion products were removed by ultrafiltration using Vivaspin (Sartorius, Goettingen, Germany) 2 mL centrifuge concentrators with 30 kDa molecular weight cut off polyethersulfone membranes. Large volumes of up to 1 L were processed using Sartocon-Micro cross flow units (Sartorius, Goettingen, Germany) as described in Chapter 2 by washing with 10 volumes of buffer.

4.2.10 Anion-Exchange Chromatography

Anion-exchange separations were performed using either a prepacked Mono Q HR 10/10 column or 50 mL of Q Sepharose Fast Flow with a 10cm bed height in a XK26 column (Pharmacia, Upsala, Sweden), equilibrated and run with piperazine buffer (20 mM) containing 150 mM NaCl, pH 6.0. Elution was performed with a gradient of the

same buffer containing 2M NaCl. Flow rates of 5mL/min were used.

4.2.11 SDS-PAGE

SDS-PAGE gels were prepared as described in Chapter 2, to monitor the digestion components.

4.2.12 Size Exclusion Chromatography

Size exclusion chromatography was performed using a FPLC system as described in Chapter 2, with columns comprising a cross linked agarose medium (Superose 12 or Superose 6 HR 10/30, volume 25 mL).

4.2.13 Serum Titre Estimation

Antibody titres directed against the venom were determined by ELISA as described in Chapter 2 using an affinity - purified (horse radish peroxidase conjugated) rabbit anti-sheep $F(ab')_2$ second antibody at a 1 in 2,500 dilution.

4.3 Results

4.3.1 The Effects of Trypsin on IgG and Serum

A ratio of trypsin to substrate of 1:50 rapidly digested all the IgG to $F(ab')_2$ after just 30 min. There was further digestion with time until only trace amounts of $F(ab')_2$ remained after 72 hr (Figure 4.1a). Albumin in the whole serum digests was rapidly cleaved after just 30 min to a band of ~ 44 kDa. However, this partially digested fragment appeared highly resistant to further proteolysis and was still intact after 72 hr, by which time the $F(ab')_2$ had been completely broken down (Figure 4.1b).

4.3.2 Pepsin Purity

Three grades of pepsin were compared by SDS-PAGE under non-reducing conditions and were all shown to consist primarily of a single band representing pepsin A with a MW of ~ 38 kDa (Figure 4.2). Pepsin A (EC 3.4.23.1) is the predominant aspartic endopeptidase found in the gastric juice of vertebrates. Commercial grade preparations can also contain gastricsin (pepsin C, EC 3.4.23.3) and a minor

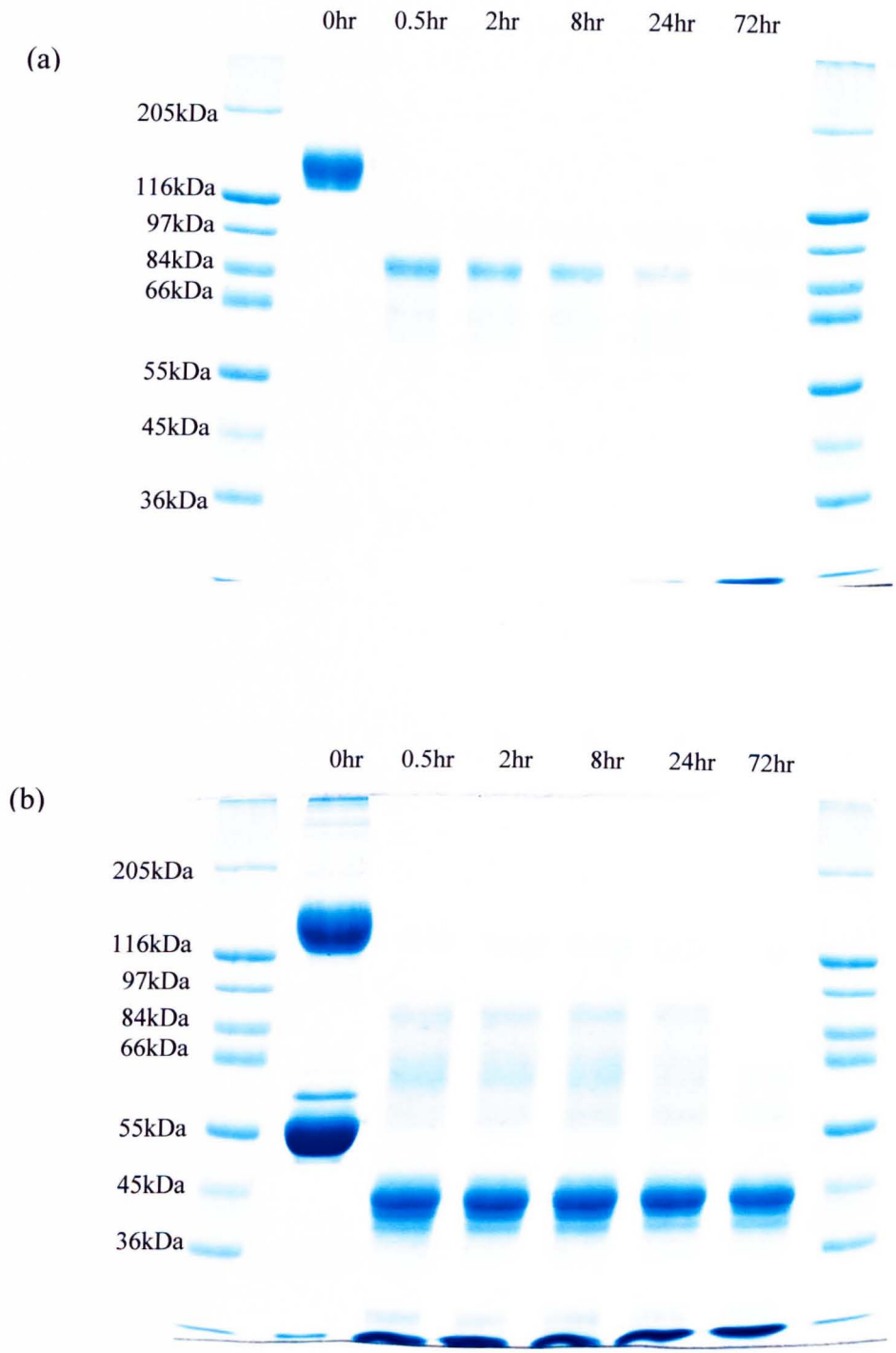


Figure 4.1 Trypsin digestions as assessed by SDS-PAGE (7.5%). The effects of digestion time on IgG (a), and serum (b).

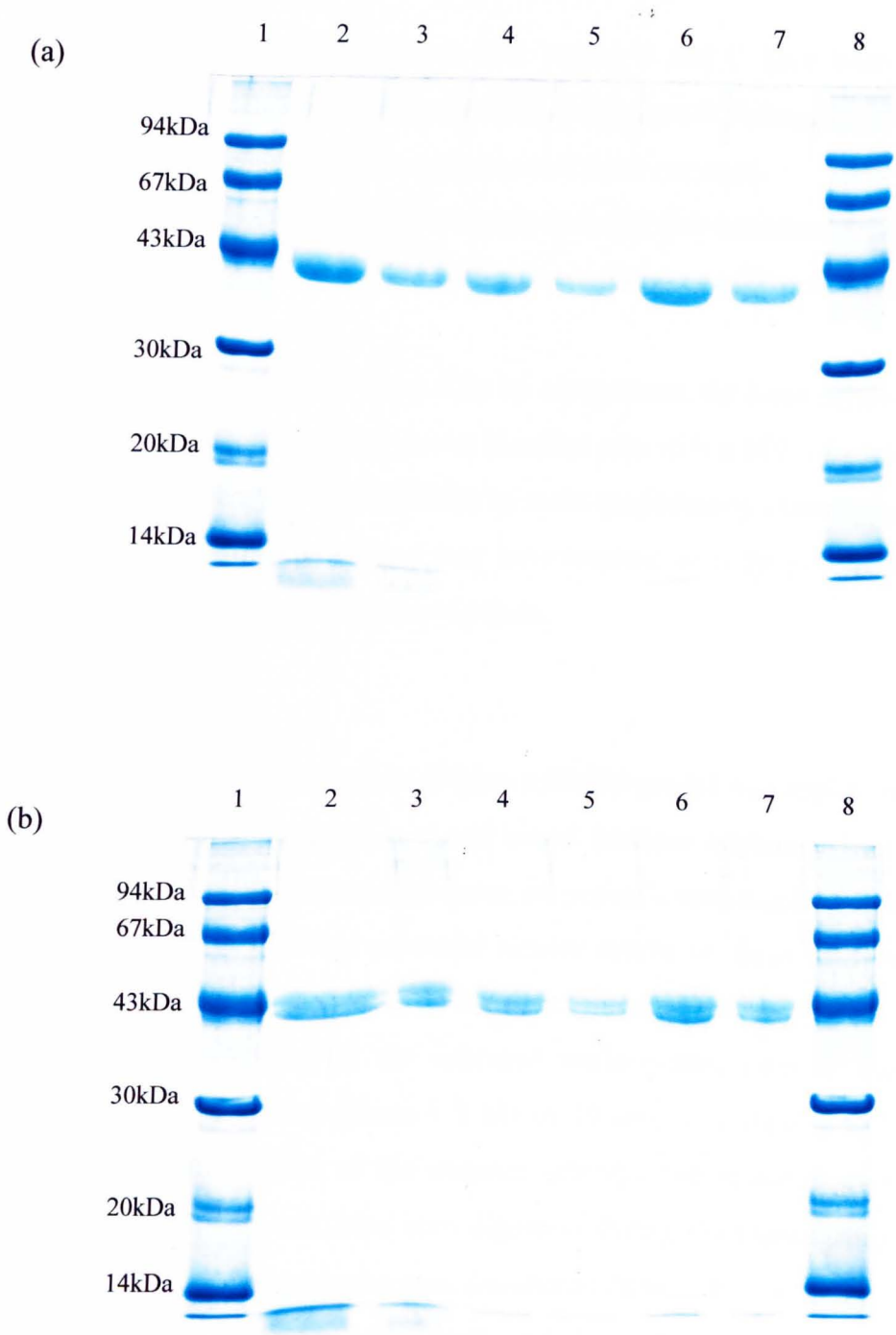


Figure 4.2 An analysis of different grades of pepsin by SDS-PAGE (12%). Merck European Pharmacopoeia grade, 200 & 100µg (lanes 2 & 3) respectively. Sigma 1:60,000 grade, (lanes 4 & 5), and Sigma highest grade, (lanes 6 & 7), 5 & 2.5µg per lane respectively. Under non-reducing (a) and reducing (b) conditions.

component called pig gelatinase (pepsin B, EC 3.4.23.2) which have more restricted specificities.

Under these SDS-PAGE conditions both pepsin B and C have been shown in the literature to be clearly resolved from pepsin A (Nielsen & Foltmann, 1995). No bands could be detected which may have represented these enzymes.

The Merck European Pharmacopoeia grade material also contained a large amount of low MW material which ran at the solvent front and was undetectable in either of the Sigma products.

Under reducing conditions (Figure 4.2), by comparison, the band representing pepsin A was resolved as two bands of almost identical size with a MW of ~ 40 kDa. Pepsin A is known to have a MW of 34,586Da by mass spectrometry (Jones *et al.*, 1995) and this was, therefore, unexpected and may have resulted from the poor incorporation of SDS due to the highly acidic nature of pepsin.

4.3.3 Pepsin Activity

When 100mg dry weight of pepsin (Sigma 1:60,000 grade) was applied to a Mono Q anion exchange column the unbound and bound fractions contained 7.3 mg and 50.2 mg by OD respectively. A standard curve of pepsin's enzymatic activity measured using a haemoglobin substrate produced similar results on three different days and was used to calculate the activities of the eluted material (Figure 4.3). No enzymatic activity could be detected in the unbound fraction and, because the minimum detectable level of the assay (mean + 2 SD of 10 zero replicates) was 1 mg/L, this fraction contained $\leq$ 0.02% of the original activity. The majority of the inactive material is thought to result from auto digestion during the manufacturing process, cell debris and/or mucosaccharides (not detected at 280nm) from the stomach mucosa (Rajagopalan *et al.*, 1966; Hristova, 1968). All the active enzyme was found in the bound fraction which contained 100% of the original enzymatic activity.

4.3.4 The Effects of pH on Pepsin Digestion

4.3.4.1 IgG

At pH 5.0, no digestion products of IgG could be detected until after 8 hr (data not shown). At pH 4.5 the first digestion products could just be detected after 0.5 hr, with

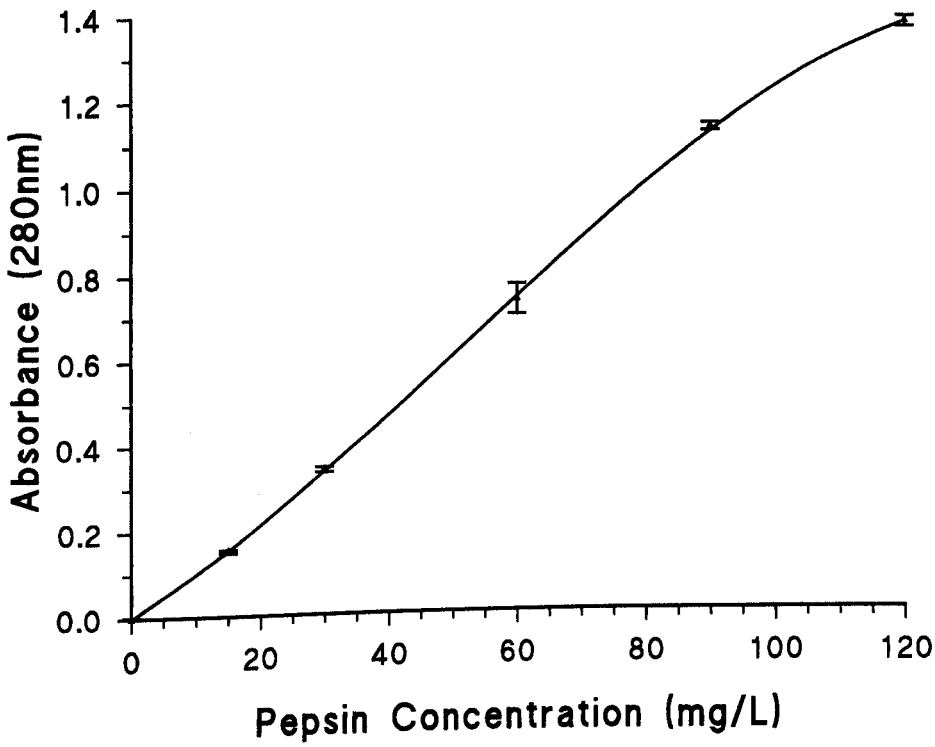


Figure 4.3 Pepsin (Sigma 1:60,000 grade) enzymatic assay standard curve using haemoglobin as substrate. Mean ±SD (n=3)

a large but incomplete proportion of the IgG digested after 72 hr to a band of ~ 90 kDa, presumably representing $F(ab')_2$ (Figure 4.4). Digestion at pH 4.0 proceeded more rapidly and after 72 hr no IgG starting material could be detected and a similar picture emerged after just 2 hr at pH 3.5. After 72 hr at pH 3.5 a slight band of ~ half the size of the $F(ab')_2$ could be seen which presumably represented the further breakdown of the $F(ab')_2$ to Fab'. At pH 3.0 all the IgG starting material had been broken down after just 0.5 hr and a progressive decrease in the intensity of the $F(ab')_2$ band was seen with time.

A slight band of ~ 52 kDa could also be detected after 8 hr at pH 4.5 and may represent intact Fc. This band was also present for the first 2 hr of digestion at pH 4.0, although it must have been rapidly degraded as it could not be detected after this time or in lower pH digests.

Because of differences in the estimated molecular weight of certain proteins under non-reducing conditions compared to their known MW, some samples were re-tested under reducing conditions in order to calculate their correct MW for identification purposes. Serum proteins were identified as transferrin (82kDa), albumin (67kDa), and IgG heavy chain (54kDa) or light chain (27-28kDa).

4.3.4.2 Serum

The pool of undiluted ovine antisera had a protein concentration of 95 g/L by BCA protein assay. The digestion process was easily followed using SDS-PAGE under non-reducing conditions (Figure 4.5). Minimal serum digestion was seen after 72 hours at pH 5.0, but a minor band which was slightly larger than albumin and thought to represent ovine transferrin, was rapidly digested after 2 hr (data not shown). At pH 4.5 a large but incomplete proportion of the IgG band was digested after 72 hr to a band of ~ 90 kDa presumably representing $F(ab')_2$, while albumin was only partially digested. Digestion intermediates were also seen over the first 24 hr with bands from ~ 45 kDa and smaller, the larger of which appeared to be digested to form lower molecular weight bands with time until, at 72 hr, only small peptides existed which ran at the electrophoresis front. At pH 4.0 the intact albumin was undetectable after 72 hr and the intermediates were once again seen during the early stages of the digestion but this time the intact IgG band was almost completely absent after 72 hr. Digestion

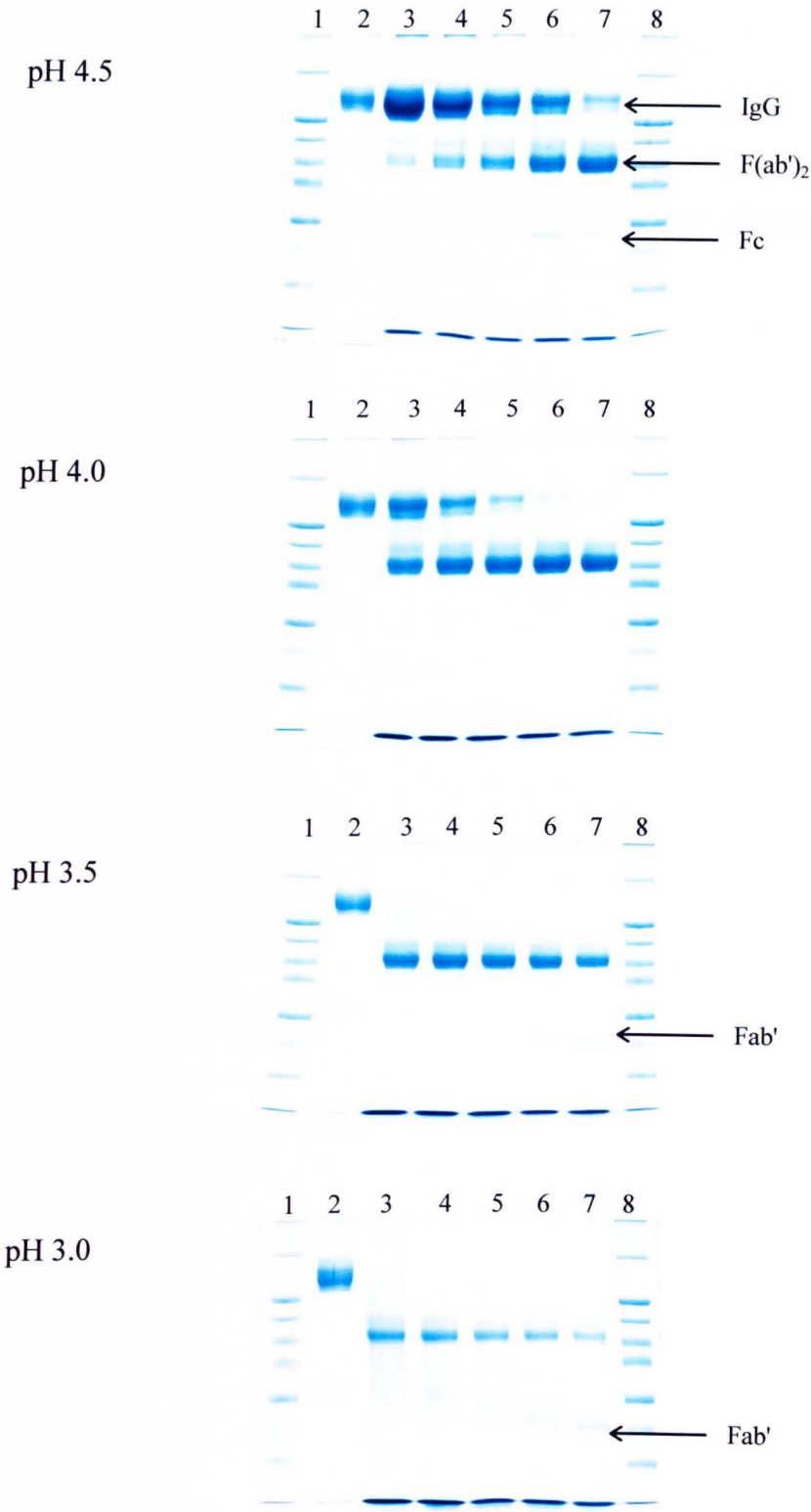


Figure 4.4 The effect of pH on the pepsin digestion of IgG, as assessed by SDS-PAGE (7.5%). Digestion samples at 0hr (lane 2), 0.5hr (lane 3), 2hr (lane 4), 8hr (lane 5), 24hr (lane 6), 72hr (lane 7), and molecular weight markers (lanes 1 & 8), 205, 116, 97, 84, 66, 55, 45 and 36kDa.

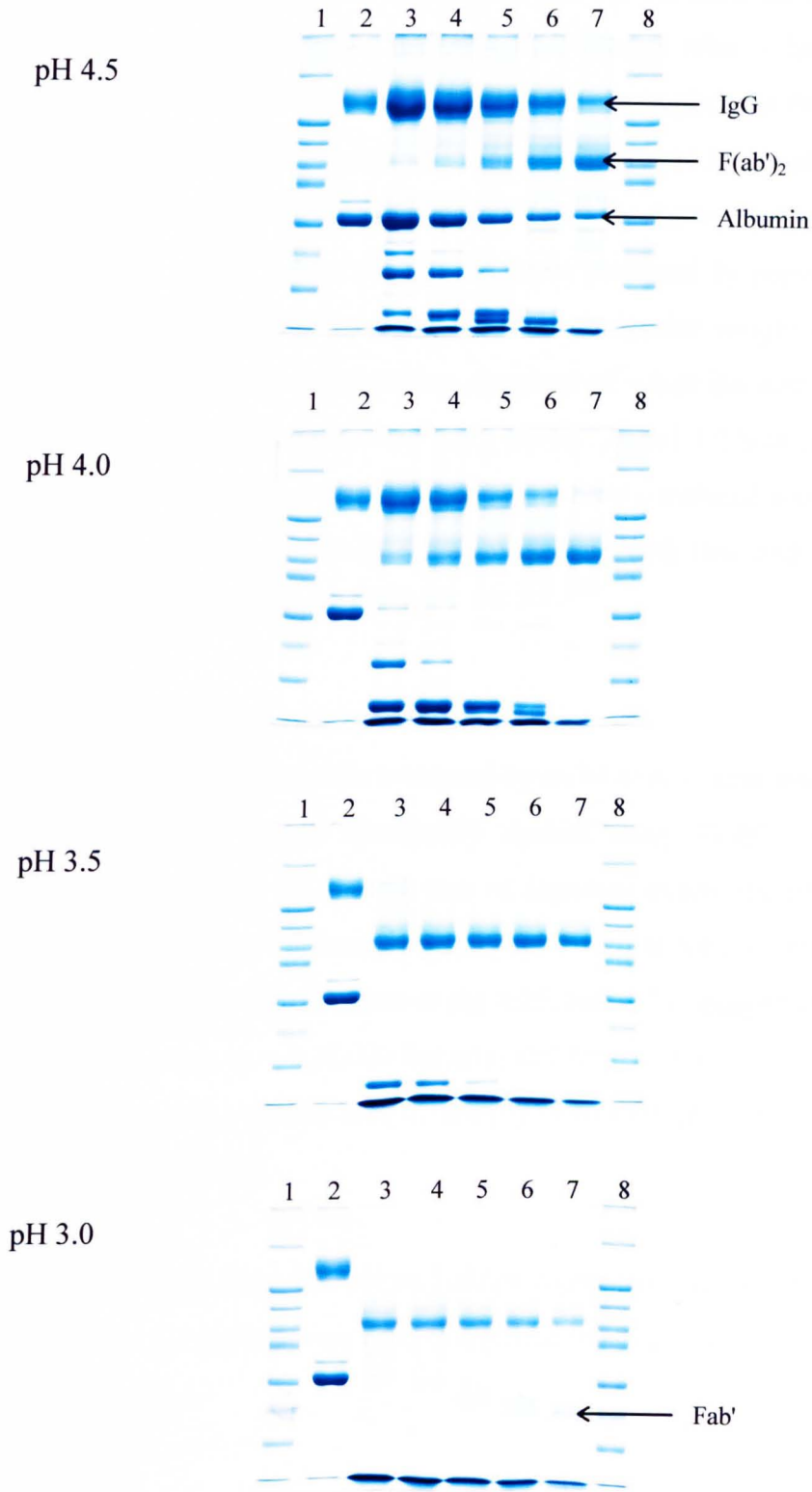


Figure 4.5 The effect of pH on the pepsin digestion of serum, as assessed by SDS-PAGE (7.5%). Digestion samples at 0hr (lane 2), 0.5hr (lane 3), 2hr (lane 4), 8hr (lane 5), 24hr (lane 6), 72hr (lane 7), and molecular weight markers (lanes 1 & 8), 205, 116, 97, 84, 66, 55, 45 and 36kDa.

at pH 3.5 resulted in the complete elimination of both the albumin and transferrin bands after only 0.5 hr and intact IgG was undetectable after 2 hr. An albumin breakdown product was seen as a band just above the electrophoresis front in the 0.5 - 8 hr samples and was found to have a molecular weight of ~ 43 kDa when re-tested on a 12% acrylamide gel under reducing conditions (data not shown), which is similar to the reported 46kDa human serum albumin fragment produced by pepsin (Bos *et al.*, 1988). At 24 hr only a $F(ab')_2$ band and very low molecular weight material were evident. However, at 72 hr a slight shadow appeared of ~ half the size of the $F(ab')_2$ representing the further digestion of the $F(ab')_2$ to Fab'. At pH 3.0 both intact albumin and IgG were undetectable after just 0.5 hr and only $F(ab')_2$ material was evident. The $F(ab')_2$ band, however, appeared to decrease in intensity with time and again a slight band appeared of ~ half its size after 24 hr.

4.3.5 Effects of Digestion on Antigen Binding

Apparent venom binding activity was measured by an ELISA system using an affinity purified second antibody raised specifically against sheep $F(ab')_2$. Samples had increased specific titres after the first 30 min of digestion except the pH 3.0 digests which showed a progressive decrease in titre from 1:18,000 for the antisera starting material to 1:7,600 (Figure 4.6). Digests at pH 3.25, and 3.5 displayed an increase in titre over the first 8 hours to ~ 1:28,000 but after this time diverged, so that the lower pH digests demonstrated a greater loss of activity with time (e.g. at 72 hr, pH 3.25 digest had a titre of 1:12,700).

4.3.6 The Effect of Post Digestion pH on Soluble Aggregate Formation

A range of different pH buffers were used to equilibrate a Superose 12 size exclusion chromatography column.

4.3.6.1 IgG digests

When the IgG digest was run on a size exclusion chromatography column equilibrated with pH 4.0 buffer, two major peaks were evident, the first representing the $F(ab')_2$ (~100 kDa) while the second presumably represents an Fc fragment (~ 8 kDa) and

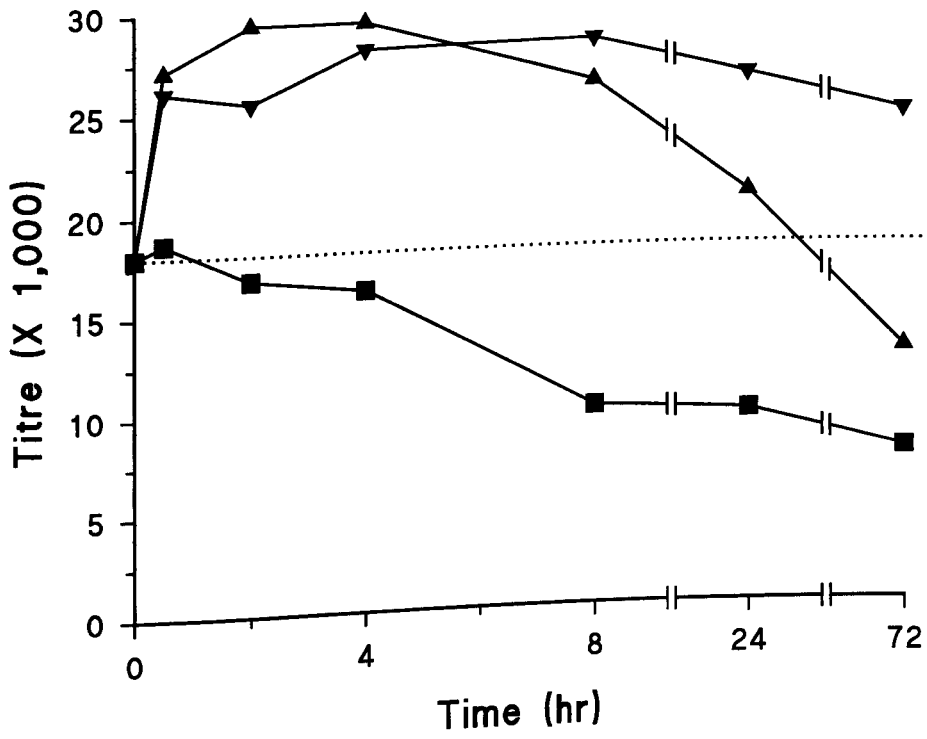


Figure 4.6 The effect of antiserum digestion on ELISA titre. pH 3.5 (▼), 3.25 (▲), and 3.0 (■).

was followed by several lower molecular weight minor peaks (< 8 kDa) (Figure 4.7). When the digest was run at pH 5.0 a similar profile was observed, except some of the minor low molecular weight peaks appeared closer to the second major peak. However, when the pH of the digest was adjusted to pH 6.0, 7.0 and 8.0 a small but notable and increasing amount of relatively high molecular weight material was eluted first from the column, with a large proportion apparent in the void volume (e.g. ≥ ~ 2,000 kDa). This was confirmed by repeating the run on a Superose 6 column which showed that the peak was spread over a very high and wide molecular weight range which stretched back as far as the $F(ab')_2$ peak.

There also appeared to be changes in some of the lower molecular weight peaks as the pH was changed, which may represent the aggregation process.

This relatively high molecular weight material was not observed by SDS-PAGE (Figure 4.4) which implies that it consists of aggregated low molecular weight fragments of Fc.

4.3.6.2 Serum digests

A similar picture was seen with the serum digests except that there were more low molecular weight peaks present at pH 4.0 and a new peak was evident between the two major peaks with a retention time that gave it a molecular weight of ~ 13 kDa indicating that this fragment is formed from the digestion of a non-IgG component (Figure 4.8). When the digest was run at pH 5.0 a similar profile was observed, except some of the minor low molecular weight peaks again appeared closer to the second major peak. However, when the pH of digests was adjusted to pH 6.0, 7.0 and 8.0 a small but notable and increasing amount of relatively high molecular weight material was eluted first from the column, with a large proportion apparent in the void volume (e.g. ≥ ~ 2,000 kDa). This was confirmed by repeating the run on a Superose 6 column which showed that the peak was spread over a very high and wide molecular weight range which stretched back as far as the $F(ab')_2$ peak. A larger proportion of this high molecular weight component was seen compared to IgG digests, indicating that non IgG fragments also contribute to this peak.

There also appeared to be changes in some of the lower molecular weight peaks as the pH was changed, which may represent the aggregation process.

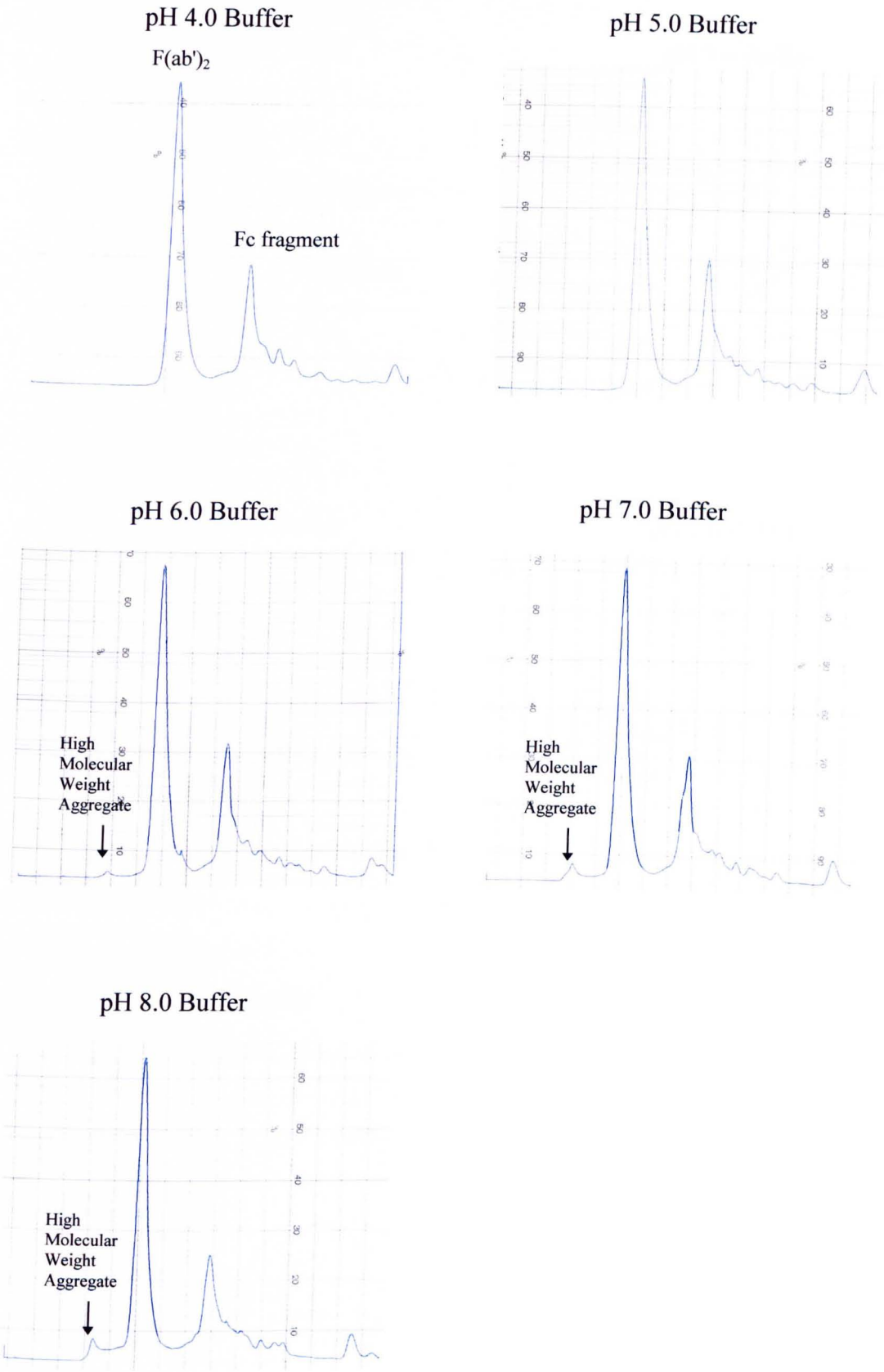


Figure 4.7 The effect of pH after IgG digestion on soluble aggregate formation as assessed by size exclusion chromatography.

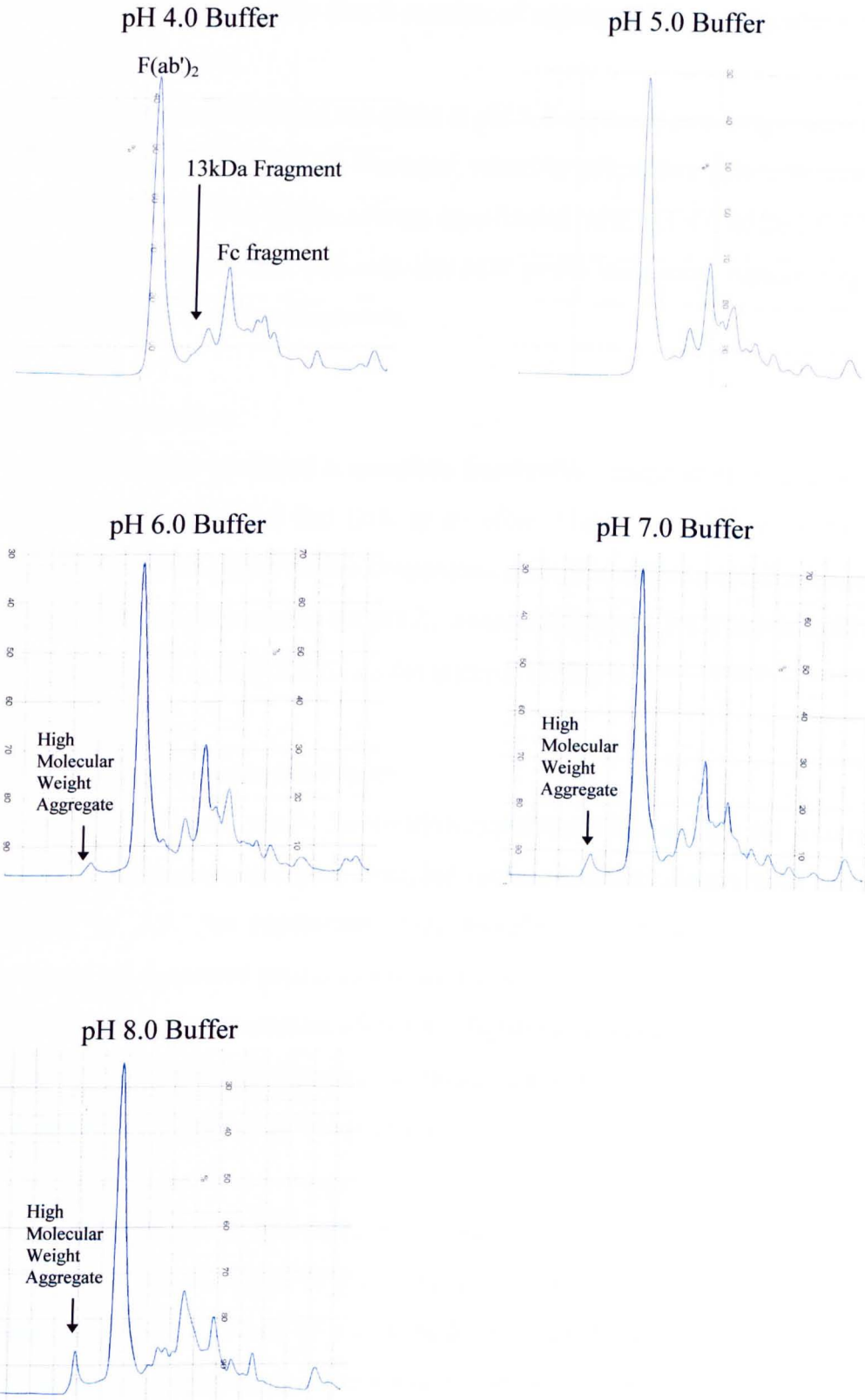


Figure 4.8 The effect of pH after serum digestion on soluble aggregate formation as assessed by size exclusion chromatography.

This relatively high molecular weight material was not observed by SDS-PAGE (Figure 4.5) which again implies that it consists of aggregated low molecular weight Fc and non IgG fragments.

When this peak was collected and run again at pH 8 it appeared as a single high MW peak without any low MW material. However, when the pH of this high MW material was adjusted to 4.0 and run on the column equilibrated with pH 4.0 buffer, the high MW peak was no longer found and only low MW peaks were seen, representing the disaggregation of the digestion fragments.

4.3.7 Pepsin Inactivation

A pH of 7.0 or higher produced a complete irreversible inactivation of the pepsin activity, while pH 5.0 and 6.0 had little or no effect (Table 4.1). However, further experiments using much higher pepsin concentrations indicated that a small amount of active pepsin was still present after the pH 7.0 treatment (2hr at 37°C) and that pH 8.0 or higher was required to fully inactivate the material (Table 4.1).

4.3.8 Precipitation of Inactivated Pepsin

It was noticed during initial pepsin inactivation experiments that at high pH solutions of inactivated pepsin remained crystal clear, but became intensely cloudy when the pH was adjusted to 3.5. An experiment was, therefore, performed to assess the precipitation of pH denatured pepsin as a potential pepsin removal step. By measuring the OD at 280nm of the supernatant after centrifugation, it was calculated that up to 76% by OD of the original pepsin was precipitated (Table 4.1). The remaining 24% in solution may represent impurities and pepsin fragments.

4.3.9 Effects of High pH on IgG and Serum Digests.

Unfortunately exposure of digests to pH 8.0 for 2 or more hr at 37°C to inactivate the pepsin resulted in the formation of a new peak of ~ 50kDa when assessed by size exclusion chromatography which corresponded with a decrease in the $F(ab')_2$ peak. This new material could just be detected as a shoulder on the $F(ab')_2$ peak after 2 hr and was clearly evident after 24 hr, and was assumed to represent the breakdown of

	pH Treatment (2 hr, 37°C)						
	5.0	**6.0**	**7.0**	**7.5**	**8.0**	**8.5**	**9.0**
Residual enzymatic activity:							
100mg/L Pepsin	1.332	1.216	0.004	nd	0.007	nd	0.027
25g/L Pepsin	nd	nd	0.111	0.074	0.012	0.010	-0.001
Precipitation on adjustment to pH 3.5:							
1g/L Pepsin	1.090	1.051	0.265	nd	0.239	nd	0.383

Table 4.1 Effects of pH on pepsin. Results expressed in terms of supernatant OD (280nm).

$F(ab')_2$ (Figure 4.9).

4.3.10 The Minimum pH Required to Stop Pepsin Activity

Serum samples adjusted to between pH 5.2 and 6.0 were assessed for digestion products by SDS-PAGE after incubation with pepsin (1:50) at 37°C for either 3 or 7 days (Figure 4.10). The transferrin band just above the unaffected albumin band in the pH matched controls was not found in any of the pepsin treated samples indicating that it is highly susceptible to pepsin cleavage. The IgG band had clearly been partially digested at the lower pH's and at 5.5 only a slight reduction in the intensity of this band was seen. At pH 6.0 the IgG band appeared identical at 3 and 7 days. Because of the detrimental effects on the product of fully denaturing all the pepsin to a permanently inactive state by high pH alone it was decided to process the digest at a pH where pepsin has minimal activity (e.g. pH 6.0).

4.3.11 Effects of Salt Concentration on High Molecular Weight Aggregate

Serum digestions were routinely terminated by adding sufficient 50mM piperazine solution to raise the pH to 6.0 and the precipitate formed during digestion removed by centrifugation or filtration. When buffer containing 150mM NaCl was used to wash the precipitate in order to reduce any losses it was noticed that a larger amount of high molecular weight aggregate was seen in the digested material by size exclusion chromatography at pH 8.0. The effects of the salt concentration on soluble aggregates was, therefore, investigated.

It was assumed that serum contained 154mM NaCl and any dilution factors were taken into account. The concentration of NaCl in the crude unfiltered digest was adjusted by the addition of various ratios of high salt buffer (2M NaCl, 20mM piperazine, pH 6.0), mixed and filtered before diluting and running on a size exclusion chromatography column at pH 8.0 (Figure 4.11). Control digests that were filtered without adjusting the level of NaCl or those in which the level was further reduced by diluting with an equal volume of distilled water both contained a similar very low level of high molecular weight aggregate as assessed at pH 8.0. However when the salt level was increased to 0.2 or 0.4M a progressively increased amount of the high molecular weight aggregate was observed. This suggests that a proportion of the

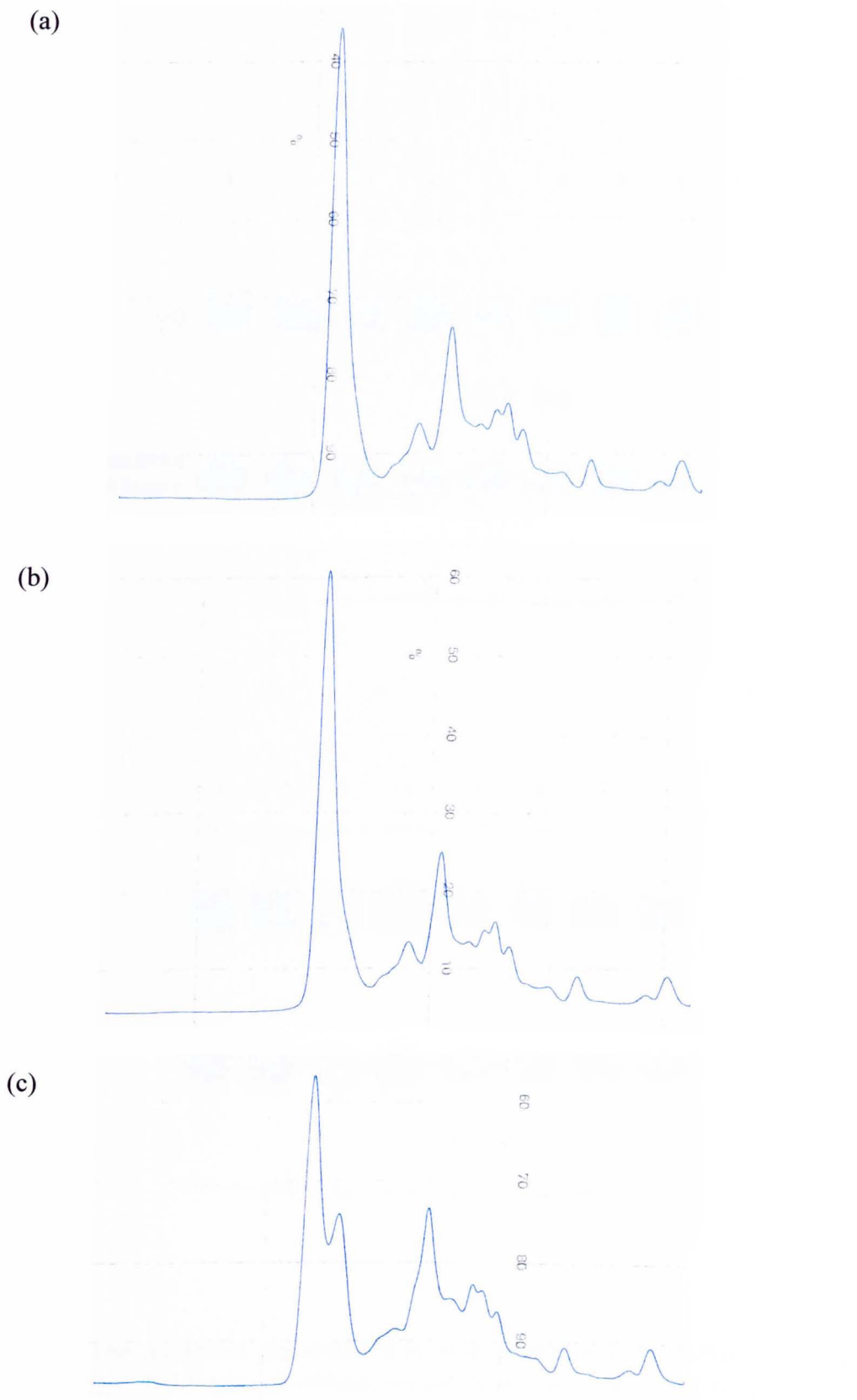


Figure 4.9 Effects of pH 8.0 treatment on a serum digest. Without treatment (a), or adjusted to pH 8.0 and incubated at 37°C for 2 hr (b), or 24 hr (c). Size exclusion chromatography (Superose 12 column, citrate/saline pH4.0).

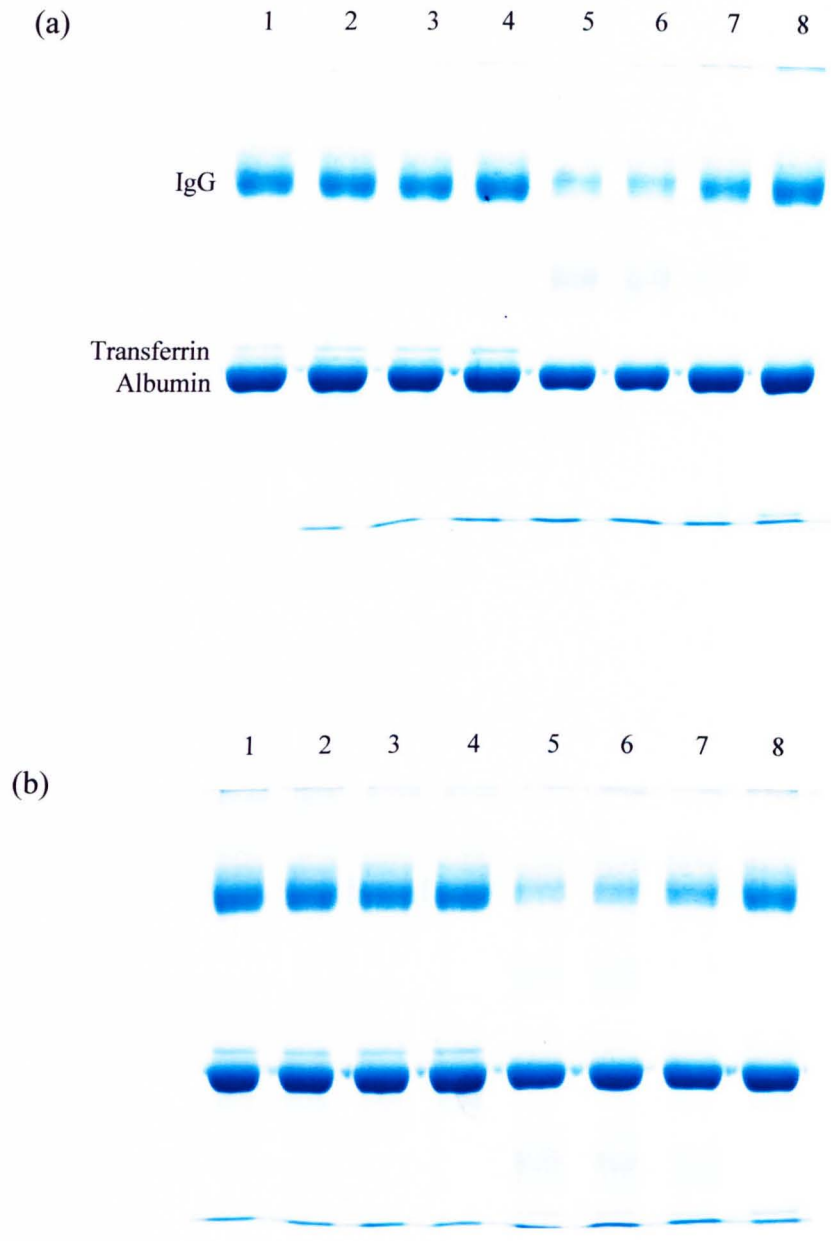


Figure 4.10 The minimum pH required to stop pepsin activity. Serum incubated (37°C) at pH 5.2, 5.3, 5.5 and 6.0 without pepsin (lanes 1-4), and with pepsin (lanes 5-8) respectively, after 3 days (a) and 7 days (b).

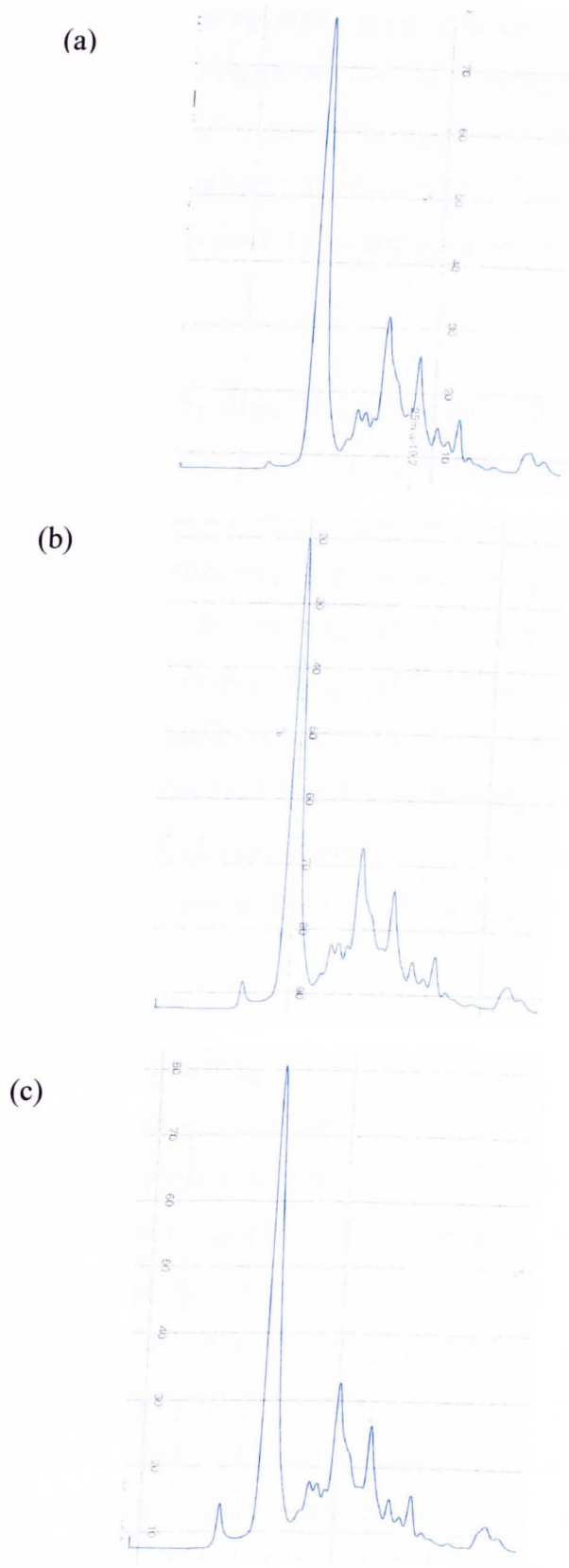


Figure 4.11 Effects of salt concentration on levels of high molecular weight aggregate. Crude digest material (pH6.0), with no extra NaCl added (a), or NaCl added to give a concentration of 0.2M (b), or 0.4M (c), before filtration. Size exclusion chromatography on a Superose 12 column, in Tris/saline pH8.0 buffer.

soluble high molecular weight aggregate material is unstable and precipitates at low salt concentrations and is then easily removed by centrifugation or filtration. For these reasons no extra salt was added to the serum digests until after they were centrifuged or filtered to remove the precipitate formed during the digestion. Following this work a low salt solution was used to wash the precipitate in order to reduce any losses.

4.3.12 Diafiltration

Cross flow diafiltration of the digested material with 10 volumes of buffer could easily be performed at pH 6.0 without any significant fouling of the membrane by precipitated material unlike at a lower pH. All the material with a lower molecular weight than the $F(ab')_2$ as assessed by size exclusion chromatography at pH 8.0 passed through the ultrafilter to waste, leaving principally $F(ab')_2$ and a small quantity of high molecular weight aggregate (Figure 4.12). The amount of protein material was assessed by OD(280nm) and yields calculated. A 50kDa ultrafilter gave yields of 13.2 and 14.5g/L of serum for serum batch I and II respectively, while a 30kDa ultrafilter produced yields of 17.7 and 21.9g/L respectively. Therefore the 30kDa ultrafilter increased yields by ~ 30% compared to a 50kDa membrane despite producing products of the same purity.

4.3.13 Ion-Exchange Chromatography

4.3.13.1 Preparative scale (Q Sepharose FF)

Diafiltered digests in 20mM piperazine, 150mM NaCl, pH 6.0 were separated into 3 peaks by anion exchange chromatography with a salt gradient (Figure 4.13). The first, which passed straight through the column and constituted ~ 90% of the material, contained the $F(ab')_2$. The second peak eluted in ~ 0.25M NaCl and contained the high molecular weight aggregate material as assessed by size exclusion chromatography. The third peak eluted in ~ 0.5M NaCl and contained the pepsin (see below). The material from the first peak was then concentrated with a 30kDa MWCO ultrafilter and gave a final yield of 19.3g $F(ab')_2$ / L of serum. It was free from high molecular weight aggregate (Figure 4.14).

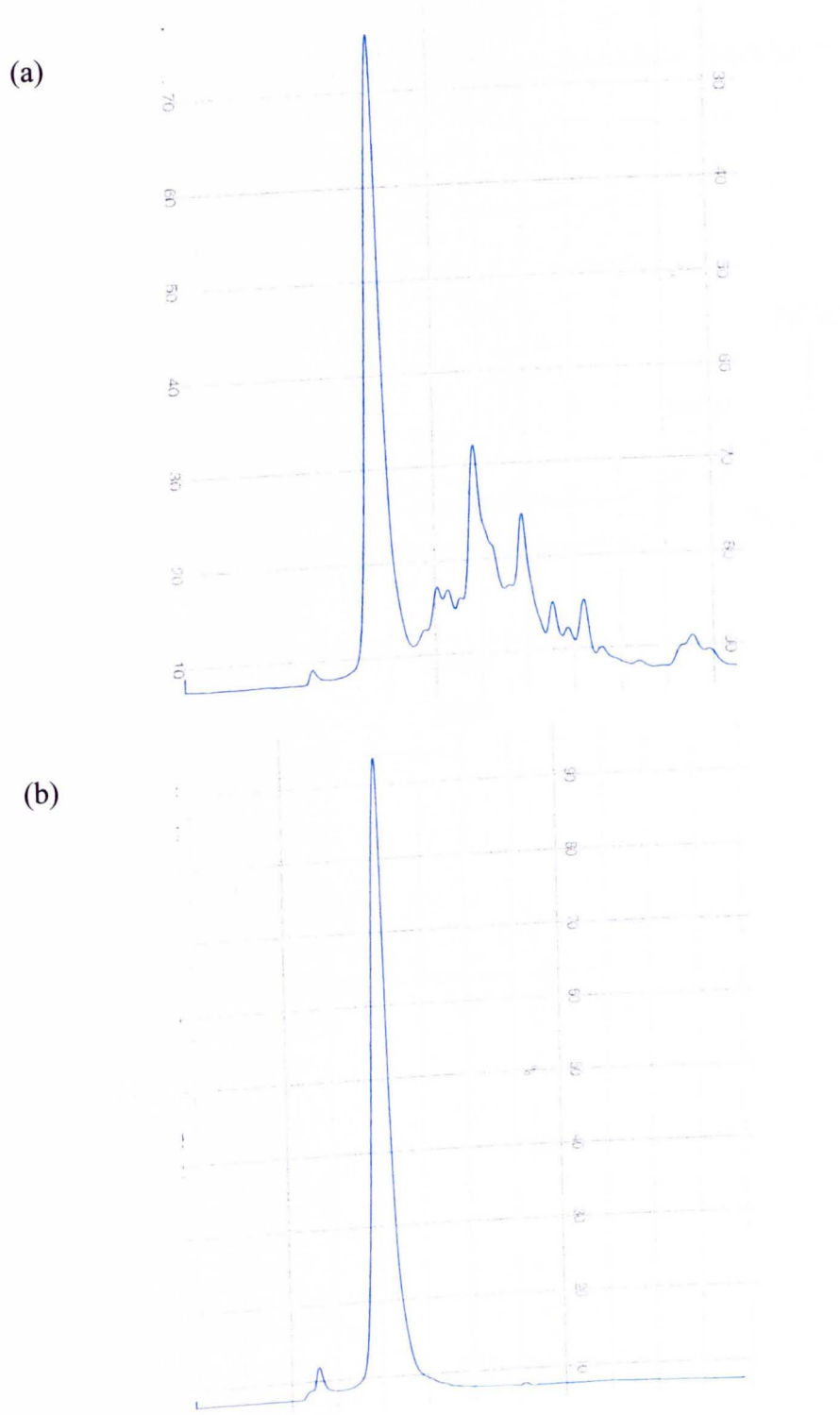
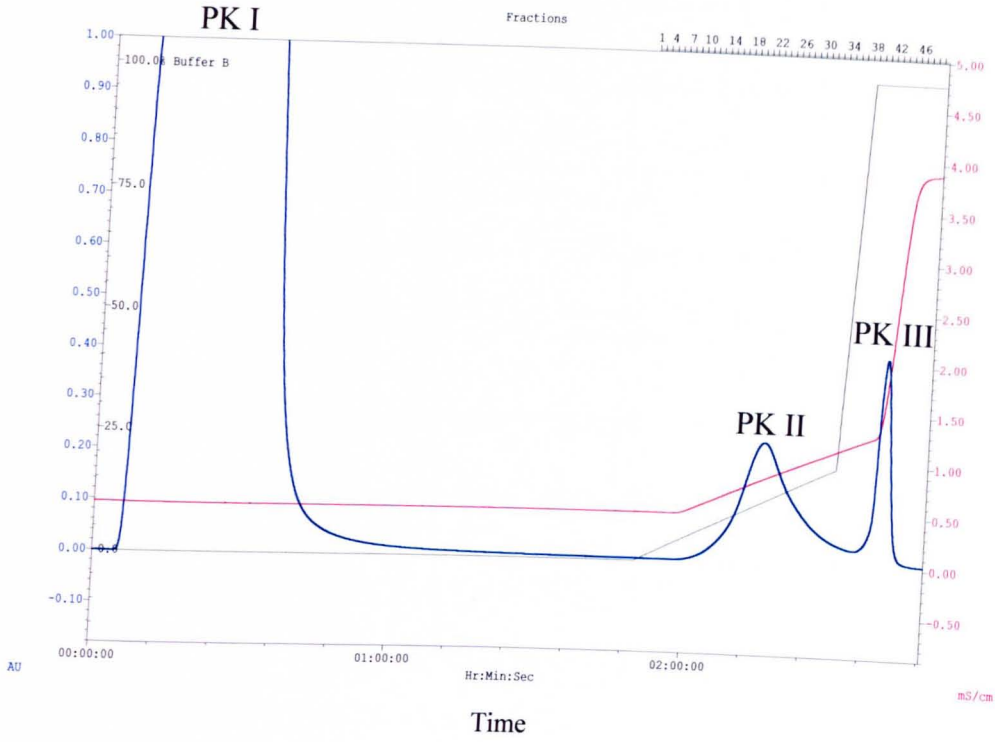


Figure 4.12 The removal of low molecular weight fragments by diafiltration. Serum digest before (a), and after diafiltration (b), using a 30K MWCO cross flow ultrafilter and 20mM piperazine, 150mM NaCl, pH6.0. As assessed by size exclusion chromatography (Superose 12, Tris/saline pH8.0).

(a)



(b)

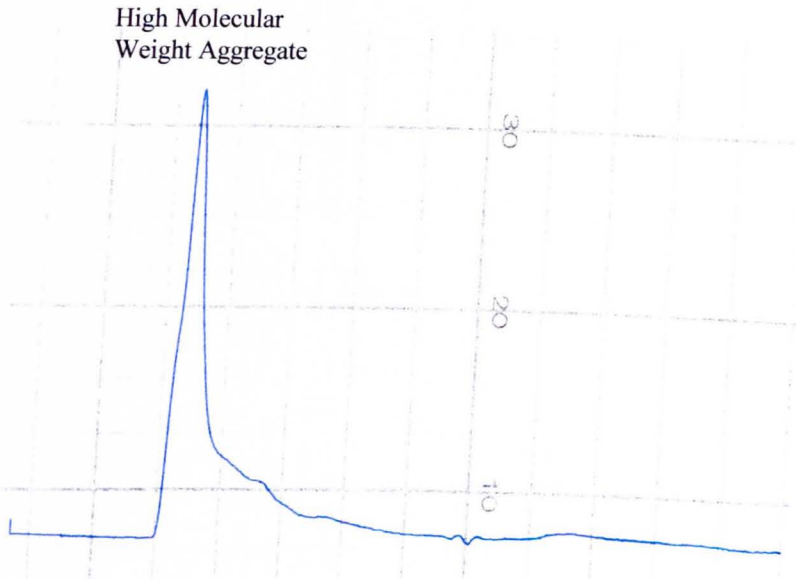


Figure 4.13 Removal of high molecular weight aggregate and pepsin by anion exchange chromatography. Q-Sepharose FF ion exchange separation of a diafiltered pepsin digested antiserum (a). Peak I, $F(ab')_2$, II high molecular weight aggregate, and III pepsin. Size exclusion chromatography of peak II (b) at pH8.0.

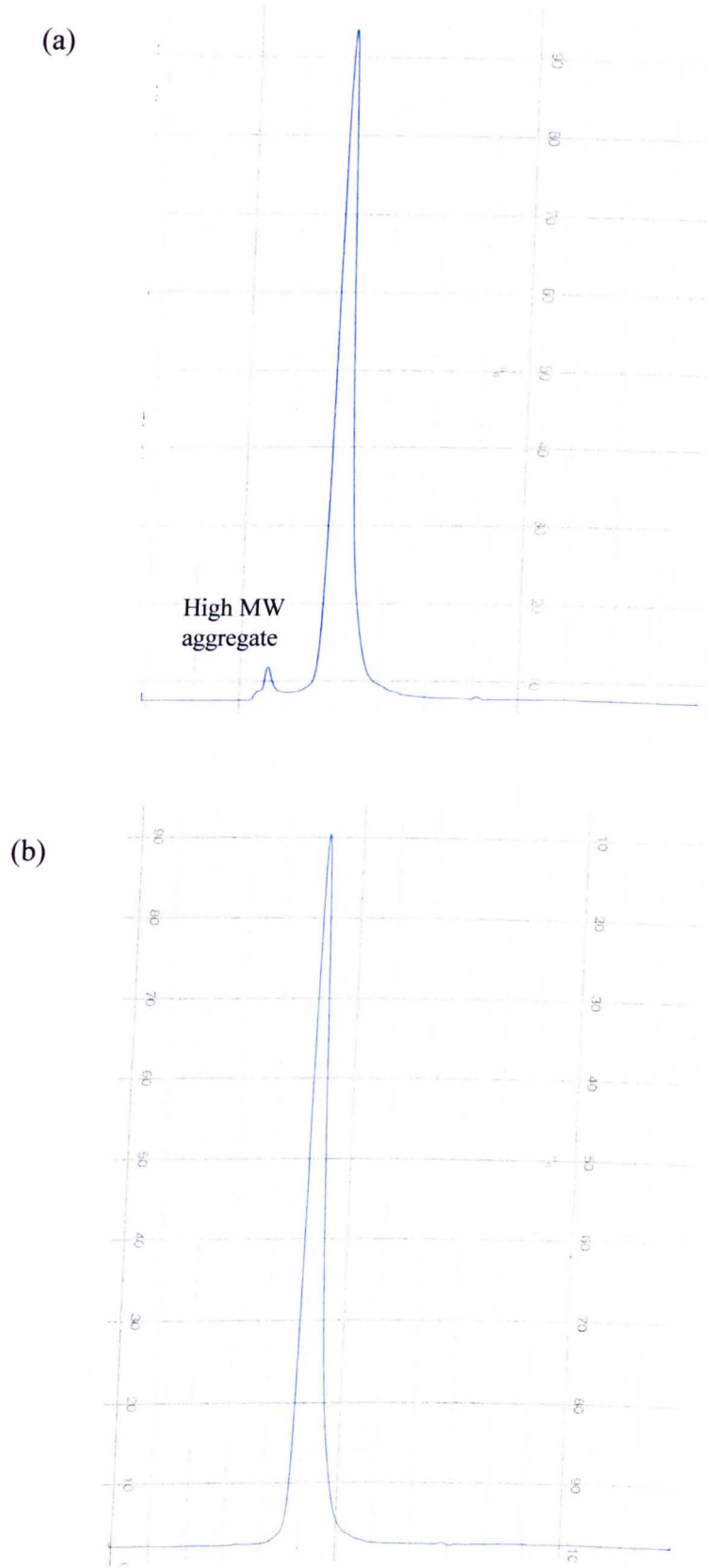


Figure 4.14 Removal of high molecular weight aggregate by anion-exchange chromatography. A diafiltered serum digest before (a), and after ion-exchange chromatography (b). As assessed by size exclusion chromatography (Superose 12, Tris/saline pH8.0).

4.3.13.2 Analytical scale (Mono Q)

A high resolution Mono Q HR column was used to check that all the highly acidic components (pepsin and high molecular weight aggregates) had been removed. The Sigma 1:60,000 grade pepsin used in these studies contained two peaks, the smaller first peak passed straight through the column and consisted of impurities and a second well defined sharp peak which eluted in ~0.5M NaCl containing the pepsin. Since 1.9g of pepsin was added to each litre of antisera, from which 19.3g of the final $F(ab')_2$ product was produced, if no pepsin was lost during the processing the product would contain 10% pepsin. For these reasons 0.2mg of pepsin was compared with 2 mg of $F(ab')_2$ preparations (Figure 4.15). Serum digests diafiltered using either a 30 or 50kDa MWCO ultrafilter still contained large, roughly equivalent, amounts of pepsin along with an extra peak which eluted with ~ 0.25M NaCl and represented the high molecular weight aggregate component. Pepsin was undetectable in the final $F(ab')_2$ product and none of the final product material was bound by the column indicating that it was free from highly acidic components, such as the high molecular weight aggregate.

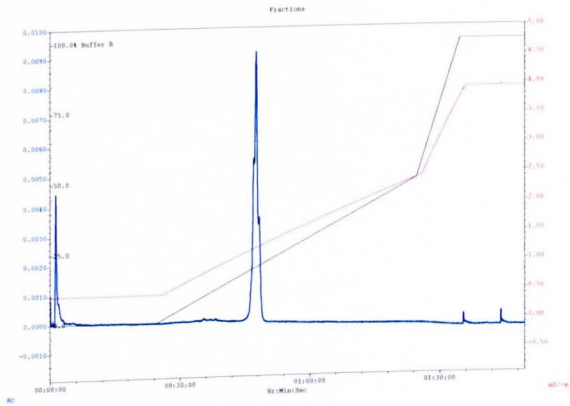
4.3.14 Processing Yields.

Two different batches of antisera were processed by identical methods and the yields calculated (Figure 4.16). Lower yields were evident from antiserum I compared to antiserum II.

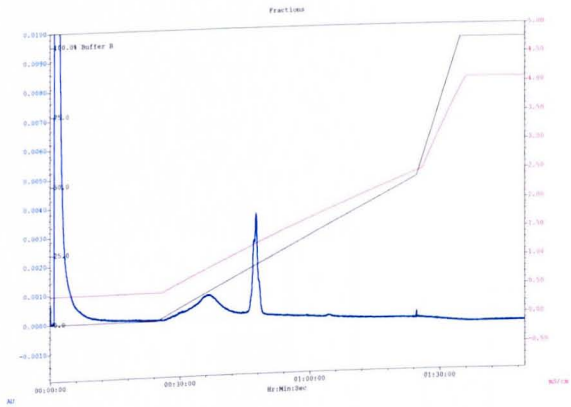
4.3.15 A Comparison of Purity with Commercially Available Antivenoms.

Size exclusion chromatography of a commercially available antivenom (CSL tiger snake) at pH 8.0 revealed the presence of a small quantity of high molecular weight material. However, when this was repeated under low pH (4.0) conditions the high molecular weight component could no longer be seen (Figure 4.17). This antivenom also contained a small peak with a molecular weight of ~ 280kDa. SDS-PAGE analysis of this antivenom showed that it along with other commercial antivenoms contained trace amounts of high molecular weight (>205kDa) and low molecular weight (~36kDa) components but were free from undigested IgG (Figure 4.18). An impurity was also present with a molecular weight of ~70kDa. The ovine material had

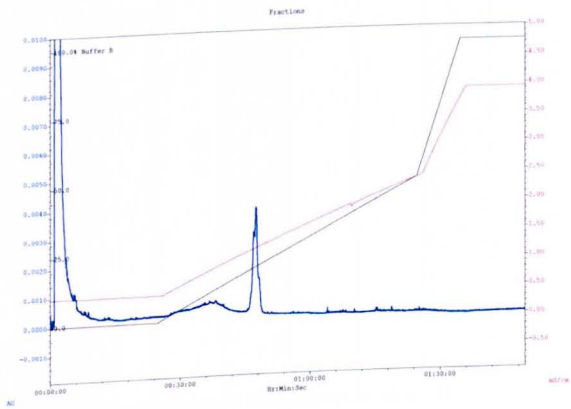
(a)



(b)



(c)



(d)

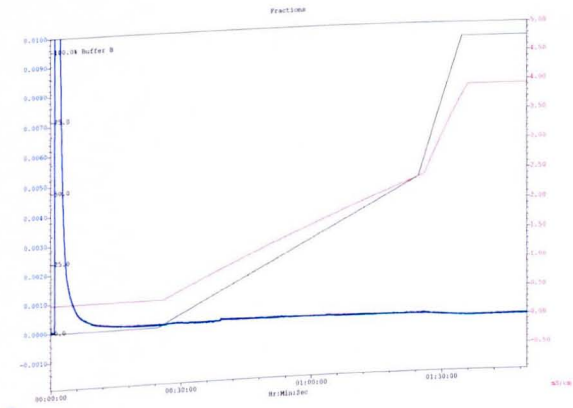


Figure 4.15 Residual pepsin as determined with an analytical Mono Q chromatography column. Pepsin (0.2mg) control (a), or 2mg of each of the following: Pepsin digested serum following diafiltration with a 50kDa MWCO ultrafilter (b), or a 30kDa MWCO ultrafilter (c), or following diafiltration (30kDa MWCO) plus ion-exchange chromatography (d).

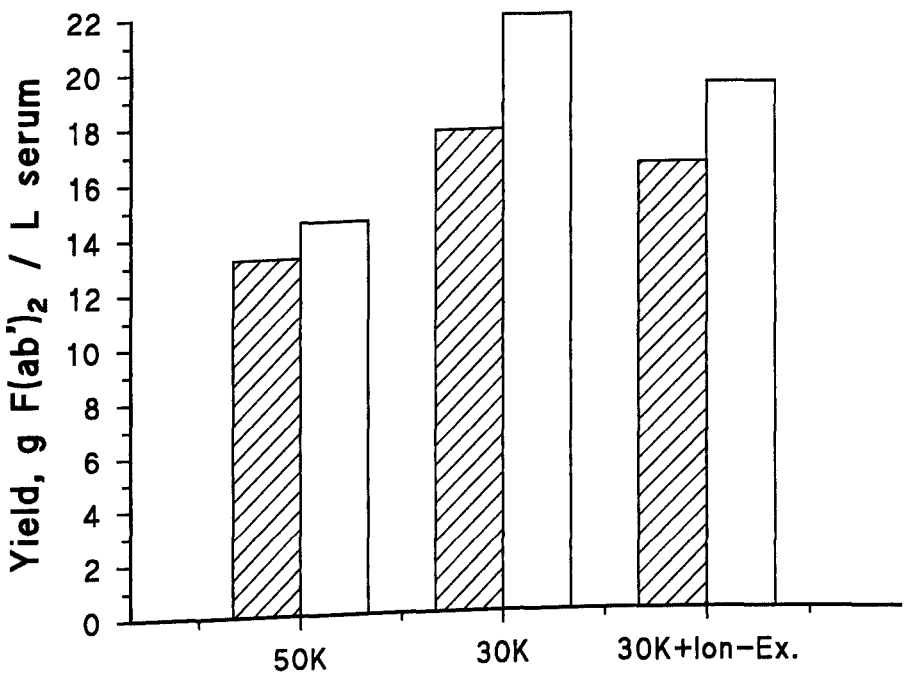
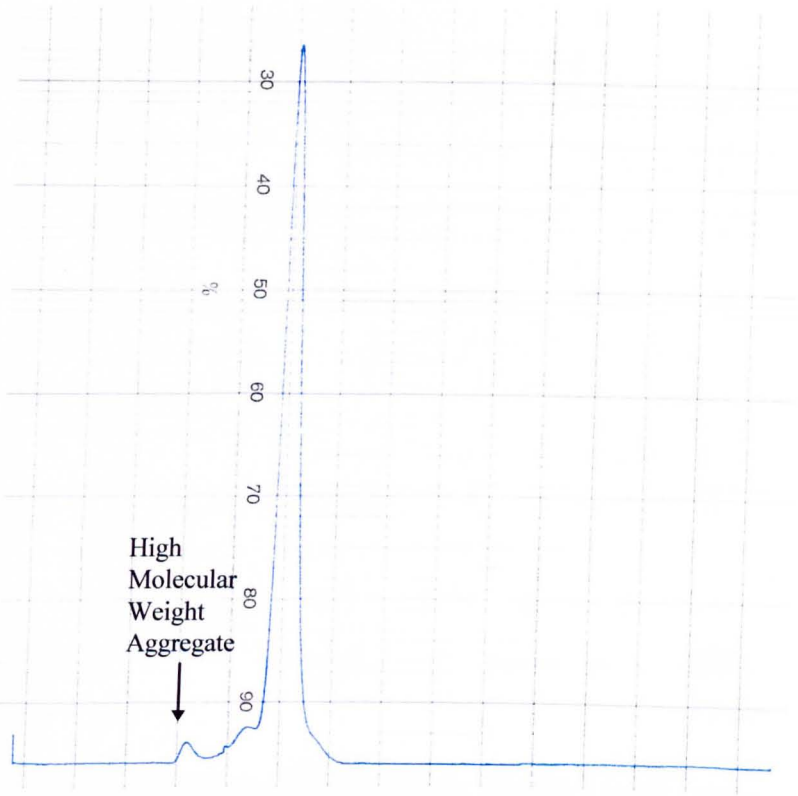


Figure 4.16 The effect of process on yield. Using 50kDa and 30kDa MWCO ultrafilters or diafiltration + ion-exchange chromatography. Serum batch I (hatched bars), or II (open bars).

(a)



(b)

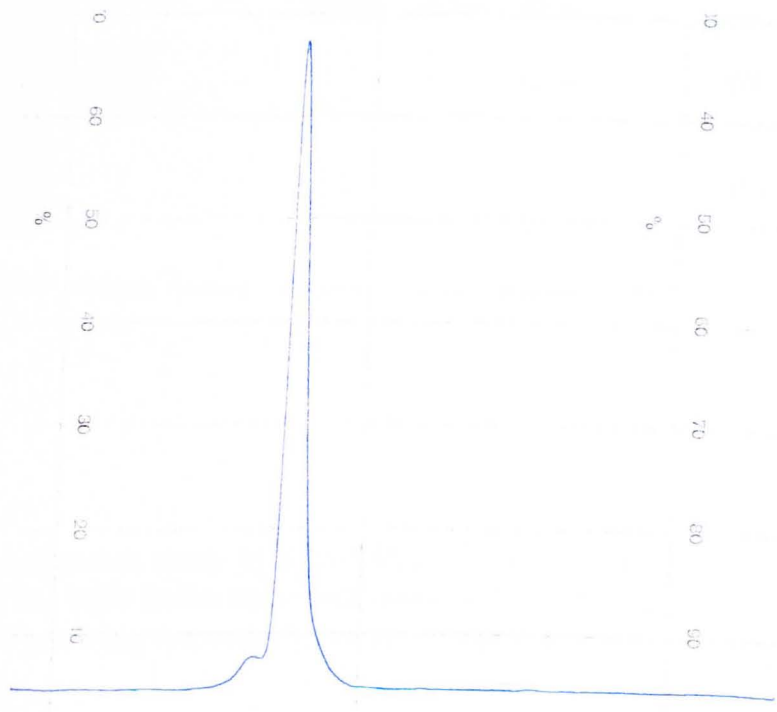


Figure 4.17 Size exclusion chromatography of a commercially available antivenom. CSL Tiger snake antivenom at pH8.0 (a), and pH4.0 (b).

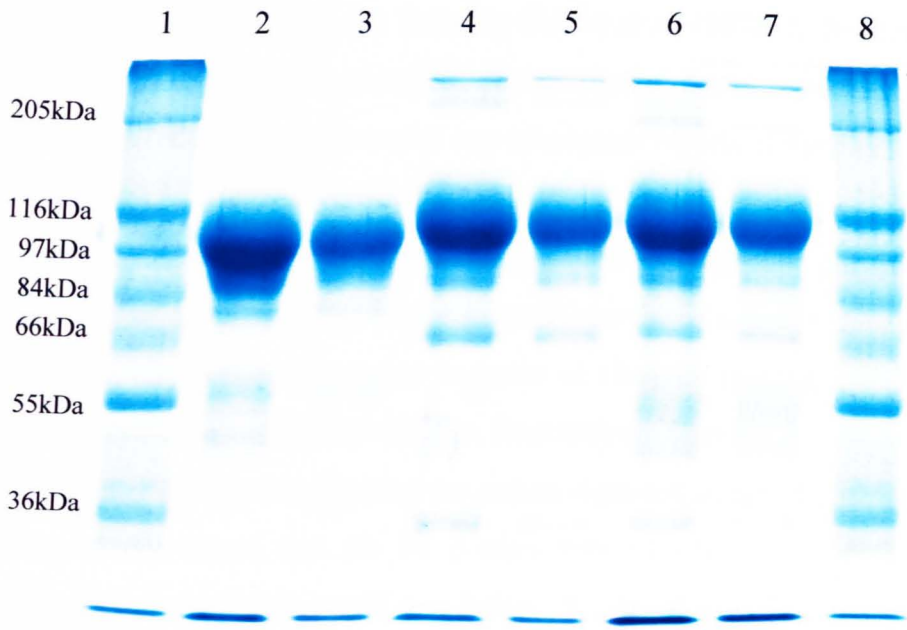


Figure 4.18 Antivenom purity by SDS-PAGE (7.5%, non-reducing). Ovine $F(ab')_2$ (lanes 2 & 3), CSL Tiger Snake antivenom (lanes 4 & 5), Behring Europe antivenom (lanes 6 & 7), 20 and 10μg respectively and molecular weight markers (lanes 1 & 8).

less impurities and the $F(ab')_2$ was of a slightly smaller size than the equine $F(ab')_2$, which is in agreement with calculations from size exclusion chromatography. The main impurity in the ovine material had a molecular weight of ~ 56kDa which was also found in one of the other antivenoms.

4.4 Discussion

The production of antitoxins for therapeutic use must ideally result in a 'highly pure' immunoglobulin fragment, without any Fc or other contaminating proteins or aggregates which may lead to side effects.

Sheep IgG (IgG1 and IgG2) was found by Davies *et al.* (1978) to be rapidly digested by trypsin to $F(ab')_2$ but only slowly by pepsin at pH 4.7 with residual IgG still intact after 48 h at 37°C. We are unaware of any subsequent reports of the digestion of sheep IgG (or serum) by these enzymes.

Although trypsin was confirmed to be highly effective at digesting purified sheep IgG in these studies, when tested on whole serum it was found to preferentially digest the $F(ab')_2$ rather than a highly resistant fragment of albumin, making it unsuitable as a digestion enzyme for antitoxin production from antisera directly.

Similar to other groups investigating the pepsin digestion of IgG from sheep or other species, we also found that the Fc portion was rapidly broken down into low molecular weight fragments at pH 4 or below. At pH 4.5, low levels of protein of a size similar to intact Fc could be seen in the early stages of digestion. After a prolonged digestion, especially at pH 3.0, Fab' was seen as noted by Haber and Stone (1967) using rabbit antibody at pH 2.5. Therefore control of the digestion time was an important factor for the complete digestion of the material without loss of $F(ab')_2$.

Studies of the pepsin digestion of whole ovine serum showed that ovine $F(ab')_2$ is markedly more resistant to digestion by pepsin at a pH of 3.5 than the other serum proteins, allowing their breakdown without any substantial loss of $F(ab')_2$ or of its ability to bind antigen following up to 24 hr digestion. Digestions were, therefore, subsequently performed for between 14-24 hr at pH 3.5. Large increases in ELISA titre seen in the first hour of digestion are most likely due to the exposure of a part of the $F(ab')_2$ around the hinge region previously masked by the Fc domain allowing the

labelled second antibody to recognise the entire molecule and, therefore, represent an artefact of the assay system (Carrico *et al.*, 1974).

It is common practice when performing pepsin digestions to terminate the reaction by inactivating the enzyme with alkali to pH 7.4 - 8.0 (Hudson & Hay, 1989; Stanworth & Turner, 1978; Lin *et al.*, 1993). When high concentrations of pepsin were used, a pH of ≥ 8.0 was required to permanently inactivate all the enzyme after 2 hr at 37°C. Unfortunately if the pH of serum or IgG digests was raised to ≥ 8.0 for 2hr or more at 37°C to inactivate the enzyme, a large amount of a new product was formed with a molecular weight of ~ 50kDa which appeared to represent the breakdown of $F(ab')_2$ to Fab by an unknown mechanism. Because of the high pH required to denature all the pepsin irreversibly to an inactive form and the detrimental effects of these conditions on the $F(ab')_2$, this approach was abandoned in favour of stopping the digestion using conditions under which pepsin had little or no enzymatic action, e.g. pH 6.0-7.0, and then physically removing the pepsin from the product.

It was discovered during the course of these studies that after an optimal digestion at pH 3.5 a small proportion of the low molecular weight digestion products aggregated or polymerised to form high molecular weight components at pH 6.0 - 8.0 as assessed by size exclusion chromatography. These soluble high molecular weight aggregates were found in both IgG and serum digests but with a larger proportion in serum digests, indicating that they consist of aggregated small fragments of both Fc and non IgG material. Aggregation could not be observed with the SDS-PAGE technique which is presumably due to the anionic detergent (SDS) causing the dissociation of any aggregated protein. Therefore, the ability to run a size exclusion chromatography column over a wide range of pH has allowed a useful insight into the aggregation process seen after a digestion, and allowed a process to be devised which removes these troublesome components.

Although aggregates and complement activation have been implicated in adverse reactions to antivenom, the exact cause remains elusive. However, numerous studies have been performed to elucidate the cause of human intravenous IgG reactions. Different types of aggregates have been shown to either cause complement activation or produce a hypotensive response in a rat model which results from the activation of phagocytes via Fc receptors (Bleeker *et al.*, 1987; 1989; 2000). It has also been shown

by Lobatto *et al.* (1987) that small quantities (10μg/kg body weight) of soluble IgG aggregates experimentally injected i.v. in man are rapidly cleared ($t_{1/2}$, 26min) from the circulation predominantly via the liver. Thus it now appears likely that reactions to human IgG may be due to the release of inflammatory mediators caused predominantly by phagocyte activation in the liver through the interaction of IgG dimers or aggregates with Fc receptors (Bleeker *et al.*, 1987; 1989; 2000; Lobatto *et al.*, 1987; Jenei *et al.*, 1991; Sylvestre *et al.*, 1996). It is conceivable that aggregated animal Fc fragments found in varying concentrations in antivenoms could produce similar effects. Some antivenoms are potent activators of the complement system which may be linked to their aggregate concentration. However, clinical studies have failed to find evidence of *in vivo* complement activation following antivenom treatment indicating that the amounts used are insufficient to result in a measurable effect (Sutherland, 1977; Malasit *et al.*, 1986; Waldesbuhl *et al.*, 1970; Ramasamy *et al.*, 1997). Therefore, it seems unlikely that complement activation alone could account for the clinical reactions produced by antivenoms. A combination of these effects may, however, be responsible for the non IgE mediated reactions produced by i.v. antivenom administration.

European Pharmacopoeia guidelines outline the use of size exclusion chromatography to measure aggregate / polymer levels in human intravenous immunoglobulin preparations, which should represent not more than 3% of the total area of the chromatogram, and also stipulate that anticomplementary activity should be less than 1CH50 per mg of immunoglobulin (European Pharmacopoeia, 1997).

If the salt concentration of the digest was reduced to ≤ 60mM NaCl by dilution, it was found that a large proportion of the aggregating material became unstable, precipitated, and was then easily removed by centrifugation or filtration. This reduction in the amount of aggregating material helped in its elimination, as diafiltration at pH6.0 was ineffective in this respect.

Following the preliminary results it seemed logical to utilise a purification step such as diafiltration which takes advantage of the large difference in size between the unwanted non-aggregated low molecular weight fragments and the $F(ab')_2$, found in the pepsin-digested serum. Because of results showing that the crude digest was free from high molecular weight aggregates at a low pH, attempts at diafiltration were

initially successfully performed at this pH on a small scale using 30kDa MWCO centrifuge concentrator units at 4-8°C. However, because of residual pepsin activity and the unstable nature of some of the fragments, attempts to process larger quantities of material using a cross flow ultrafilter at RT over several hours were unsuccessful, as a large amount of precipitate was formed which rapidly clogged the membrane. A range of progressively higher pH conditions were tested and eventually pH 6.0 was adopted at which the enzyme is virtually inactive and at which the cross flow ultrafiltration could be performed unhindered with minimal precipitate formed during the course of diafiltration. High yields of $F(ab')_2$ were produced with a membrane of the correct pore size (30kDa MWCO) under these conditions, but 34% extra losses were produced when a 50kDa MWCO membrane was used. This is particularly relevant in the large scale production of therapeutic products where cross flow ultrafiltration units are routinely used. Smaller bench scale volumes (<20mL) could also be processed easily using the centrifugal concentrator units.

Unfortunately diafiltration was found to be particularly ineffective at removing pepsin even when a 50kDa MWCO ultrafilter was used, which may reflect pepsin's ability to bind or form complexes with the digestion products (Ryle & Porter, 1959).

Although the material purified by digestion and diafiltration alone was reasonably pure (94% by size exclusion chromatography) and in high yield (21.9g/L serum), a further ion exchange step was developed to remove the highly acidic contaminants (pepsin and residual aggregated material).

Because of the exceptionally low (2.2) isoelectric point of pepsin (Jonsson, 1972), it will bind to an anion exchange matrix in the presence of 150mM NaCl at pH 6.0. Since this pH is below the isoelectric range for most of the $F(ab')_2$ these fragments passed straight through the column at this salt concentration. Just as importantly, other acidic residual fragments including the high molecular weight aggregates were also bound to the column under this concentration and were removed.

A new commercial process recently introduced by Aventis-Pasteur utilises two ion exchange steps to improve the potency, purity and safety (4% side effects) of its equine based pepsin digested products, removing the majority of both the low molecular weight contaminants and the high molecular weight aggregates previously found (Grandgeorge *et al.*, 1996; Pepin-Covatta *et al.*, 1997; Chippaux *et al.*, 1999).

This anion exchange step was, therefore, used as a final step to remove any residual aggregating fragments and pepsin, and resulted in a product of over 96% purity (by size exclusion chromatography) and a yield of 19.3g $F(ab')_2$ / L serum. These yields compare favourably with the 6-14g $F(ab')_2$ / L equine plasma reported by Benanchi *et al.* (1988) and the 97% ($F(ab')_2$ + Fab') purity by size exclusion chromatography described by Grandgeorge *et al.* (1996) for the new Aventis-Pasteur antivenom, and 87% for the old IPSER Europe antivenom.

As well as producing a highly refined $F(ab')_2$ at high yield, the new method also avoids any salt precipitation steps which are time consuming; difficult to perform on a large scale under sterile conditions; and may result in large (~18%) losses and the formation of aggregates (Schultze *et al.*, 1965; Friesen, 1987).

To summarise, a new simple high yield method of processing serum to a highly pure $F(ab')_2$ was developed which should be suitable for the large scale production of therapeutic antibody fragments. This consisted of:

i) Adjusting the pH of sheep antiserum to 3.5 with 0.36 M HCl.

ii) Adding pepsin at an enzyme to substrate ratio of 45.2 U/mg substrate (1:50 w/w) and incubating at 37°C for 14 - 18 hr.

iii) Stopping the digestion by adjusting the pH to 6.0 with 50mM piperazine and removing the resulting precipitate by centrifugation or filtration.

iv) Diafiltration (washing) with at least 10 volumes of piperazine buffer (20mM piperazine, 150mM NaCl, pH 6.0) using a 30 kDa molecular weight cut off membrane.

v) Passing the material through an anion exchange column (Q Sepharose FF) equilibrated with the piperazine buffer.

vi) Concentration and formulation.

CHAPTER 5 :

LATRODECTUS VENOM AND THE DEVELOPMENT OF A NEW ANTIVENOM

5.1 Introduction

Spiders of the genus *Latrodectus* are distributed widely across the World and there are four major species of 'black widow' spiders found in the United States: *Latrodectus hesperus*, *La.mactans*, *La.variolus* and *La.geometricus*. About 2,500 bites by this genus are reported each year to the US poison control centres, which probably represents a considerable underestimate of the true number (Litovitz *et al.*, 1991 - 2000).

Poisoning caused by the bite of this spider can run a serious and dramatic course, although the victims' lives are not generally in danger. Nonetheless, before the introduction of antivenom therapy, and partly due to the underreporting of less severe cases, the mortality rate was estimated at 5% (Ingram & Musgrave, 1933).

A variety of signs and symptoms have been noted but principally consist of agonising and often unbearable pain both locally and in parts remote from the bite site, cramping pain or spasms, muscular stiffness, sweating, lacrimation, bronchitis, rhinitis, hypertension, tachycardia, bradycardia, nausea and vomiting, erythema, oedema, and 'pins and needles'. The most severe effects usually start to dissipate after about 24 to 36 hours, but a full recovery is often protracted and may take up to four weeks (Maretic & Stanic, 1954; Southcott, 1976; Banham *et al.*, 1994; White *et al.*, 1995).

All the effects of envenoming are thought to be produced by a 130 kDa molecular weight venom component called α-latrotoxin which comprises ~ 3 - 6% of the venom and specifically binds with high affinity ($K_d \sim 10^{-10}$M) to the cell surface proteins, latrophilin and neurexin, found on presynaptic neuronal membranes and, thereby, stimulates release of neurotransmitters and peptides from neuronal and endocrine cells (Grasso, 1976; Gorio *et al.*, 1978; Scheer & Meldolesi, 1985; Meldolesi *et al.*, 1986; Rosenthal & Meldolesi, 1989; Grasso & Mastrogiacoma, 1992; Pashkov *et al.*, 1993; Grishin, 1998; Lang *et al.*, 1998; Liu & Misler, 1998). This is thought to be brought about by modulation of the secretory machinery by the toxin bound receptor causing an enhancement of the depolarisation-evoked secretion as well as spontaneous release

(Liu & Misler, 1998; Rahman *et al.*, 1999; Saibil, 2000). Recent results using cryo-electron microscopy have shown that, in a solution containing Ca^{2+} or Mg^{2+}, the toxin forms active tetramers at concentrations above 0.3nM (39μg/L) with an axial channel and, using their hydophobic base, can insert into lipid bilayers containing the receptor protein to form a pore allowing the influx of calcium ions and the efflux of neurotransmitters (Ashton *et al.*, 2000; Orlova *et al.*, 2000; Volynski *et al.*, 2000).

Treatment of latrodectism is principally directed at relieving the severe pain using narcotic analgesics or antivenom, but no large scale controlled studies have been performed to determine their relative efficacy (Miller, 1992). Consequently, therapy in the United States as elsewhere, has evolved largely from anecdotal experience with relatively few patients. Other therapies that have been recommended include hot baths, and the administration of calcium gluconate, muscle relaxants, and benzodiazepines (Timms & Gibbons, 1986; Miller, 1992; Maretic, 1983; Zukowski, 1993; Barron, 1960; Clark *et al.*, 1992; Blair, 1934).

The use of narcotic analgesics together with benzodiazepines has been advocated for pain relief, but requires hospital admission for observation as repeated large doses are normally required (Miller, 1992; Clark *et al.*, 1992). However, as severe latrodectism can cause breathing difficulties the use of drugs which induce respiratory depression may be contra-indicated (Muller, 1993; Zukowski, 1993).

In contrast, the use of antivenom has repeatedly been found to produce spectacular results, with a complete recovery often occurring after just 30 min (Wiener & Fraser, 1956; Sutherland & Trinca, 1978). Indeed antivenom has been reported to be highly effective even when administered many days (90 and ≥120 days) after the bite in victims with an exceptionally prolonged sequela (Southcott, 1961; O'Malley *et al.*, 1999; Sutherland, 1992; Banham *et al.*, 1994).

Because the only antivenom available in the United States comprises unrefined horse serum its use is limited to patients with signs of severe envenoming or with a high risk of developing complications e.g. age under 16 or over 60 years, respiratory difficulty, marked hypertension, pregnancy, or distress that does not respond to other measures (Miller, 1992; Russell, 1996). This limited use reflects the high incidence of life-threatening and, occasionally, fatal reactions that may be associated with the systemic administration of the available Merck product (Miller, 1992; Clark *et al.*, 1992;

Zukowski, 1993; Kobernick, 1984). Nevertheless, the response is usually dramatic, with full and lasting relief of symptoms without the need for further antivenom or hospital admission, unless reactions to the antivenom occur (Clark *et al.*, 1992; Miller, 1992; Zukowski, 1993; Timms & Gibbons, 1986; Sutherland, 1992).

Stringent restrictions on antivenom use are not found in countries such as Australia, where antivenom is widely available and consists of a refined equine $F(ab')_2$ product (CSL Red back spider antivenom). Indeed it is used more than any other antivenom in this region with relatively few reactions, none of which have proved fatal (Sutherland & Trinca, 1978; Sutherland & Lovering, 1979; Sutherland, 1992).

Highly refined ovine Fab-based therapeutic products such as Digibind™ have an excellent safety record with an incidence of adverse reactions of less than 1% (Kirkpatrick, 1991). Such products clearly demonstrate that a safe antibody-based treatment could be developed for black widow spider envenoming in North America. The current studies were, therefore, carried out in response to urgent requests by clinicians for such a safe product (Zukowski, 1993).

5.2 Methods

5.2.1 Venom Extraction Methods

Due to the perceived problems in obtaining and maintaining a spider colony in the UK, the electrically stimulated venom and intact venom glands were obtained from a specialised supplier in America (Spider Pharm Inc.).

5.2.1.1 Electrical stimulation

Spiders were anaesthetised with carbon dioxide to the point where they could be picked up without danger to the handler and without trauma to themselves. A small suction tube was then placed in the spider's mouth to remove any secreted juices or vomit that may be produced during stimulation. Mild electrical stimulation was applied and venom extruded from the fang tips collected into a micro-manipulated pipette. The volume of pooled venom from a batch of spiders was then measured and the pool frozen. When required, batches of venom were thawed, pooled further and

diluted in PBS and the protein concentration determined using the BCA assay. The pooled batches were sub-aliquoted and frozen.

5.2.1.2 Venom gland extract

Venom glands (15 pairs) obtained from adult female *La.hesperus* spiders were ground up in 1 mL of PBS using a pestle and mortar, centrifuged and the protein concentration of the supernatant determined using the BCA protein assay. A further 15 pairs of glands were gently squeezed using a glass rod in 1 mL of PBS and the protein concentration of the supernatant determined.

5.2.2 SDS-PAGE Analysis

Venoms and antivenoms were assessed by SDS-PAGE as described in Chapter 2, using either non-reducing or reducing conditions in which samples were treated with mercaptoethanol and heated at 95°C for 10 min.

5.2.3 Immunisation of Sheep

5.2.3.1 *Latrodectus hesperus* venom immunogen

Antibodies were raised by immunising nine sheep in groups of three with up to a maximum of either 0.25mg (low dose group), 1mg (medium dose group) or 2mg (high dose group) every 28 days of *La. hesperus* venom. Blood samples were taken at set time intervals (Table 5.1).

5.2.3.2 *Latrodectus tredecimguttatus* venom and α latrotoxin immunogen

Antibodies were also raised to *La. tredecimguttatus* venom and the purified component α-latrotoxin using immunisation doses of up to 2mg and 10μg per sheep respectively.

5.2.3.3 Mixed *La. hesperus* and *Loxosceles reclusa* venom immunogen

Three sheep were immunised with 0.25 mg of *La. hesperus* venom mixed with up to 8μg of *Loxosceles reclusa* venom per sheep (Table 5.2) as *Loxosceles* venom has been reported to have an adjuvant type effect (Barbaro *et al.*, 1994; Mota & Barbaro, 1995).

		Immunogen dose (mg) per sheep		
Immunisation / Sample / Bleed	**Weeks Post Primary Immunisation**	**Low Dose Group**	**Medium Dose Group**	**High Dose Group**
Pre-Immunisation Sample	0			
Primary Immunisation	0	0.25	0.25	0.25
Re-Immunisation	4	0.25	0.5	0.5
Sample	6			
Re-Immunisation	8	0.25	1.0	1.0
Sample	10			
Re-Immunisation	12	0.25	1.0	2.0
Sample	14			
Re-Immunisation	16	0.25	1.0	2.0
Sample	18			
Re-Immunisation	20	0.25	1.0	2.0
Sample	22			
Re-Immunisation	24	0.25	1.0	2.0
Bleed	26			
Re-Immunisation	28	0.25	1.0	2.0
Bleed	30			
Bleed	34			
Re-Immunisation	36	0.25	1.0	2.0
Bleed	38			
Bleed	42			
Hold	44			

Table 5.1 *La. hesperus* venom immunisation protocol.

Procedure	Weeks Post Primary Immunisation	Immunogen (μg) per sheep	
		La.hesperus Venom	*Lo.reclusa* Venom
Pre-Immunisation Sample	0		
Primary Immunisation	0	250	2
Re-Immunisation	4	250	4
Sample	6		
Re-Immunisation	8	250	8
Sample	10		
Re-Immunisation	12	250	8
Sample	14		
Re-Immunisation	16	250	8
Sample	18		
Re-Immunisation	20	250	8
Sample	22		
Re-Immunisation	24	250	8
Bleed + Sample	26		
Re-Immunisation	28	250	8
Bleed + Sample	30		

Table 5.2 Mixed venom immunisation protocol

5.2.4 ELISA for Specific Antibody Levels to Whole Venom or α Latrotoxin

ELISA plates were coated with 2μg/mL of whole venom and different concentrations of α latrotoxin and antiserum samples assessed for specific antibody as described in Chapter 2.

5.2.5 Enzyme Digestion and Processing of Antiserum

Papain-digested Fab fragments were prepared as described in Chapter 3 and pepsin digested $F(ab')_2$ fragments by the method developed in Chapter 4.

5.2.6 Phrenic Nerve Hemidiaphragm Preparation

Left phrenic nerve-hemidiaphragm preparations were prepared as described in Chapter 2.

The ability of the antivenom to neutralise the effects of the venom was assessed by premixing a fixed venom concentration (10mg/L) with antivenom and incubating at 37°C for 30 min before addition to the hemidiaphragm preparation (t_0). The tissue was washed with fresh Krebs buffer after 30 and 60 minutes.

The ability to reverse the neurotoxicity was assessed by washing out the venom after 30 min and adding antivenom.

5.2.7 *In vivo* Neutralisation

Murine LD_{50} and ED_{50}s were performed as described in Chapter 2.

5.3 Results

5.3.1 Venom Extraction Methods

The protein concentration of electrically stimulated *La.hesperus* venom was found to vary between 50 - 80 μg / μL with different batches.

Ground *La.hesperus* venom glands produced 1.75 mg of protein from 15 pairs of glands (or 117 μg per spider) compared to 890μg from 15 pairs of gently squeezed venom glands (or 59 μg per spider).

The venoms extracted using the three different methods were analysed by SDS-PAGE under reducing conditions and compared with a sample of regurgitated fluid obtained

from the mouth of *La.hesperus* spiders during electrical stimulation (Figure 5.1). The electrically stimulated venom contained 7 bands, of which the first 4 ran closely on the gel with molecular weights of between 100 - 130 kDa and were the predominant venom components. The other components had molecular weights of 82, 47 and 44kDa. The regurgitated material consisted of 5 bands, of which the predominant one had a molecular weight of 67 kDa. This 67kDa protein was completely absent from the electrically stimulated venom, indicating that it consisted of pure uncontaminated venom. The two venom gland preparations appeared identical and contained more than 13 bands. The 6 bands seen in the electrically stimulated venom were all present in these preparations although in different proportions and numerous new bands were found, the predominant one with a molecular weight of 67 kDa.

5.3.2 A Comparison of *Latrodectus* Venoms and Antivenoms from Around the World.

5.3.2.1 *Latrodectus* venoms

All the venoms from the different species tested by SDS-PAGE contained a major band of the same molecular weight as α-latrotoxin (Figure 5.2). Only minor differences were evident between the two North American species *La. hesperus and La. mactans* with *La. mactans* containing a larger proportion of α-latrotoxin sized material. These two venoms were obtained by electrical stimulation from the same supplier. The *La. hasselti* and *La. tredecimguttatus* venoms were both obtained from venom gland homogenates and had a high background compared to the electrically stimulated venoms, a smaller proportion of the α-latrotoxin sized material and some major bands not seen in the North American spider venoms.

5.3.2.2 *Latrodectus* antivenoms

The commercially available North American Merck & Co., Inc. antivenin (Lot 0948J, Exp. 17 May 2002) consisted of freeze dried material containing 1:10,000 thimerosal (mercury derivative preservative) and was reconstituted with the water for injection provided (2.5mL) and rapidly dissolved to give a clear viscous liquid. The material had a protein concentration of 106 mg/mL using the BCA protein assay with a BSA standard and, therefore, each vial contained 265 mg which is sufficient for the

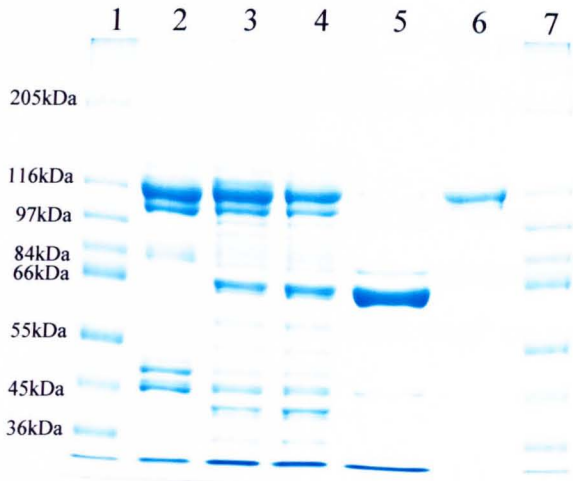


Figure 5.1 *La.hesperus* venom extraction methods, as assessed by SDS-PAGE (7.5%) under reducing conditions. Electrically stimulated venom (lane 2), venom gland homogenate (lane 3), squeezed venom glands (lane 4), regurgitated juices (lane 5), α latrotoxin (lane 6), and molecular weight markers (lanes 1 & 7).

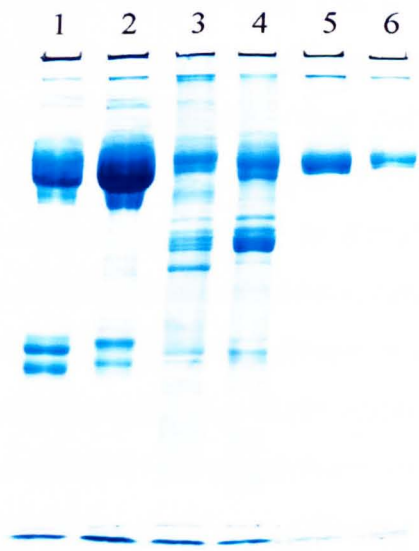


Figure 5.2 A comparison of *Latrodectus* venoms from around the World by SDS-PAGE (10%) under non-reducing conditions. *La.hesperus* (lane 1), *La.mactans* (lane 2), *La.hasselti* (lane 3), *La.tredecimguttatus* (lane 4), and α latrotoxin, 5 or 2.5μg (lanes 5 & 6).

treatment of a black widow spider bite. The antivenom appeared identical to normal horse serum by SDS-PAGE, with two major bands representing IgG and albumin (Figure 5.3). The Merck antivenom also appeared to contain a large (~10%) proportion of high molecular weight material by size exclusion chromatography (Figure 5.4), the majority of which presumably represents polymerised or aggregated material.

The Australian (CSL) commercially available red back spider antivenom batch 0534 03801 (like one of their snake antivenoms) had no detectable intact IgG or albumin and contained only one major band of $F(ab')_2$ and a small amount of high molecular weight material.

5.3.3. Effects of Venom Immunisation Dose on Titre Against Whole Venom

The antibody responses of individual sheep demonstrated a considerable variation to the *La. hesperus* immunogen and, initially, were not related directly to dose. Thus the maximum titre of 1:1,500,000 was produced by sheep No CN2549 in the low dose group, 10 weeks after the primary immunisation (Figure 5.5). A second high responding sheep (CN2544) in the medium dose group produced a titre of 1:1,100,000 10 weeks after the primary immunisation while all the other sheep produced titres between 1:600,000 - 1:800,000 at this time point. Due to this variability, the small number of sheep per dose group and the staggered immunisation schedule, there was no relationship between the venom immunogen dose and response until after the maximum immunisation doses were injected at week 16 (Figure 5.6). After this time the average response for each group appeared to be directly related to the dose of immunogen. Statistical analysis of the individual titres against *La. hesperus* venom by analysis of covariance (ANCOVA) using SPSS software for the complete sets of data between weeks 22 and 42 revealed a highly significant difference in response between the low and high dose groups ($P<0.001$) and a significant difference between the low and medium groups ($P=0.009$). No significant difference ($P=0.053$) was found between the high and medium dose groups.

5.3.4 Effects of Venom Immunisation Dose on Titre Against α Latrotoxin

An antigen coating concentration of 500ng/mL of α latrotoxin was chosen which

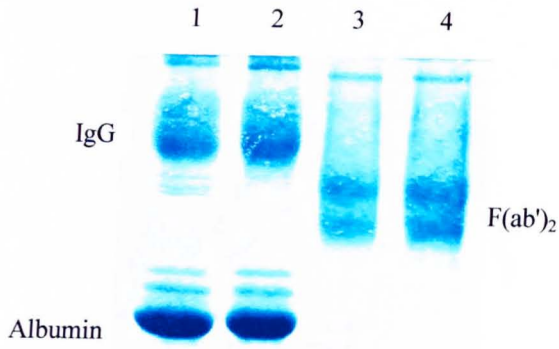


Figure 5.3 A comparison of commercial antivenoms by SDS-PAGE (10%). Normal horse serum (lane 1), Merck black widow antivenom (lane 2), CSL red back spider antivenom (lane 3), CSL brown snake antivenom (lane 4).

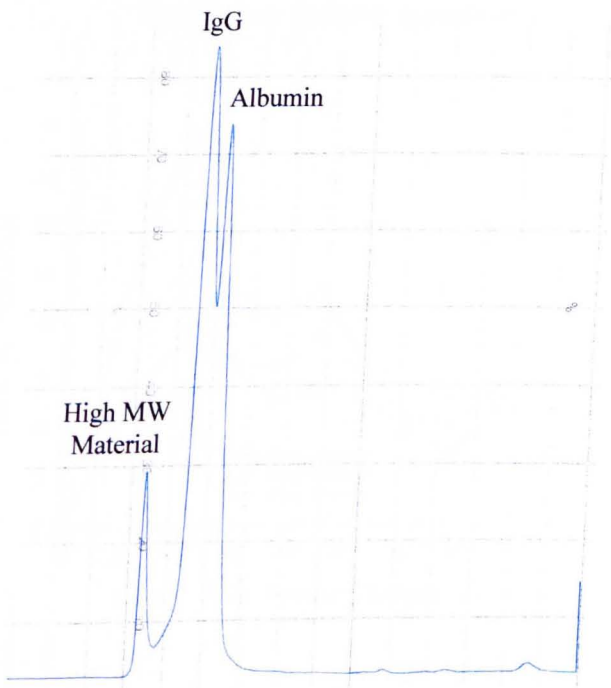


Figure 5.4 Merck Black Widow spider antivenom as assessed by size exclusion chromatography (Superose 12, Tris/saline, pH8.0).

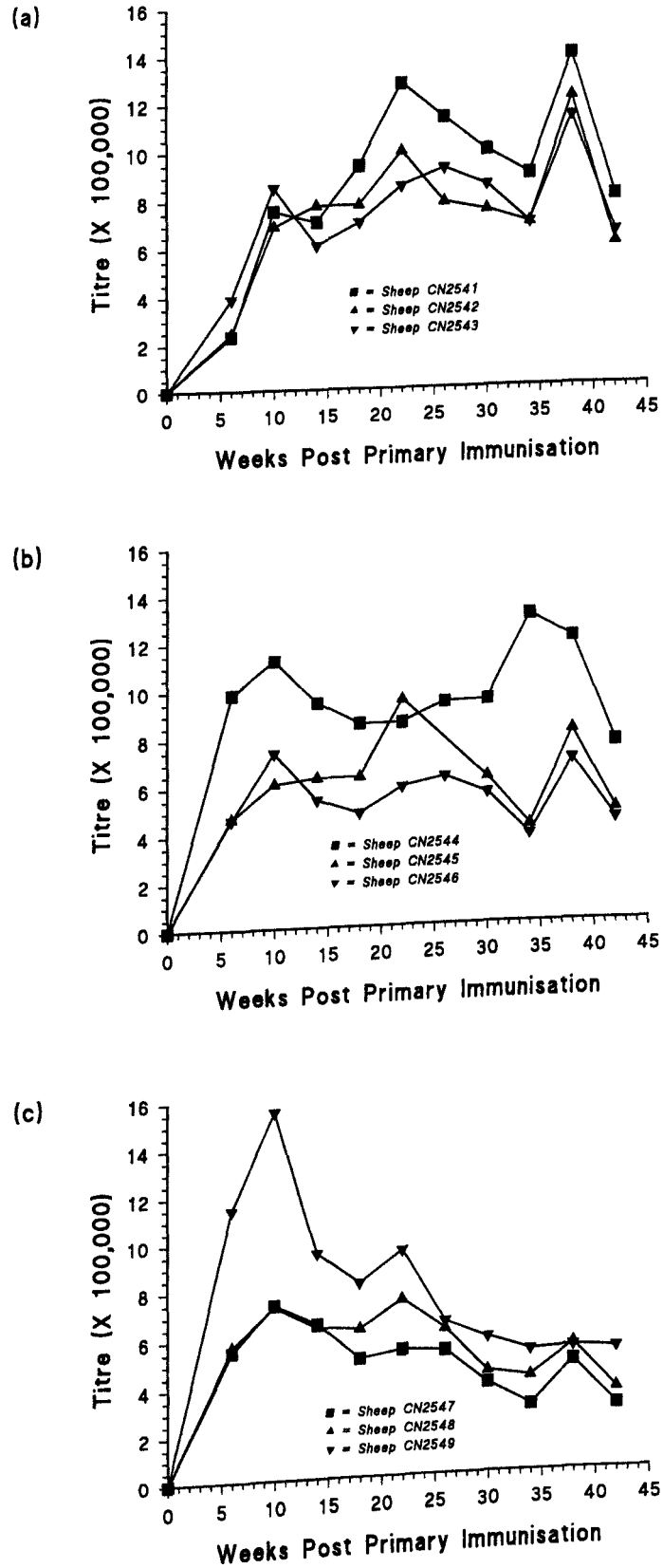


Figure 5.5 Individual anti-*La.hesperus* venom ELISA titres. High (a), medium (b) and low (c) immunisation doses.

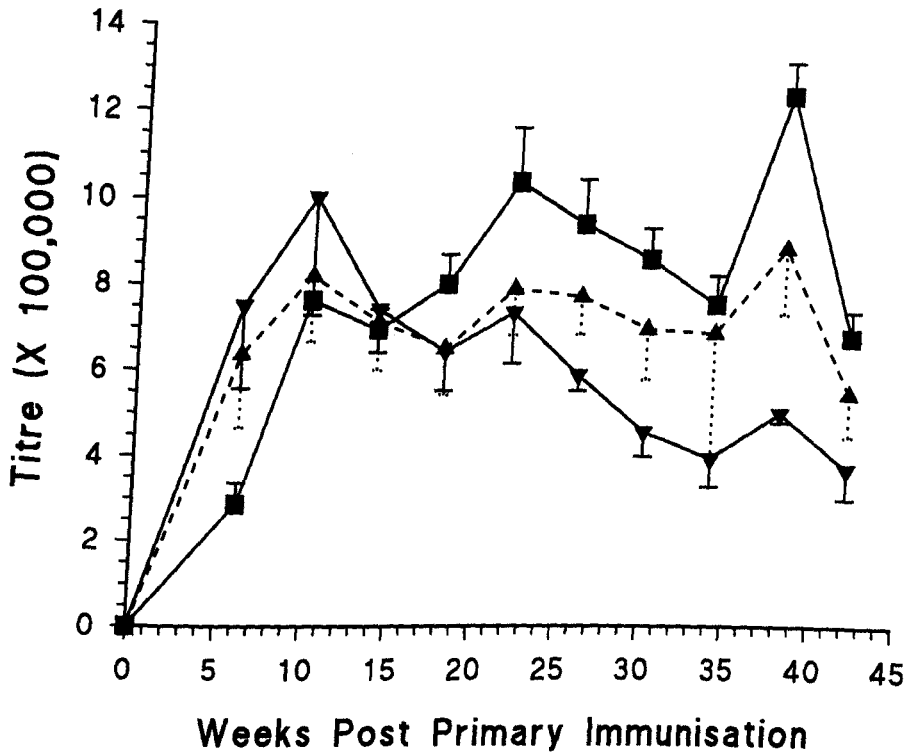


Figure 5.6 Effect of immunogen dose on titre against *La.hesperus* venom. High (■), medium (▲), and low (▼) dose immunisations ±SEM (n=3).

produced a high absorbance reading with specific antisera and relatively low non-specific binding (Figure 5.7). Maximum titres against α latrotoxin for individual sheep immunised with the low, medium and high doses of venom ranged between 1:31,000 to 1:85,000, 1:57,000 to 1:103,000 and 1:58,000 to 1:69,000 respectively (Figure 5.8). The sheep demonstrated a considerable variation in their response to α latrotoxin, with sheep No CN2549 and CN2544 from the low and medium dose groups producing the highest titres at week 10 of 1:85,000 and 1:100,000 respectively. There were, however, variations in the proportion of antibodies against the whole venom that were specific for α latrotoxin; for instance, despite two low dose group sheep No CN2547 and CN2548 producing identical titres against the whole venom at week 10, antisera from sheep CN2548 had over double the level of antibodies specific for α-latrotoxin. Due to this variability, the small number of sheep per dose group and the staggered immunisation schedule there was again no immediate relationship between the venom immunogen dose and average response until after the maximum immunisation doses were injected at week 16 (Figure 5.9). Statistical analysis (ANCOVA) of the data between weeks 18 and 30 revealed a significant (P=0.009) difference in titres to α-latrotoxin between the high and low dose groups. However, there was no significant difference between the titres produced by the medium dose group compared to either the low (P=0.297) or high (P=0.330) dose groups.

5.3.5 Effects of Mixed Venom Immunisation on Antibody Titre.

5.3.5.1 Specific titres to *La.hesperus* venom

Of the three sheep immunised with the mixed immunogen (*La.hesperus* and *Lo.reclusa* venoms), sheep No CN2568 produced the highest antibody titres of up to 2,460,000 against *La. hesperus* venom (Figure 5.10a). No significant (ANCOVA, P=0.307) difference overall could be found in specific titres to *La.hesperus* venom produced by sheep immunised with either *La. hesperus* venom alone or the mixed immunogen, although the titres for week 26 and 30 were significantly (P=0.025) greater in the mixed immunogen group (Figure 5.11a).

5.3.5.2 Specific titres to α latrotoxin

Of the three sheep immunised with the mixed immunogen, sheep No CN2568

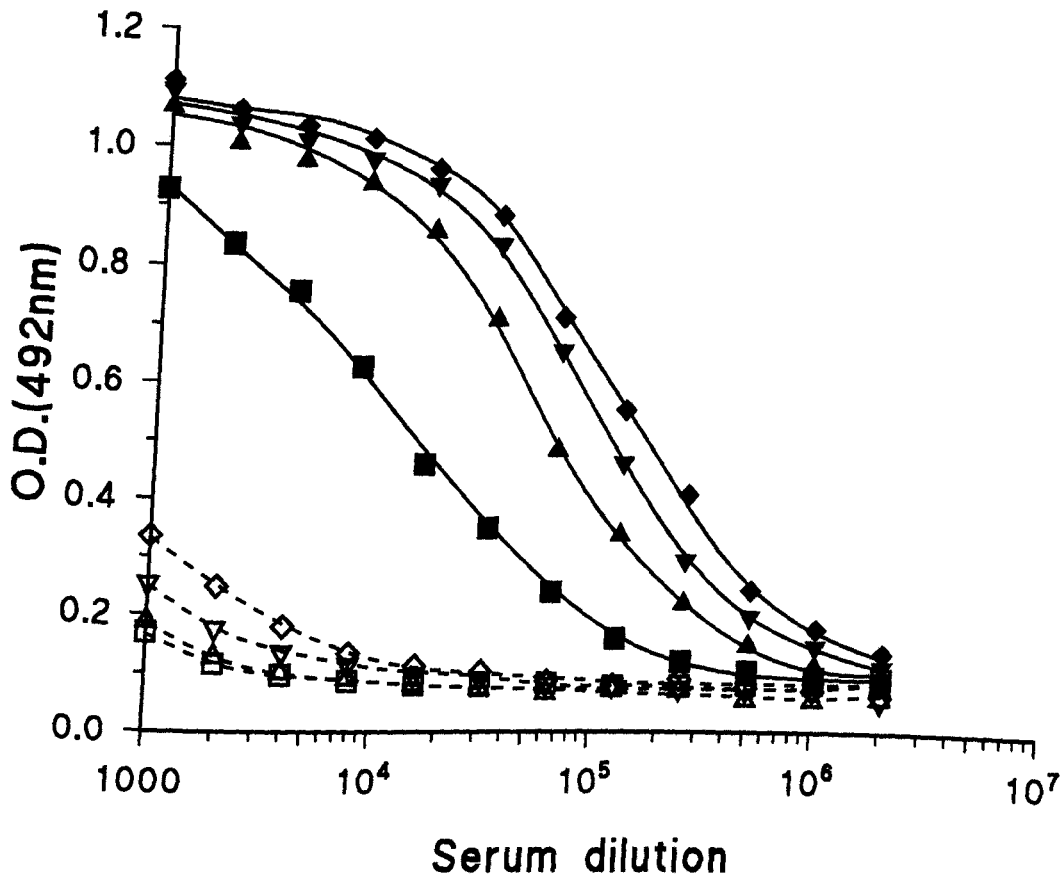


Figure 5.7 Effects of antigen coating concentrations on an anti-α latrotoxin ELISA. 100ng/mL (■), 500ng/mL (▲), 1μg/mL (▼), 2μg/mL (◆), α latrotoxin. Anti-*Latrodectus tredecimguttatus* (solid symbols), NSS (open symbols).

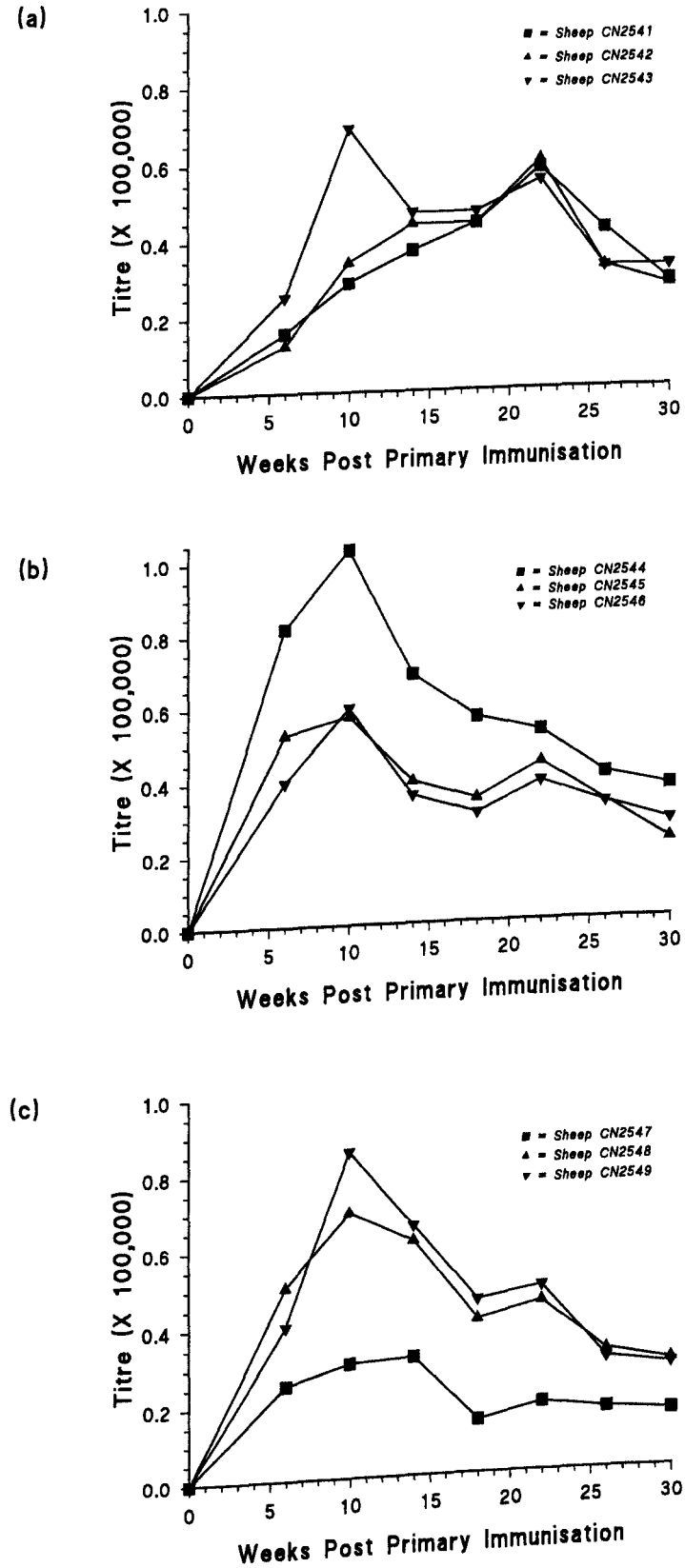


Figure 5.8 Individual anti-α latrotoxin ELISA titres. High (a), medium (b) and low (c) immunisation doses.

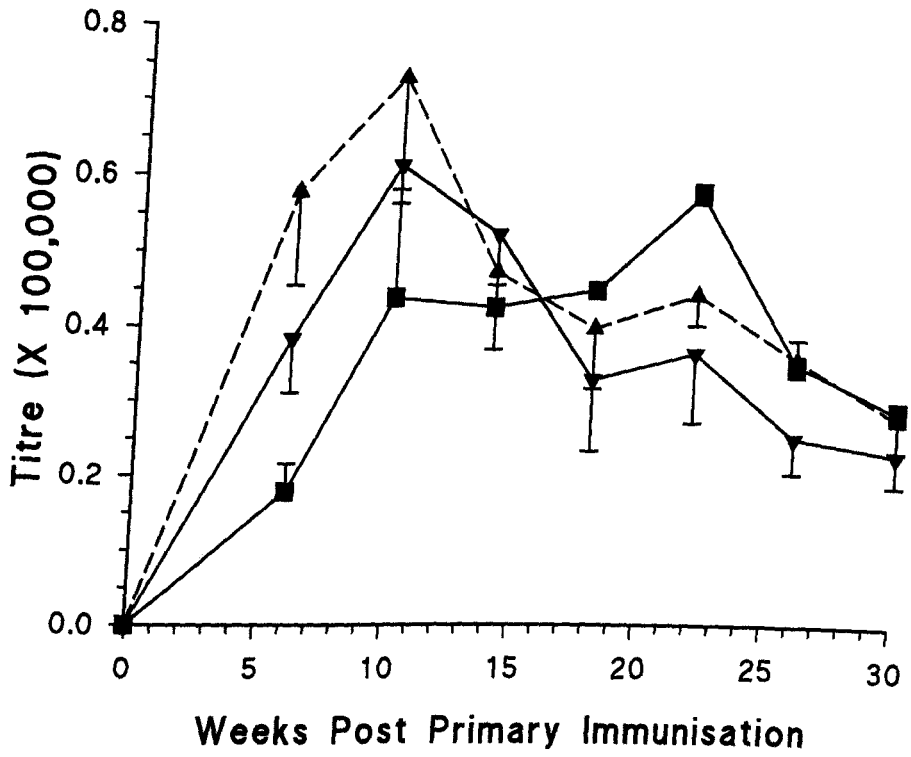


Figure 5.9 Specific antibody levels in sheep against α latrotoxin. High (■), medium (▲) and low (▼) immunisation dose ± SEM(n=3).

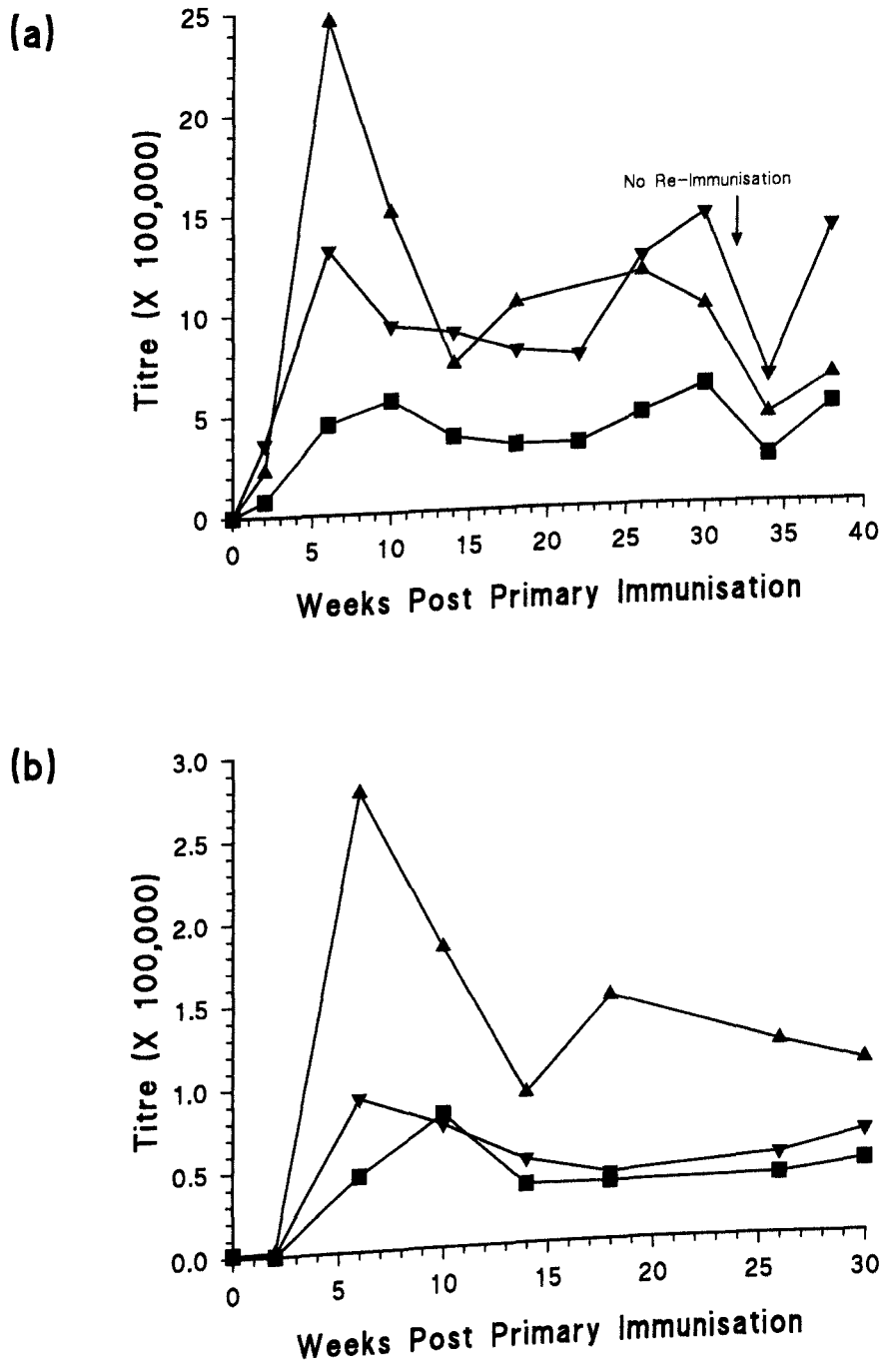


Figure 5.10 Mixed immunogen ELISA titres against *La.hesperus* venom (a), and α latrotoxin (b). Sheep No. CN2567 (■), CN2568 (▲), and CN2569 (▼).

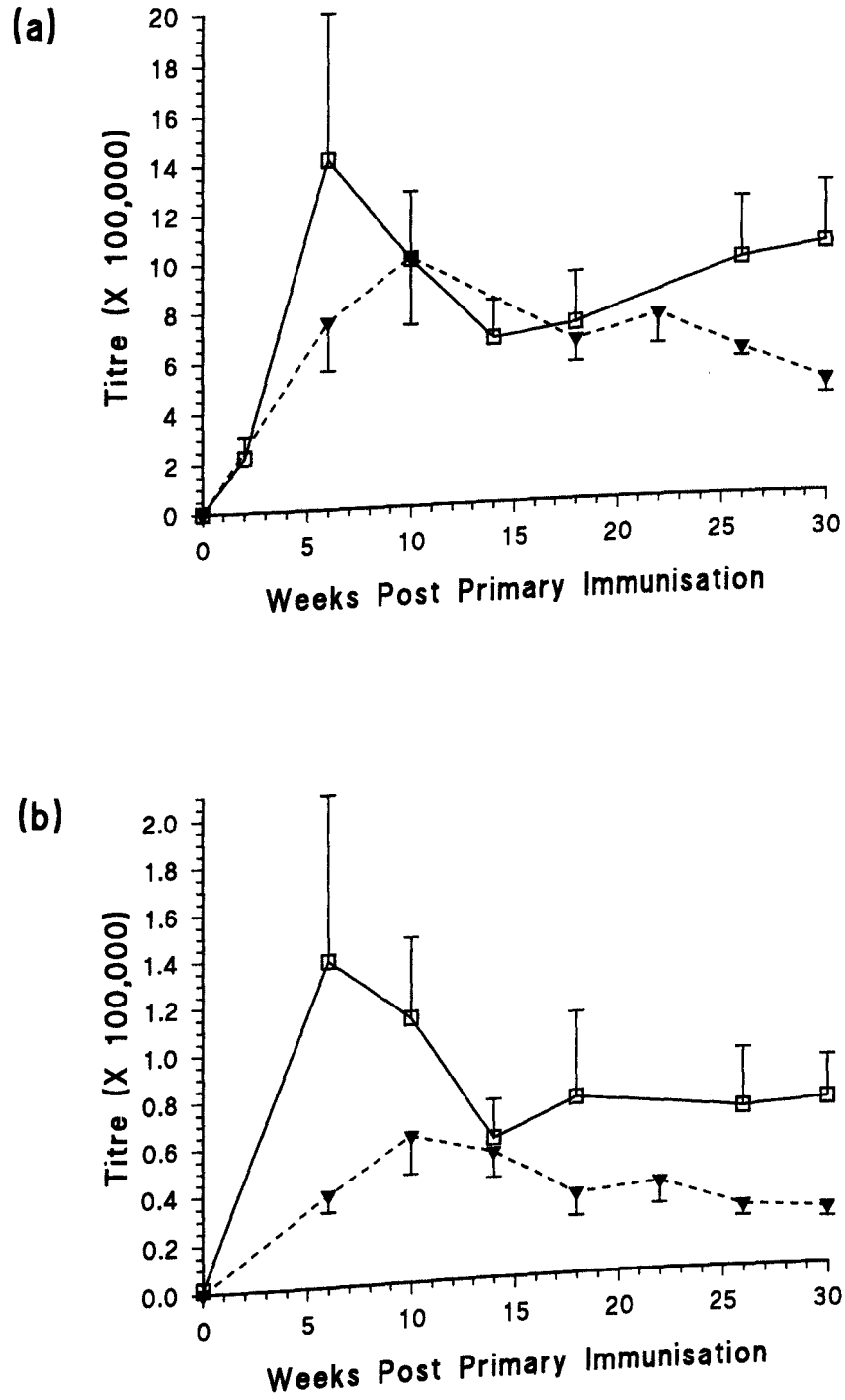


Figure 5.11 The effects of a mixed immunogen on specific ELISA titres. Titres to *La.hesperus* venom (a), or α latrotoxin (b). Sheep immunised with either, 0.25mg *La.hesperus* venom (▼), or *La.hesperus* venom plus up to 8μg *Lo.reclusa* venom (□). ±SEM (n=3).

produced the highest antibody titres of up to 277,000 against α-latrotoxin (Figure 5.10b). Significantly (P=0.007) greater antibody titres overall were found against α-latrotoxin using the mixed immunogen compared to sheep immunised with the same dose of *La. hesperus* venom alone (Figure 5.11b).

5.3.6 Antivenom Processing

5.3.6.1 Sodium sulphate precipitation

Following the sodium sulphate precipitation step it was estimated by OD (using an extinction coefficient of 1.5) that the week 30 high dose antiserum contained ~ 37 g/L of IgG. This however represents an over estimate as it is known that salt precipitation alone results in an impure IgG (Otero *et al.*, 1999; Rojas *et al.*, 1994).

5.3.6.2 Papain digestion

The 24 hr papain digestion of the above purified IgG fraction resulted in material principally consisting of Fab plus some high molecular weight dimers / aggregates and some smaller fragments which may represent over digested Fab or Fc fragments (Figure 5.12). A yield of 17.8 g per L of serum was calculated from the OD but due to the relatively impure nature of the material this represents an overestimate of the Fab yield.

5.3.6.3 Pepsin digestion

Following the new pepsin method of digesting the antiserum for 14 hr and washing the material using a cross flow ultrafiltration unit (30kDa MWCO), a yield of 17.4g of $F(ab')_2$ per L of antiserum was obtained with a purity of 95% by size exclusion chromatography (Figure 5.13a). A similar yield of 17.5g/ L was produced following digestion for 18 hr and, therefore, the 14 hr digestion was adopted. This material still contained a small quantity of high molecular weight aggregate but due to the highly acidic nature of this component it was removed along with pepsin on passage through an anion exchange column at pH 6.0 in the presence of 150mM NaCl. The unbound $F(ab')_2$ material was then concentrated on a cross flow ultrafiltration unit with a resultant overall yield of 16.1g of $F(ab')_2$ per L of antiserum with a purity of 98% by size exclusion chromatography (Figure 5.13b).

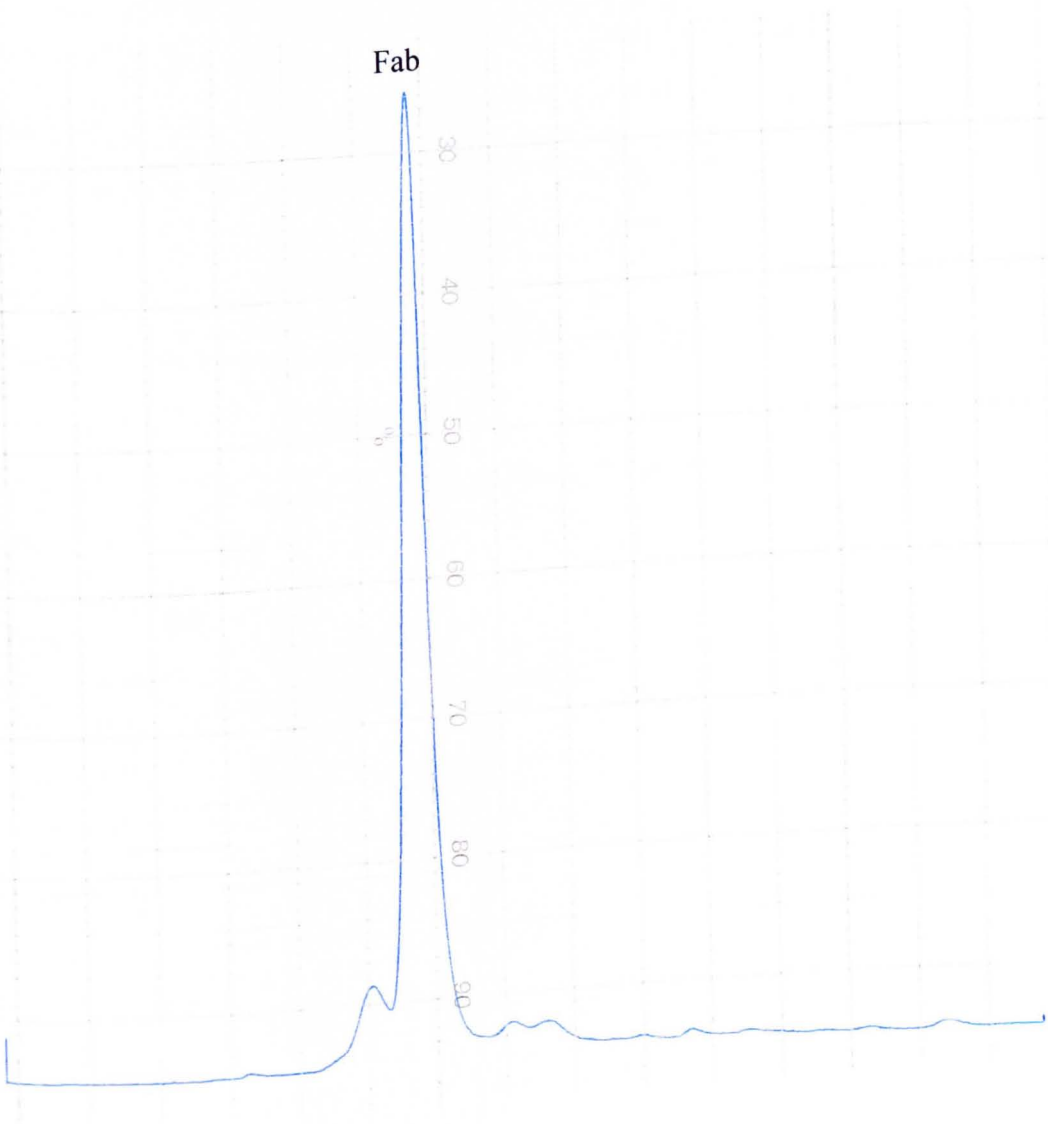


Figure 5.12 Anti *La.hesperus* (high dose) Fab as assessed by size exclusion chromatography (Superose 12, Tris/saline pH8.0).

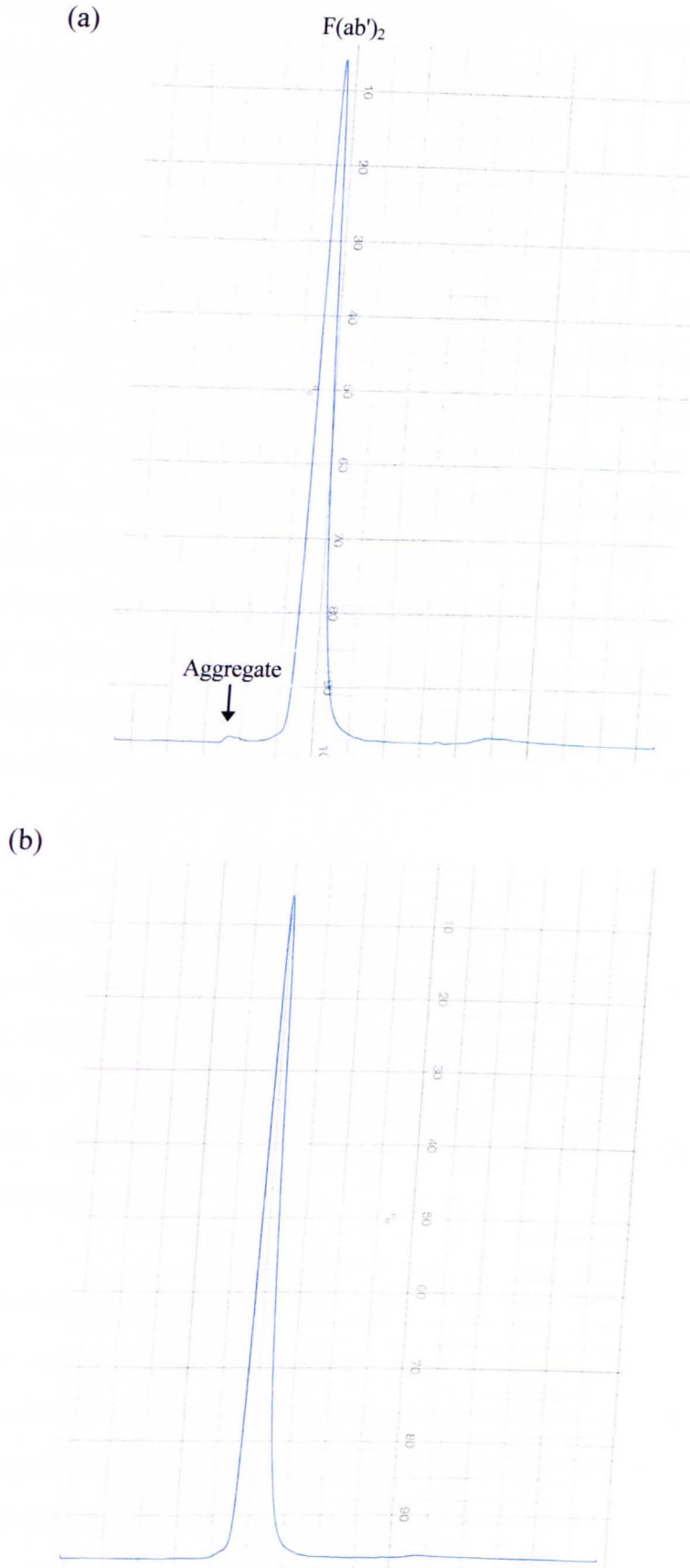


Figure 5.13 Purity of $F(ab')_2$ as assessed by size exclusion chromatography. After diafiltration (a), followed by ion-exchange chromatography (b).

Pepsin was undetectable in this product as assessed with an analytical ion exchange (Mono Q) chromatography column (Figure 5.14).

5.3.7 Effects of Venom and Antivenom on a Nerve Muscle Preparation

5.3.7.1 Venom

The nerve stimulated response was potentiated by 1mg/L of *La.tredecimguttatus* venom reaching a maximum of 115% at 30 min and the amplitude of the twitches became less regular with signs of occasional spontaneous partial twitches (Figure 5.15a). A transient initial potentiation was produced by 3.3 mg/L followed by a slight inhibition. A 10 mg/L concentration produced a rapid blockade within 30 min with an 82% maximum blockade (Figure 5.15a).

A significant potentiation of the directly stimulated muscle contractions was produced over the first 65 min compared to controls by 10 mg/L (ANCOVA, P=0.034), 3.3 mg/L (P=0.002) and 1mg/L (P=0.001) of *La. tredecimguttatus* venom. These effects were of an immediate but transient nature and most pronounced at 35 min (Figure 5.15b).

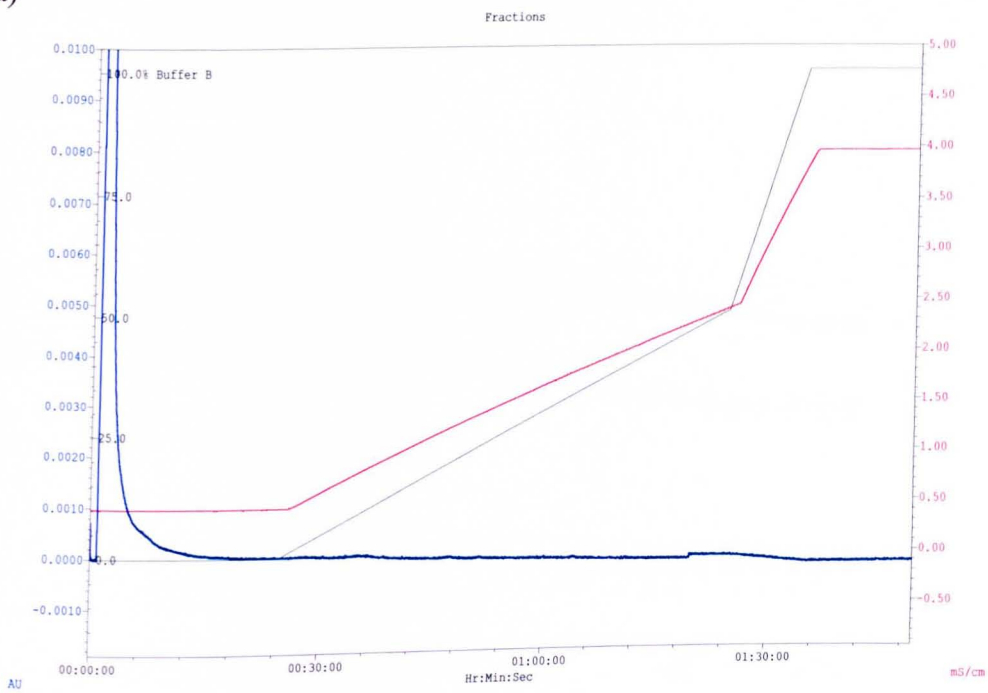
5.3.7.2 Effects of high calcium Krebs on the venom

In the presence of a high calcium concentration (4 mM) the venom (10 mg/L) resulted in a significant (ANCOVA, P<0.001) increase in blockade of the nerve stimulated response with up to 96% inhibition compared to 82% for 1.9 mM calcium (Figure 5.16). There was no significant difference (P=0.971) between the effects on the directly stimulated preparation.

5.3.7.3 Antivenom neutralisation

The effects of the venom (10mg/L) were almost fully neutralised by pre-mixing with 50mg/L of anti-*La.hesperus* high dose Fab and a smaller amount of neutralisation was produced by the medium dose Fab (Figure 5.17). However, there was transient initial potentiation which suggests (Figure 5.15) that neutralisation of the 10mg/L venom was not complete. Only an approximately 50% neutralisation was produced by the low dose Fab. Effects of the venom (10mg/L) were, however, fully neutralised by pre-mixing with 200mg/L of Fab raised against *La.hesperus* venom using either the high

(a)



(b)

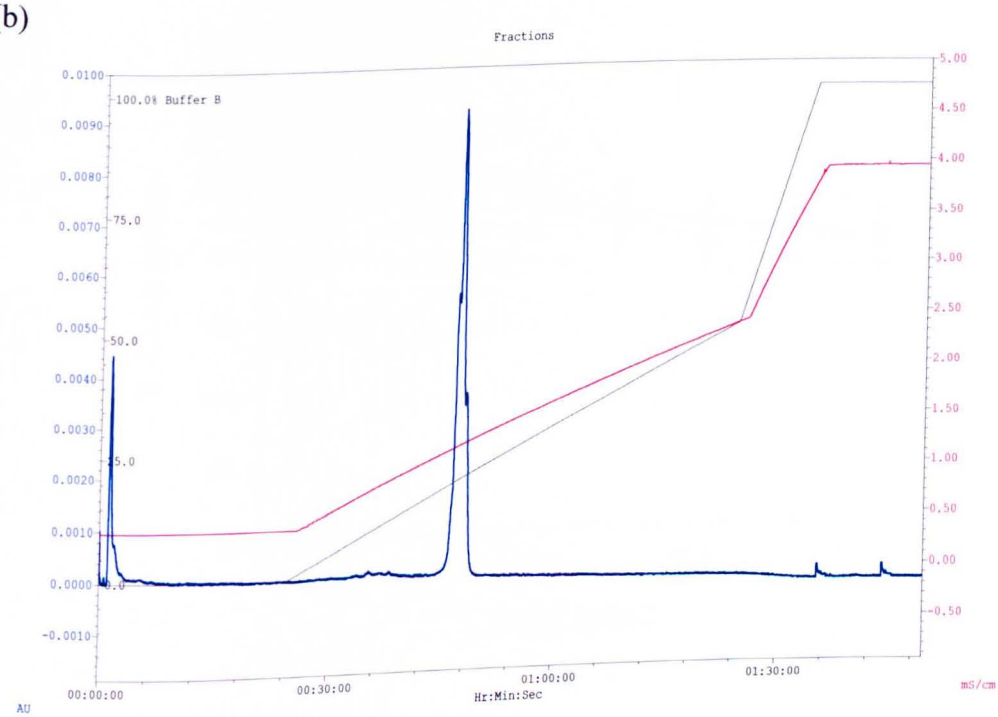


Figure 5.14 Analytical anion-exchange chromatography using a Mono Q column. Anti-*La.hesperus* (high dose) $F(ab')_2$, 2mg (a), or pepsin, 0.2mg (b). Buffer A: 20mM piperazine, 150mM NaCl, pH6.0 and buffer B: 20mM piperazine, 2M NaCl, pH6.0.

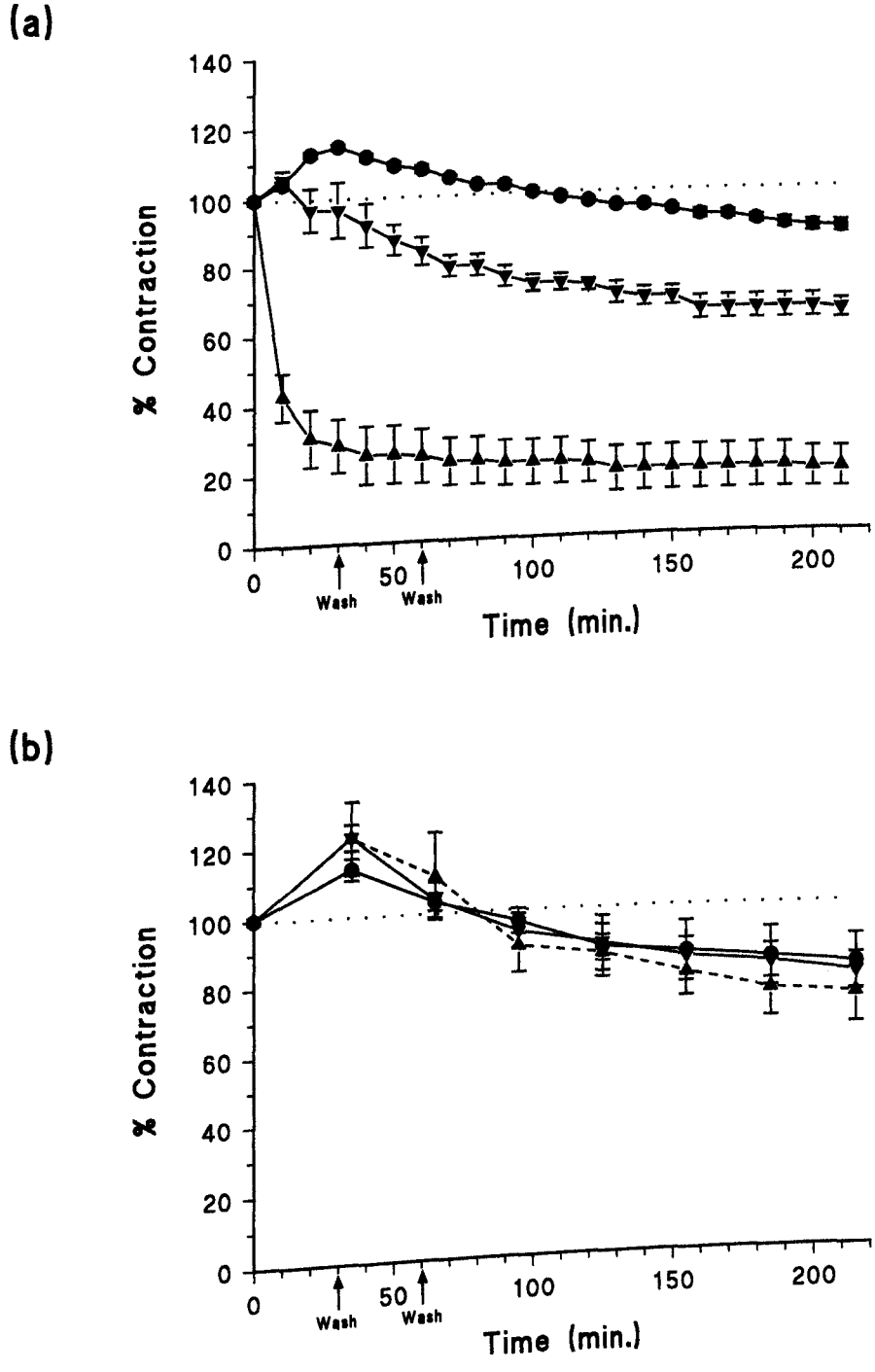


Figure 5.15 Effects of *La. tredecimguttatus* venom on the mouse hemidiaphragm. Nerve stimulation (a), or direct muscle stimulation (b), using 1 (●), 3.3 (▼), or 10mg/L (▲) venom. ±SEM (n=4)

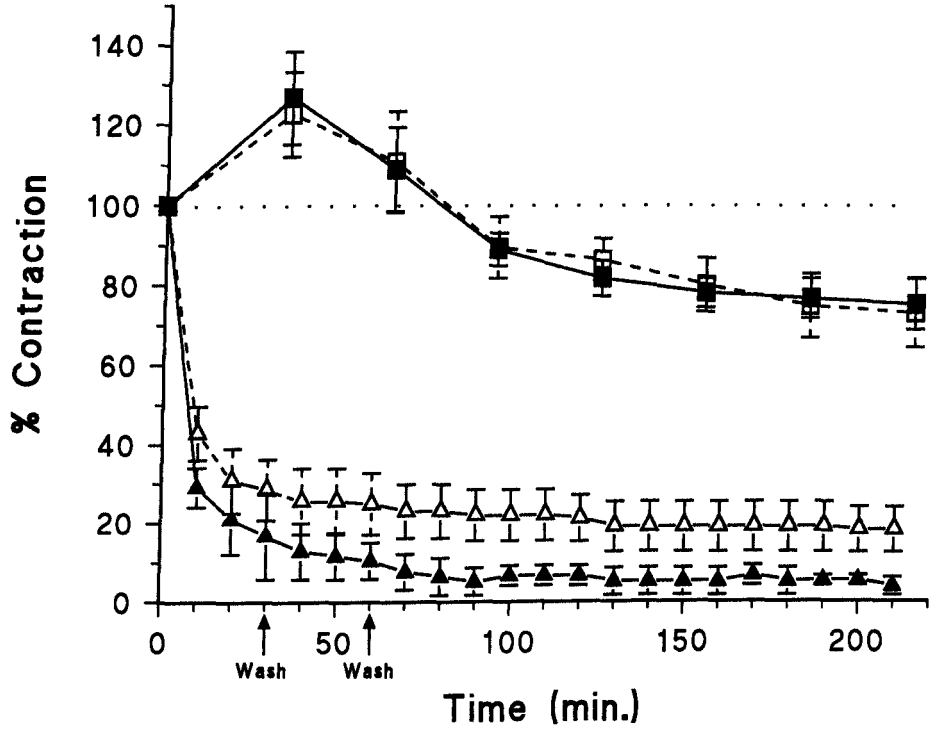


Figure 5.16 The effect of calcium concentration on *La.tredecimguttatus* venom (10mg/L). Normal (1.9mM) calcium (open symbols) and high (4.0mM) calcium (closed symbols) concentrations. Direct muscle (squares) and nerve (triangles) stimulation ±SEM (n=3-4).

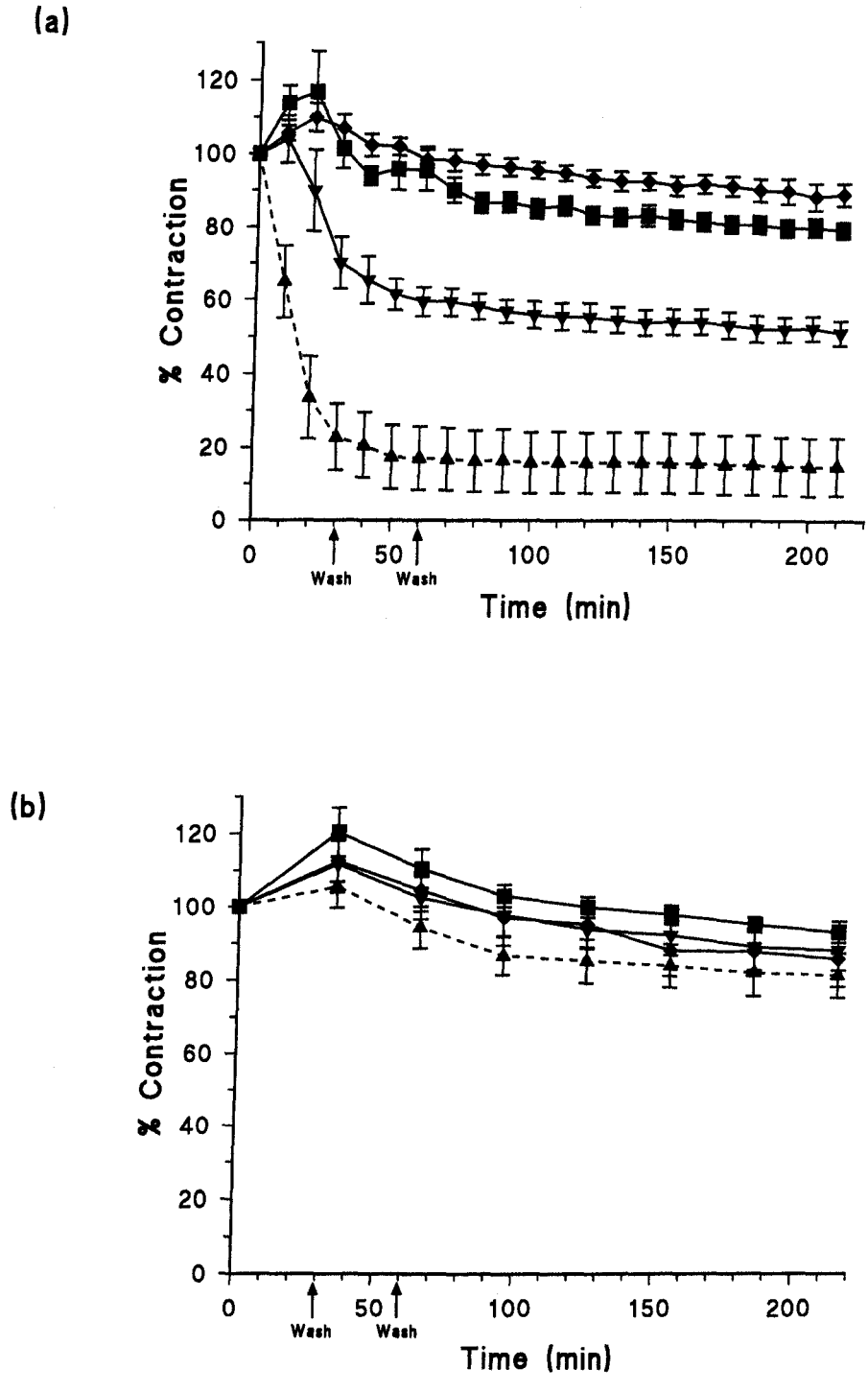


Figure 5.17 The effect of immunisation dose on specific neutralising antibody levels as assessed on nerve diaphragm preparations. Venom (10mg/L) only (▲), or premixed with 50mg/L of anti-*La. hesperus* high dose Fab (◆), medium dose Fab (■), or low dose Fab (▼) ±SEM (n=4-6). Nerve stimulated (a), or direct stimulation (b).

or low immunisation schedules (Figure 5.18).

Fab antibody fragments (200 mg/L) raised against *La. tredecimguttatus* venom neutralised the inhibitory action of the venom and caused a transient potentiation over the first 30 min of the nerve stimulated response, but venom effects were fully neutralised by a 400mg/L concentration (Figure 5.19). Anti-α-latrotoxin Fab was also shown to neutralise the venom.

5.3.7.4 Effect of mixed immunogen

No significant (P=0.497) difference could be found between the neutralising ability of 50mg/L of Fab antivenom produced by immunising with either *La.hesperus* venom alone or combined with *Loxosceles* venom (Figure 5.20).

5.3.7.5 A comparison of the neutralising properties of Fab and $F(ab')_2$ with a commercial antivenom

A 50mg/L concentration of the Merck black widow antivenom was found to neutralise the effects of the venom (10mg/L) to the same extent as the high dose *La.hesperus* Fab at the same concentration (Figure 5.21). When just 25mg/L of the antivenoms were tested approximately 50% neutralisation was found, although the commercial antivenom had a slightly weaker neutralising effect. However, $F(ab')_2$ produced from the same high dose *La. hesperus* antisera at a concentration of 25mg/L neutralised the venom to about the same extent as 50 mg/L of the other antivenoms.

5.3.7.6 Delayed antivenom treatment

Antivenom (200mg/L or 1000mg/L, high dose Fab) added 30 min after 10mg/L of venom had no effect on the established nerve blockade over 3 hr (Figure 5.22). However, the potentiation produced by 1mg/L of venom over the first hour was rapidly inhibited when 200mg/L or more of antivenom was added after 30 min (Figure 5.23).

5.3.8 *In vivo* Toxicity

La.tredecimguttatus venom batch PA734 had an LD_{50} of 0.59 mg/kg (11.2 μg/mouse, 95% confidence limits from probit analysis 10.8 - 11.8) and batch PB301 had an LD_{50}

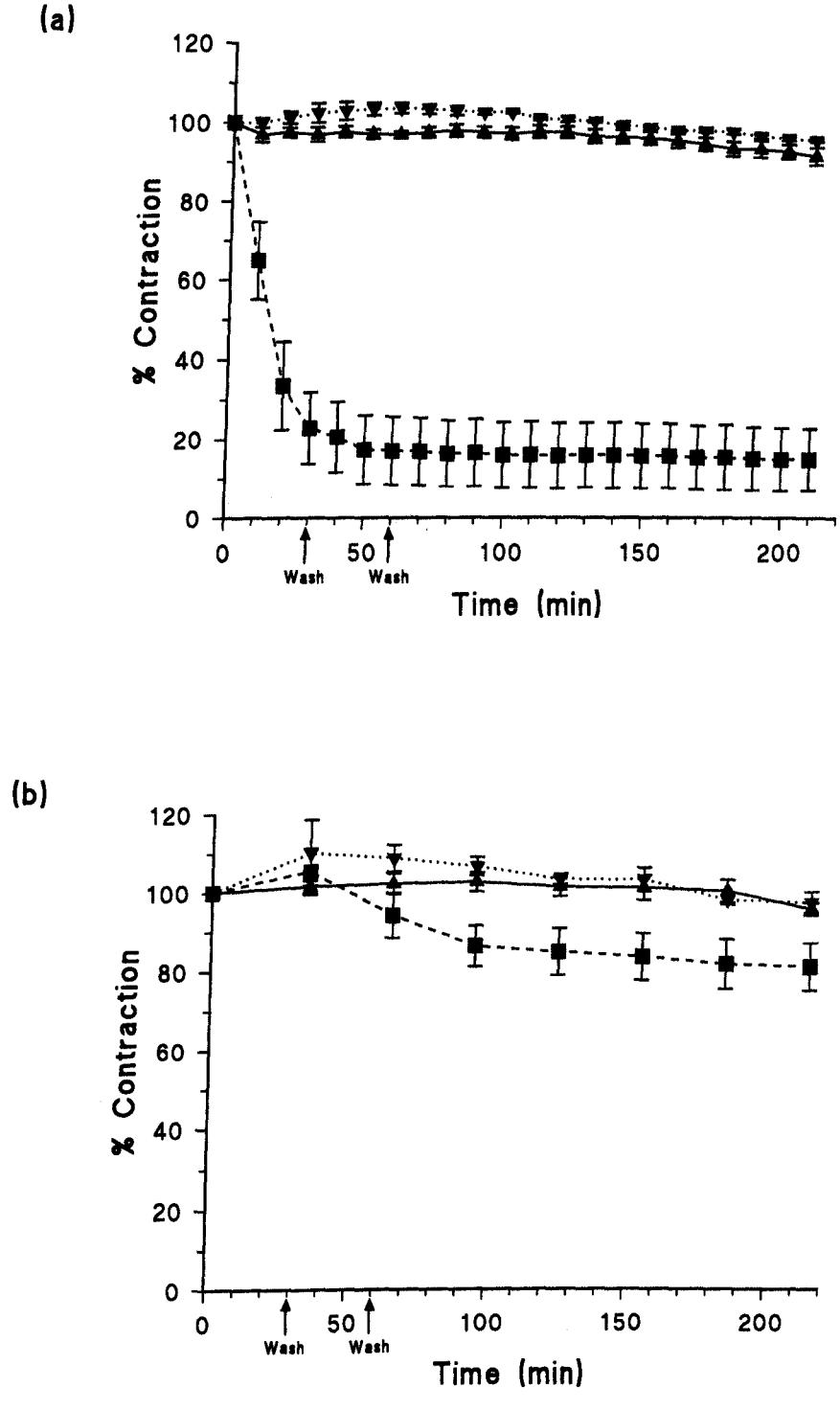


Figure 5.18 Antivenom neutralisation of *La.tredecimguttatus* venom (10mg/L). Venom only (■), plus anti-*La.hesperus* high (▲), or low (▼) dose Fab (200mg/L). Nerve (a) and direct (b) stimulation ±SEM (n=4-6).

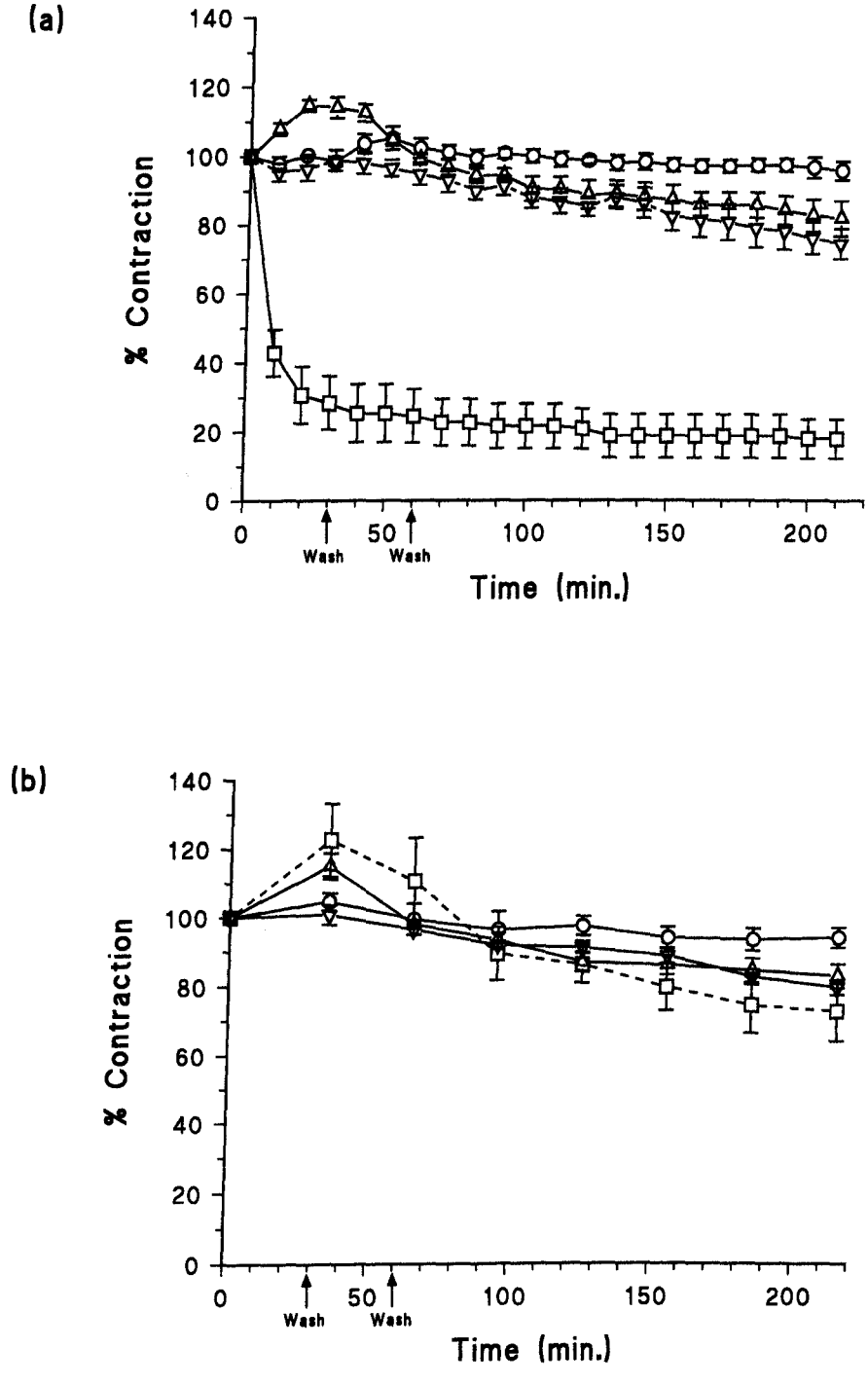


Figure 5.19 Neutralising effects of antibodies raised against *La.tredecimguttatus* venom and α latrotoxin. Diaphragm preparations, bathed in 10mg/L venom only (□), or plus anti-*La.tredecimguttatus* Fab, 200mg/L (Δ), 400mg/L (∇), or anti-α latrotoxin Fab, 400mg/L (O). Nerve (a), or direct muscle (b) stimulation. ±SEM (n=3-4).

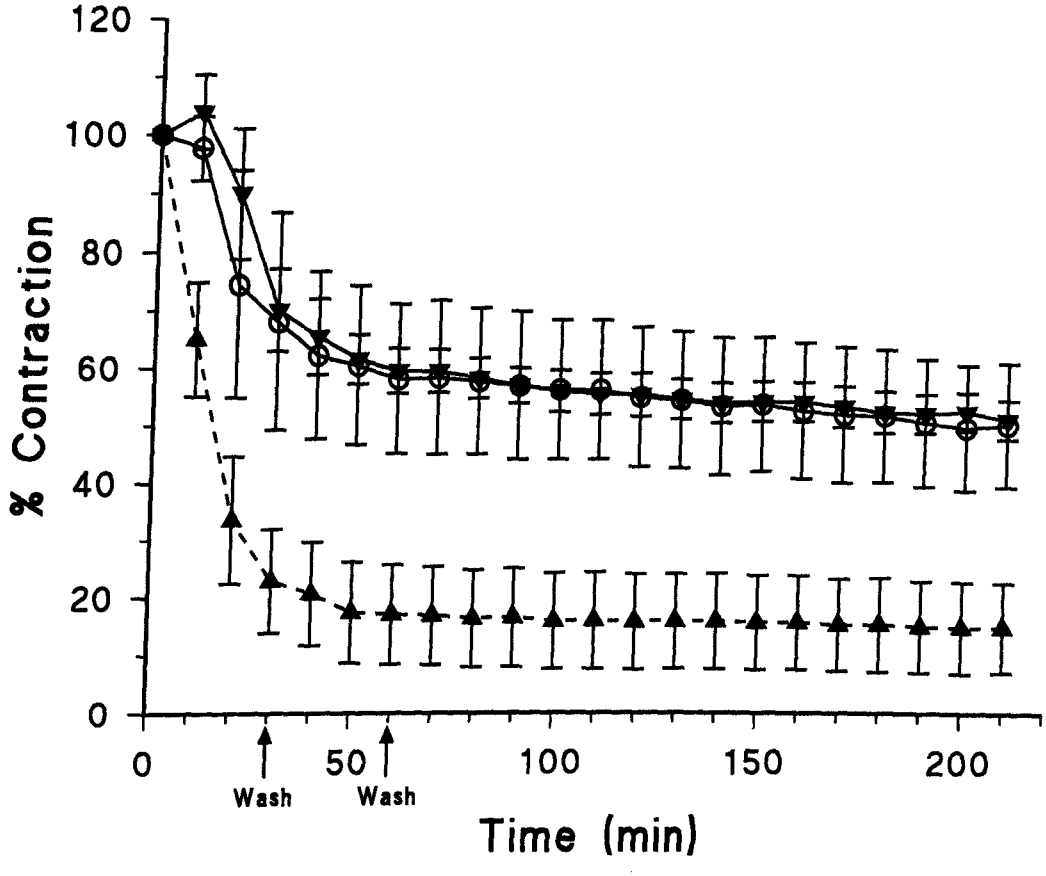


Figure 5.20 The effect of a mixed venom immunogen on specific neutralising antibody levels against *Latrodectus* venom. Nerve stimulated diaphragm preparations, bathed in 10mg/L venom only (▲), ±SEM (n=6), or plus 50mg/L anti-*La.hesperus* low dose Fab (▼), or mixed immunogen Fab (O) ±SEM (n=4).

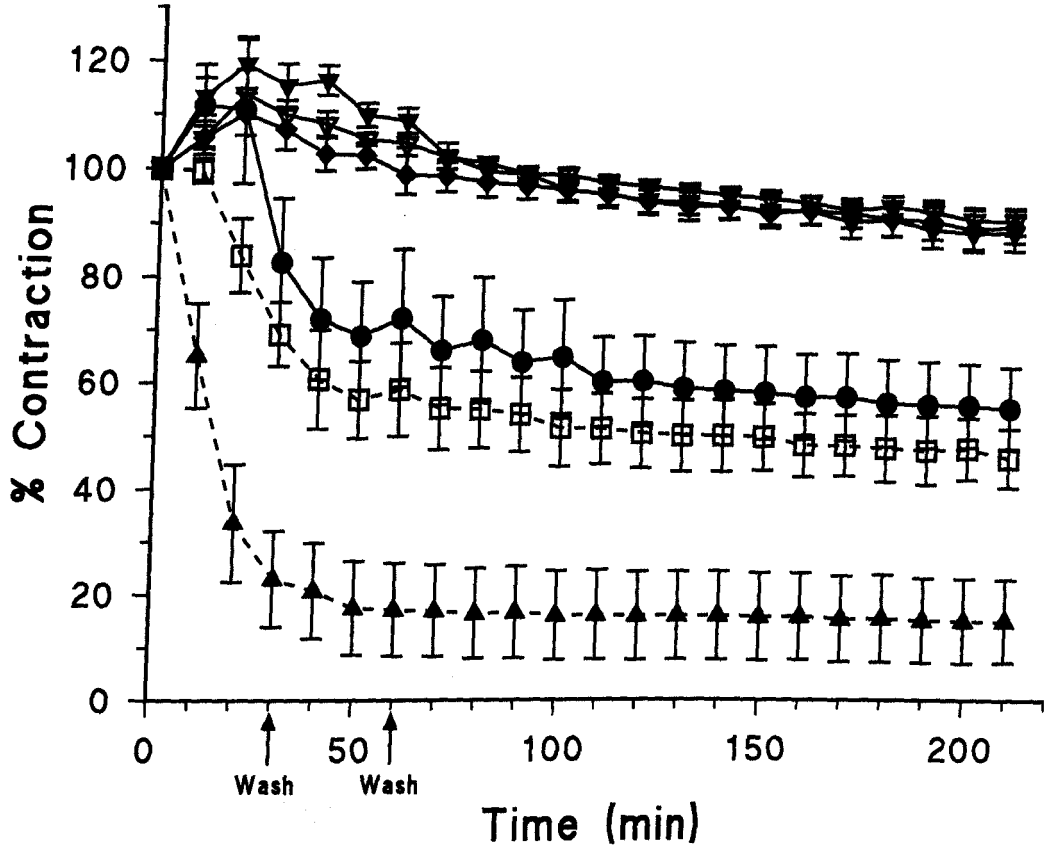


Figure 5.21 A comparison of the neutralising properties of Fab and $F(ab')_2$ with a commercial antivenom. Venom only (▲), (n=6), or plus 25mg/L (●) and 50mg/L (◆) of anti-*La.hesperus* (high dose) Fab, or 25mg/L (▼) $F(ab')_2$, or plus 25mg/L (□) and 50mg/L (▽) of Merck antivenom (±SEM n=4).

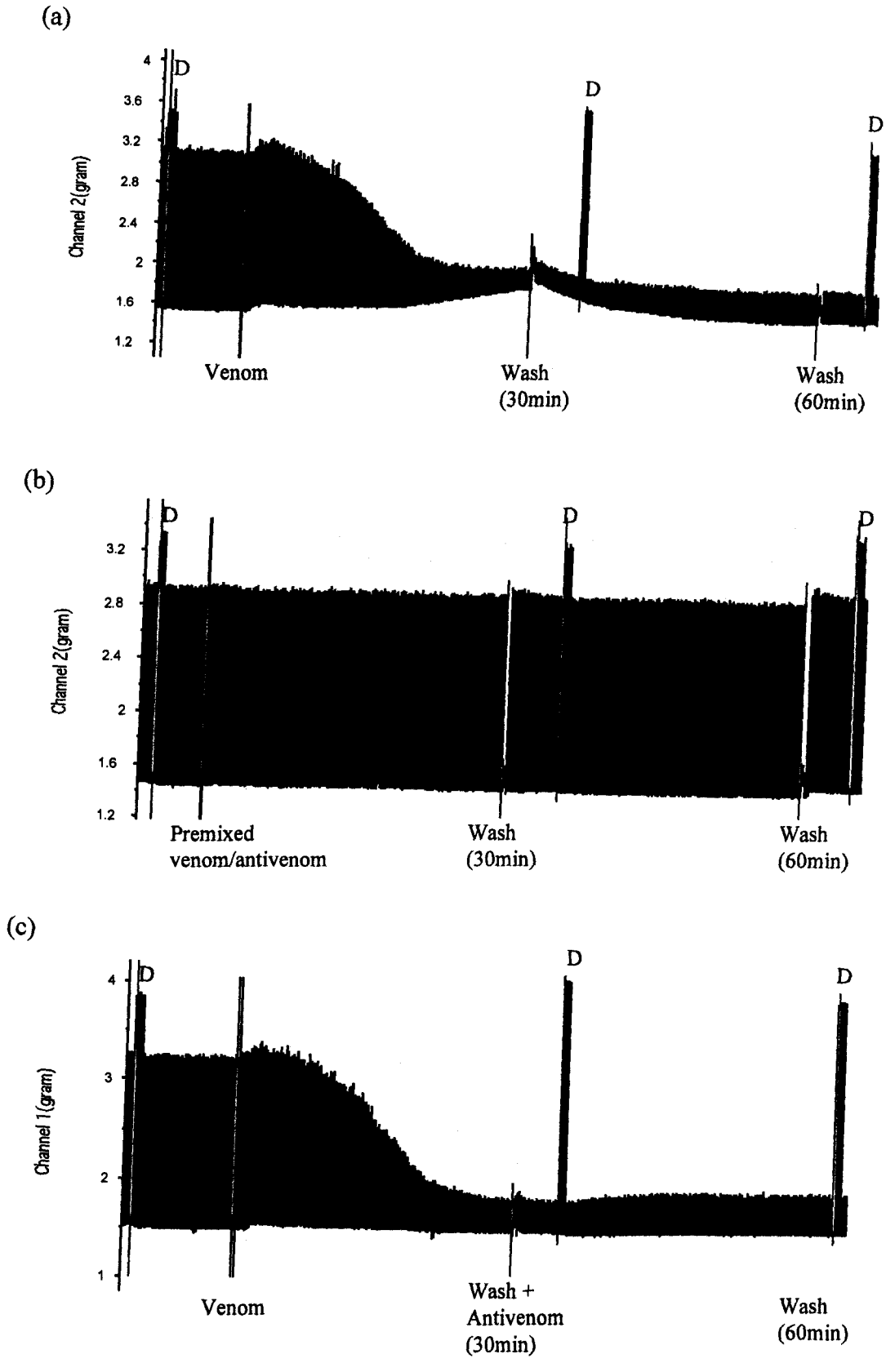
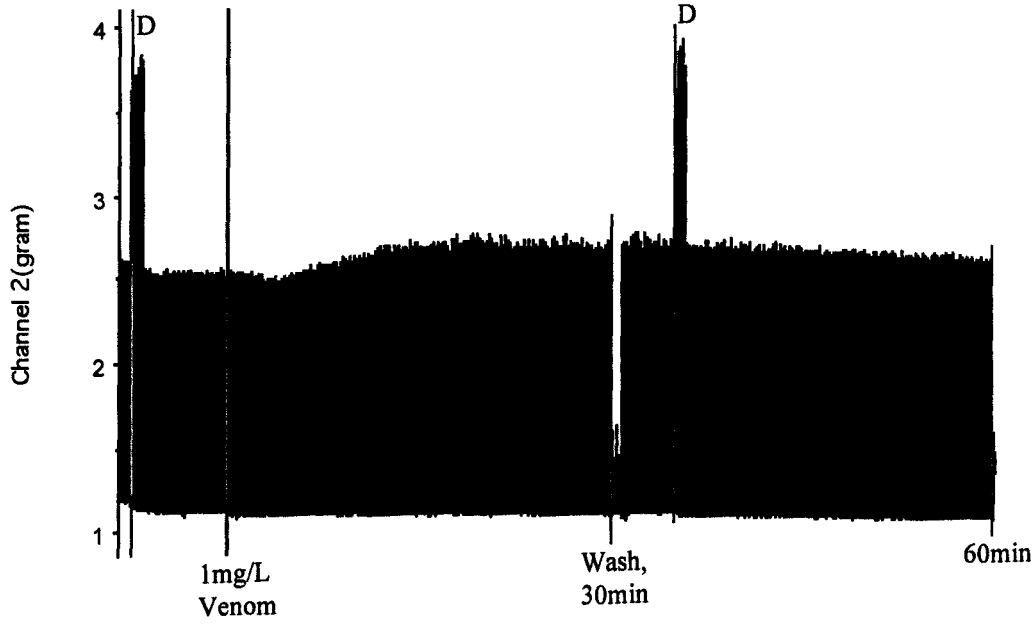


Figure 5.22 The irreversible nerve blockade produced by *La.tredecimguttatus* venom (10mg/L). The effects of washing (a), or premixing with 200mg/L *La.hesperus* high dose Fab antivenom (b), or the delayed addition of the antivenom (c). Direct stimulation (D).

(a)



(b)

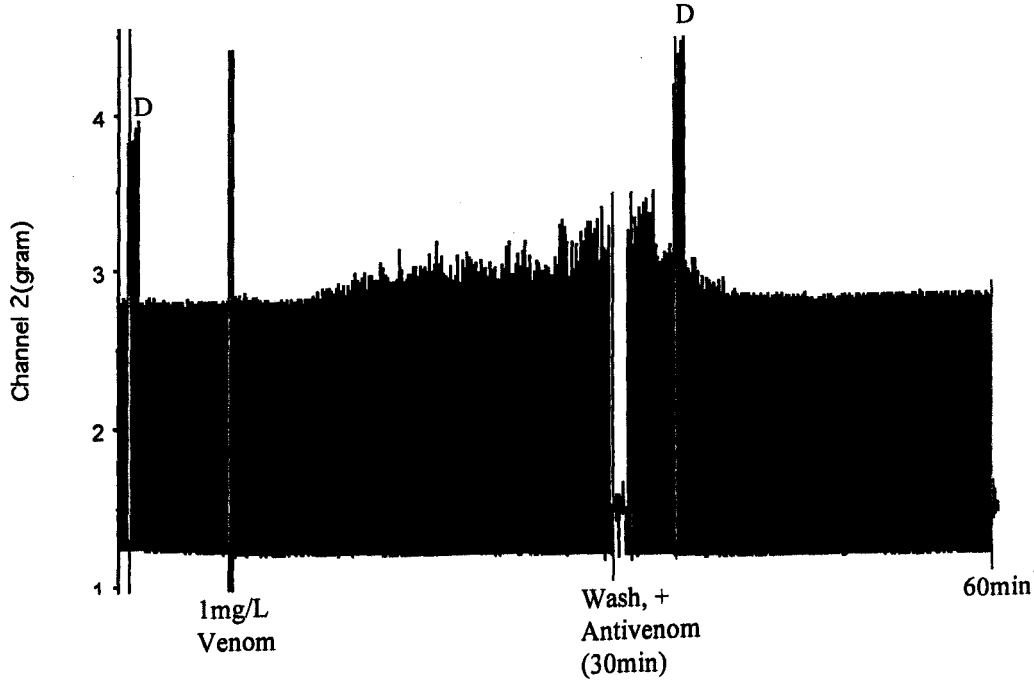


Figure 5.23 Effects of the delayed addition of antivenom on the neurotoxic action of 1mg/L of venom. Venom control (a), and the addition of 200mg/L of *La.hesperus* high dose Fab, after 30 min (b). Direct stimulation (D).

of 1.19 mg/kg (22.7μg/mouse, 95% confidence limits from probit analysis 16.6 - 29.4).

Animals rapidly displayed signs of envenoming with a change in behaviour and their general appearance. Within an hour profuse sweating could be clearly seen in some animals, their fur appeared wet and the eyes were closed (Figure 5.24a). Some animals displayed a marked piloerection and some were heavily lacrimating. Isolated muscular spasms were evident from time to time and movement had an unnatural jerky appearance with eventual hind limb paralysis in the higher dose groups. Despite the freely available food and water none of the animals were observed to eat or drink during the 24hr period and they lost weight (~20%). The ear veins also appeared dilated and by the end of the experiment the animals were almost totally inactive with stiffened bodies / increased muscle tone and often irregular or laboured breathing. Despite the array of severe effects, death appeared to be due to respiratory paralysis as indicated by the irregular or laboured breathing. As all the animals displayed these severe early effects they could not be used as an early indication of mortality and most of the animals that died did so in the latter stages of the 24 hr. The only exception to this was in the preliminary dose finding study where excessively large doses induced hyperactivity leading to convulsions and a rapid death.

5.3.9 *In vivo* Neutralisation by an Antivenom

The *La.hesperus* (high dose), $F(ab')_2$ antivenom was substantially more potent by ED_{50} testing than any of the other antivenoms and the relative order of potency for the other antivenoms was: *La.hesperus* (high dose), $F(ab')_2$ > Merck > *La.hesperus* (high dose), Fab > *La. tredecimguttatus*, Fab > α-latrotoxin, Fab > *La.hesperus* (mixed venoms), Fab / *La.hesperus* (low dose), Fab (Table 5.3).

The week 30 *La.hesperus* (high dose) antiserum from which the Fab and $F(ab')_2$ were processed had an ED_{50} value of 1.64μL per mouse (95% confidence limits from probit analysis 0.90 - 2.59). From this data and the potency of the purified antibody fragments the neutralising yield could be calculated as 78 and 37% for the $F(ab')_2$ and Fab preparations respectively (Table 5.4).

Despite the neutralising effect of the antivenoms, all the animals within the ED_{50} dose range still displayed severe signs of envenoming. Groups of two animals given higher

(a)

(b)

Figure 5.24 Effects of *Latrodectus tredecimguttatus* venom *in vivo*. (a) Displaying profuse sweating, closed eyes, dilated ear veins and inactivity, four hours after the iv injection of 10.5μg and (b) a control mouse.

	ED_{50} against $2LD_{50}$ of *La. tredecimguttatus* venom		
Antivenom	**mg/kg**	**μg/mouse**	**95% confidence limits**
Merck Black Widow A/V	3.36	64	51 - 75 μg/mouse
La.hesperus (High), $F(ab')_2$	1.80	34	19 - 59 μg/mouse
La.hesperus (High), Fab	4.13	78	56 - 107 μg/mouse
La.hesperus (Low), Fab	9.92	189	171 - 214 μg/mouse
La.hesperus (Mixed), Fab	9.84	187	130 - 233 μg/mouse
α-latrotoxin, Fab	7.37	140	69 - 244 μg/mouse
La. tredecimguttatus, Fab	6.68	127	106 - 145 μg/mouse

Table 5.3 Neutralisation of lethal venom effects *in vivo*. (95% confidence limits from probit analysis.)

	Yield	
	Number of Mouse ED_{50} Units / L Serum Processed	% Original Neutralising Activity
Antiserum	609,756	100.0
Fab	228,205	37.4
$F(ab')_2$	473,529	77.7

Table 5.4 Antivenom neutralising yields for Fab and $F(ab')_2$ processed from a batch of week 30 anti-*La.hesperus* (high dose) serum.

antivenom doses (600, 2,500 or 5,000μg/mouse) premixed with two LD_{50} of venom (22.4μg), were closely observed for signs of envenoming (Table 5.5). An antivenom dose of 600μg per mouse of both the Merck and $F(ab')_2$ antivenoms completely neutralised any signs of envenoming, or a ratio of 27μg of antivenom to each μg of venom. These animals were observed feeding and drinking normally and appeared completely unaffected.

5.4 Discussion

Although the venoms from different *Latrodectus* species displayed slight differences they all appeared to contain an identical band representing α-latrotoxin. It has been shown by various authors that antivenoms raised against different subspecies from around the world are all highly effective in cross neutralising the toxic effects of other *Latrodectus* venoms both in mice and in man with the complete relief of all symptoms (Wiener, 1961; Keegan, 1955; Finlayson & Hollow, 1945; Graudins *et al.*, 2001). Venoms obtained by electrical stimulation had a cleaner appearance on SDS-PAGE with less background protein than those obtained from venom gland homogenates. The use of such a clean venom preparation, free from regurgitated mouth part juices or cell debris, for immunisation may have helped the sheep to produce such high levels of specific antibody.

Commercial antivenoms currently available for the treatment of envenoming by *Latrodectus* spiders are raised in horses and vary in purity from unrefined antiserum such as the Merck antivenom tested here, to purified $F(ab')_2$ preparations such as C.S.L.'s Red Back Spider Antivenom. Horses are highly sensitive to this venom, producing severe life-threatening effects if too much venom is administered initially (Maretic & Stanic, 1954; Wiener, 1961). However, no untoward effects have been noted during this or other work raising antibodies in sheep with similar venom doses (Smith & D'Amour, 1939; Maretic & Stanic, 1954).

The individual antibody responses of sheep immunised with *La. hesperus* venom were highly variable as assessed using an ELISA detection system against either the whole venom or α-latrotoxin with maximum titres of up to 1:1,500,000 and 1:100,000 respectively. Although the highest immunisation doses generally produced higher titre

	Signs of Envenoming After 24 hr		
Antivenom	600μg Dose	2,500μg Dose	5,000μg Dose
Merck Black Widow Antivenom	-	-	ND
La.hesperus (High), $F(ab')_2$	-	ND	ND
La.hesperus (High), Fab	++	-	ND
α-latrotoxin, Fab	+++	+	+
La. tredecimguttatus, Fab	+/-	-	ND

Table 5.5 Neutralisation of the signs of envenoming by two LD_{50} of venom *in vivo*. No signs (-), minor signs (+/-), definite signs (+), severe signs (++), very severe signs (+++), and not done (ND).

antisera against the whole venom than those sheep immunised with lower doses, this difference became less clearly defined when titres against α-latrotoxin were compared.

The mouse phrenic nerve-diaphragm preparation was used as a specific assay for neurotoxicity. It showed the potentiating (stimulatory) effects of low venom concentrations (1mg/L) but nerve blockade by higher concentrations (10mg/L) presumably due to the rapid depletion of neurotransmitter following α latrotoxin pore formation. The neurotoxic effects of the venom were neutralised by 200 - 400 mg/L of all the antivenoms tested and the relative order of potency produced by immunising with different *La.hesperus* venom doses was high dose (2mg/sheep) > medium dose >> low dose. Concentrations as low as 50 mg/L of both the commercial Merck antivenom and the *La.hesperus* high dose Fab or just 25 mg/L of $F(ab')_2$ produced using the new process neutralised the nerve blockade produced by 10mg/L of venom. These results show that an equally effective antivenom could be processed from sheep antisera using an established papain digestion procedure, and a significantly more effective $F(ab')_2$ antivenom with about twice the activity using the new process. Despite these neutralising effects, antivenom was incapable of reversing the nerve blockade produced by the 10mg/L venom concentration *in vitro*. This may be a reflection of the high venom concentrations which are in excess of the maximum levels expected clinically and which produce a nerve blockade by depleting the nerve terminal of neurotransmitter containing vesicles. The stimulatory type effects seen at low venom concentrations (1mg/L) are more likely responsible for the symptoms of envenoming as opposed to lethality and could be rapidly reversed by high concentrations of antivenom (200mg/L).

The venom produced a mixed array of signs of envenoming in mice, with some animals sweating profusely while others were unaffected in this respect; this may reflect the use of out-bred mice. An array of different signs and symptoms have also been noted clinically in man, again with a profuse sweating seen in some individuals but absent in others (Maretic, 1983; Clark *et al.*, 1992). This may reflect differences in the relative density of α latrotoxin receptors found on different nerve types (Sugita *et al.*, 1999), with the unnatural spontaneous stimulation of these nerves producing the various effects. The predominant and consistent clinical feature is severe pain, which

may indicate that sensory nerve endings have a high receptor density and are, therefore, highly sensitive to the venom. A direct effect on these sensory nerve endings has been clearly demonstrated experimentally by various groups both *in vivo* and *in vitro* (Queiroz & Duchen, 1982; Waterman & Maggi, 1995).

The venom had a toxicity of 0.59mg/kg as assessed using the mouse LD_{50} assay over 24hr with most of the animals dying in the latter part of the 24 hr indicating that the lethal effects of the venom were of slow onset. Other groups assay this venom over 8 days with most animals dying after 2 to 3 days, indicating that a higher toxicity could be found by assaying the venom over a longer time period (Wiener, 1956; Finlayson & Hollow, 1945). Before the introduction of antivenoms, human fatalities following *Latrodectus* envenoming have occurred 3 - 30 days after the bite in adults, but just 36 or 6 hr later in 3 year or 3 month old victims respectively (Ingram & Musgrave, 1933).

It could be calculated from the relative ED_{50} values that 141mg of $F(ab')_2$ produced using the new process would be equivalent to one vial of the Merck antivenom. As each vial of commercial antivenom is produced with a potency expressed in terms of LD_{50} neutralisation, and, therefore, assuming that this measure correlates with the clinical efficacy of the material it would also represent sufficient material for the treatment of a single black widow spider bite. As this material was processed with a yield of 16.1g of $F(ab')_2$ per L of antisera, each litre of antisera could theoretically produce ~ 114 treatments. A high yield process such as this which retains 78% of the original ED_{50} neutralising power of the serum is particularly important in order to reduce the production costs and compares with a 37% yield produced by a papain digestion method. The increase in yield alone would be expected to halve the total production cost, and the elimination of the salt precipitation step would also be expected to reduce the processing time and costs. This increase in yield would halve the number of sheep required for immunisation and therefore the amount of venom needed which is extremely difficult and expensive (~$155/mg) to obtain in large quantities.

A commercial Squibbs antivenom (Anti-Black Widow Spider Serum) produced in the 1930's in America was also raised in sheep, by immunising each animal with the venom gland extracts from 3,000 spiders over a 6 month period (Smith & D'Amour,

1939). They obtained 126μg of venom per spider (D'Amour *et al.*, 1936) which is similar to the 117μg per spider found here. From this it can be calculated that 378mg of venom was used to immunise a single sheep over 6 months or ~63mg per month and resulted in an antiserum of which 1mL could neutralise 45 rat LD_{50} or 5.7mg of venom (D'Amour, 1936). This hyper-immunisation with ~ 32 times more venom than was used in the high dose group here, may have been necessary if no adjuvant was used, and produced a less potent antivenom than the high dose group, which could neutralise 610 mouse LD_{50} or 6.8mg of venom per mL of serum.

It was also reported that 3.5 hr after the subcutaneous injection of a rat with the venom from two spiders (~252μg), one mL of Squibbs (week 10) antiserum was required intra-peritoneally in order to produce a full recovery, and indicates that 23 times more antivenom is required to reverse the signs of envenoming compared to preventing lethality by premixing (D'Amour *et al.*, 1936; D'Amour, 1936).

The present experimental results show that with premixing, only 6 times more Merck antivenom is required for neutralising the blockade of the phrenic nerve *in vitro* or preventing the lethal effects in mice *in vivo*. However 9 times the ED_{50} is required to completely neutralise all the signs of envenoming *in vivo*. One vial of the Merck product contained ~265mg of protein which is known to be sufficient for the treatment of a single black widow spider bite and from the ED_{50} assay calculated to be capable of neutralising 46mg of venom. The large excess in this antivenom may, therefore, be necessary clinically to reverse the signs and symptoms of neurotoxicity if only a small proportion of the antibodies are specific for the critical region of the neurotoxin responsible for clinical features such as pain and which is left exposed on the nerve terminal membrane allowing antibody binding. Some excess of antivenom is also likely to be required clinically to allow for dilution in the circulation and antivenom clearance. An amount of antivenom ~ 100 times the weight of venom delivered may typically be required for snake antivenoms.

Latrodectus venom contains 7 high molecular mass (110-140kDa) toxic proteins (α-latrocrustatoxin, α, β, γ, δ, and ε-latroinsectotoxin, and α-latrotoxin) which contain some highly homologous amino acid sequences. For instance α-latrotoxin shares 37 and 34% of its sequence with δ-latroinsectotoxin and α-latroinsectotoxin respectively (Grishin, 1998). The presence of these identical amino acid sequences on most of the

venom components would be expected to induce a strong immune response and may help to explain the exceptionally potent nature of the antivenoms when premixed, as assessed by either neurotoxicity neutralisation or ED_{50}, and suggest perhaps the binding to a common structural component necessary for pore formation (Pescatori & Grasso, 2000). However, the continued presence of severe signs of envenoming using concentrations of ~ 8 times the *La.hesperus* (high dose) Fab ED_{50} suggest that the toxin was still capable of binding its receptor and producing an effect despite carrying a bound antibody fragment which prevented the lethal effects.

Evidence for at least four functional domains (1. Receptor binding, 2. Alteration of plasma membrane calcium permeability, 3. Acceleration of neurotransmitter release, 4. Bilayer lipid membrane ion-channels) of the α latrotoxin molecule have been demonstrated with monoclonal antibodies. One such antibody (4C4.1mAb) neutralised the toxicity of both α latrotoxin and whole venom in mice without inhibiting the toxin's ability to bind its receptors by interacting with a structural component involved in pore formation (Pashkov *et al.*, 1993; Cattaneo & Grasso, 1986; Pescatori & Grasso, 2000). However, this antibody appears incapable of modifying the toxin's action after binding (Cattaneo & Grasso, 1986; Pescatori & Grasso, 2000). Interestingly several of these monoclonal antibodies will not bind the denatured toxin, implying the need for active non-denatured toxin in the immunisation procedure (Pashkov *et al.*, 1993). Further evidence for the multi -functional effects of the toxin have been demonstrated indirectly by the venom's precipitation with methyl alcohol which could subsequently only produce a temporary hind limb paralysis without any of the other normally severe effects in rats (D'Amour *et al.*, 1936).

Clinically, low blood calcium levels are known to be associated with symptoms including muscle aches, seizures and spasms as well as tetany (general stiffening and spasms of the muscles) and are treated with calcium injections or supplements. Because of the similarity of these clinical features with some of those produced by *Latrodectus* envenoming, intravenous calcium gluconate has long been recommended for pain relief after envenoming (Maretic, 1983; Timms & Gibbons, 1986). However, in the current studies it has been shown that a high calcium Krebs solution enhances significantly *in vitro* venom neurotoxicity, consistent with the work of Palmer (1975) using the rat phrenic nerve diaphragm. This finding agrees with several clinical

investigators who have had inconsistent results with calcium injections (Key, 1981; Russell, 1962; Clark *et al.,* 1992). For instance Key (1981) found calcium gluconate to be effective in only six of 13 patients and, since the symptoms found following *Latrodectus* envenoming are known to "wax and wane", many patients may appear to improve regardless of treatment if they are observed for only a limited period after envenoming (Maretic, 1983; Clark *et al.*, 1992).

An investigation of potential adjuvant type effects produced by *Loxosceles* venom in sheep revealed about 2- to 3-fold higher specific ELISA antibody titres against α latrotoxin. However, no significant difference was found in the neutralising effect when assessed on nerve muscle preparations or *in vivo*. These findings clearly indicate differences between specific antibody binding levels and neutralising antibody levels and highlight why bioassay neutralisation is a critical component in the testing of antivenoms. The lethal effects of this venom in mice appeared to be due to respiratory paralysis, which may account for the good correlation found between ED_{50} results and those with the isolated phrenic nerve diaphragm preparation. The use of the phrenic nerve diaphragm preparation may, therefore, provide a much needed more humane alternative to the standard mouse ED_{50} testing of these antivenoms which induce considerable pain and suffering in the test animals.

Observations made during the ED_{50} tests showed that none of the animals were free from the severe signs of envenoming and as the practical use of the antivenom is in ameliorating symptoms and not predominantly used to prevent the lethal effects, the use of the ED_{50} test does not appear very relevant to the clinical use of this particular antivenom. With larger antivenom doses of greater than 18 times the ED_{50} any signs of envenoming were prevented by all except the anti-α latrotoxin Fab. The use of a test system which measures the neutralisation of all the signs of envenoming would, therefore, have a greater relevance to the clinical setting. However, a humane *in vitro* equivalent would provide a more ethically acceptable test system.

The $F(ab')_2$ produced using the new process had ~ twice the potency of the Fab prepared from the same batch of antiserum. This is a reflection of the highly pure nature of the $F(ab')_2$ compared to the Fab which would still be expected to contain most of the 16% papain used in the digestion plus an unknown amount of Fc. Salt precipitation may also have caused the loss of some of the neutralising activity, and it

is now widely accepted that salt precipitation can damage the IgG and lead to the formation of aggregates which may induce reactions in the clinical setting (Otero *et al.*, 1999; Rojas *et al.*, 1994). Because of the small difference in size (23kDa vs 46kDa for papain as compared with Fab) and the high isoelectric point of papain (pI 8.75), the two cannot be efficiently separated by either diafiltration or during the conventional anion-exchange step used to remove Fc (Allen, 1996; Glazer & Smith, 1971: Hristova, 1968). Also papain, unlike pepsin, is a known allergen in man (Quarre *et al.*, 1995; Baur *et al.*, 1995).

Although the Merck, Black Widow Spider Antivenom has repeatedly been shown to be highly effective at rapidly alleviating all the clinical features of envenoming, the high incidence of reactions despite the relatively small quantity of material administered has dramatically influenced the willingness of clinicians to use this product in North America (Moss & Binder, 1987). This product is also known to induce a large degree of *in vitro* complement activation and was found to contain a large amount of high molecular weight aggregates which have been strongly implicated in the anaphylactoid reactions produced by human intravenous immunoglobulin preparations (Sutherland, 1977; Bleeker *et al.*, 1987; 1989; 2000; Lobatto *et al.*, 1987; Jenei *et al.*, 1991; Sylvestre *et al.*, 1996).

The new ovine $F(ab')_2$ antivenom raised to *La. hesperus* venom as described here was twice as effective as the Merck material by weight, essentially free from aggregates and pepsin and may provide a much needed safe and effective alternative antivenom for use in North America.

CHAPTER 6:
FINAL DISCUSION AND CONCLUSIONS

When antivenoms were first developed over a hundred years ago they all consisted of unrefined horse antiserum which, due to the large amount of foreign protein administered, frequently caused severe serum sickness one to two weeks later. Immediate type reactions were also encountered which were assumed to result from an IgE mediated response since, before the introduction of antibiotics and effective vaccination programmes, horse antisera were routinely used for a range of infections (e.g. anti-diphtheria, tetanus, scarlet fever, poliomyelitis, meningococcus), often prophylactically (Kojis, 1942). A large number of patients had, therefore, previously been exposed to horse serum proteins which is necessary for a true IgE mediated response. However, some patients developed immediate type reactions despite no previous exposure to horse serum protein (Kojis, 1942).

Subsequently processes were developed to reduce the amount of foreign protein administered with a consequent reduction in the incidence of reactions. These included sodium and ammonium sulphate precipitation of the immunoglobulin fraction and this was followed later by the introduction of pepsin to remove the Fc part of the antibody molecule. These methods to produce purified $F(ab')_2$ preparations were adopted by most antivenom manufacturers and remain essentially unchanged. As a result, the severe serum sickness reactions once frequently seen have largely been resigned to the history books. However, the risk of immediate type reactions remains. Experimental work in the 1960's using a guinea pig model of IgE mediated anaphylaxis clearly demonstrated that crude plasma was the most potent sensitising agent followed by Fc fragments or impure $F(ab')_2$ preparations, while pure $F(ab')_2$ preparations had by far the weakest activity (Hristova, 1968). Presumably this contributed to the notion that all the early reactions were IgE mediated type I hypersensitivity reactions to equine serum proteins. However, their prevalence in populations never exposed to equine material and the absence of specific cutaneous hypersensitivity in most reactive patients prompted a search for other mechanisms (Malasit *et al.*, 1986).

Sutherland (1977) found that most antivenoms, including Merck's black widow product, were anticomplementary *in vitro*, which led him and others to recommend the slow intravenous infusion of diluted antivenom to reduce complement activation (Reid, 1980). However, it has subsequently been shown (Malasit *et al.*, 1986) that the incidence and severity of early reactions are similar whether the antivenom is given by intravenous injection over 10 min or diluted and given as an intravenous infusion over 30 min and, although the antivenom activated complement *in vitro*, there was no significant evidence of *in vivo* complement activation.

It has been found that certain ovine Fab antivenoms could induce similar early reactions in patients never exposed to ovine serum products (Meyer *et al.*, 1997; Ariaratnam *et al.*, 1999; 2001). However, Digibind™, a highly refined ovine Fab product has an excellent safety record (Kirkpatrick, 1991), indicating that a well formulated, highly refined Fab product free from contaminants should be safe. A new process, recently introduced by Aventis-Pasteur, utilises two ion exchange steps to improve the potency, purity and safety (4% side effects) of its equine-based pepsin digested products, removing the majority of both the low molecular weight contaminants and the high molecular weight aggregates previously found (Grandgeorge *et al.*, 1996; Pepin-Covatta *et al.*, 1997; Chippaux *et al.*, 1999). Reactions produced with this antivenom were all mild early events, without a single case of serum sickness (Chippaux *et al.*, 1999). This indicates that some of the minor contaminants or aggregates found in antivenoms must be exceptionally potent at stimulating immediate type reactions by an as yet unknown mechanism.

Numerous studies have been performed to elucidate the cause of early reactions produced when human immunoglobulin preparations were infused intravenously. Different types of aggregate have been shown to either cause complement activation or produce a hypotensive response in a rat model which correlates with clinical reactions and results from the activation of phagocytic cells via Fc receptors (Bleeker *et al.*, 1987; 1989; 2000; Spycher *et al.*, 1999). Aggregates which activate complement are known to be rapidly coated with C3b and the opsonised complex bound via the C3b to erythrocyte CR1 receptors. The immune complexes are then safely cleared from the circulation and neutralised in the liver following Fc receptor binding without erythrocyte destruction (Nardin *et al.*, 1999). It has also been shown

by Lobatto *et al.* (1987) that small quantities (10μg/kg body weight) of complement activating soluble human IgG aggregates experimentally injected i.v. in man are rapidly cleared ($t_{1/2}$, 26min) from the circulation, predominantly via the liver without causing any adverse effects. It now appears likely that reactions to human IgG are due to the release of inflammatory mediators caused by phagocytic cell activation through the interaction of IgG dimers or aggregates with Fc receptors (Bleeker *et al.*, 1987; 1989; 2000; Lobatto *et al.*, 1987; Jenei *et al.*, 1991; Sylvestre *et al.*, 1996).

European Pharmacopoeia guidelines outline the use of size exclusion chromatography to measure aggregate / polymer levels in human intravenous immunoglobulin preparations, which should represent not more than 3% of the total area of the chromatogram. The guidelines also stipulate that anticomplementary activity should be less than 1CH50 per mg of immunoglobulin (European Pharmacopoeia, 1997).

During the present work the Merck antivenom was found to contain a large (~10%) amount of high MW material which, in the light of the above evidence, is most likely responsible for the majority of immediate anaphylactoid reactions seen clinically with this product. Although this antivenom has a high anticomplementary level *in vitro*, so little is administered clinically (~ 265 mg) that it is unlikely that any significant large scale complement activation would be seen *in vivo*. A more likely explanation for the immediate type reactions is an interaction with Fc receptors, as shown with human intravenous immunoglobulin products.

Pepsin and papain digested antivenoms contain no intact IgG which could cause these reactions. Furthermore, as pure monomer Fab and $F(ab')_2$ preparations have been administered safely to humans, reactions must result from contaminants or aggregates. No evidence could be found in the literature for Fc receptor binding by aggregated Fab or $F(ab')_2$, and reactions are, therefore, more likely to result from minor contaminants such as low molecular weight fragments of Fc. The method of enzyme cleavage and the anion exchange step developed in the new process may provide the key to reducing immediate type reactions by removing any residual aggregating fragments of Fc.

The successful use of the hypotensive model developed by Bleeker *et al.* (1987) has allowed the identification of components in human iv immunoglobulin preparations responsible for immediate type reactions and similar work should be repeated with

some typical antivenoms in order to identify conclusively the minor contaminant(s) responsible for antivenom reactions. This would help enormously with the development of improved methods for their removal and the subsequent elimination of these life-threatening side effects.

As no specific therapy is available for the toxic effects produced by mass bee attacks (Franca *et al.*, 1994; Schumacher *et al.*, 1996), a large volume of antisera was generated and used to develop a new, simple platform technology for producing antivenoms in high yield at low cost. This newly-developed ovine *Apis mellifera* antivenom had high levels of specific antibodies to the venom which neutralised its myotoxic, phospholipase A_2 and *in vivo* activities. It also contained high levels of antibodies directed against the main toxic component of bee venom, melittin, and may provide the first specific therapy for the treatment of mass envenoming by either European or Africanised bees.

Methods of processing the ovine antisera using pepsin were investigated. By determining the conditions necessary for the preferential breakdown of Fc and serum components other than $F(ab')_2$, it was possible to avoid any time consuming salt precipitation steps which are difficult to perform on a large scale under sterile conditions and may be associated with large (~18%) losses and aggregate formation (Schultze *et al.*, 1965; Friesen, 1987). Diafiltration was then optimized to remove most of the unwanted low MW fragments and the conditions for anion-exchange chromatography optimised to remove any remaining acidic impurities such as pepsin and high MW aggregates. The whole manufacturing process resulted in a $F(ab')_2$ product with a purity comparable to the best commercial antivenoms (~97% (Grandgeorge *et al.*, 1996)) but with higher yields (up to 19.3g per L of serum) than previously reported (up to 14g per L of plasma (Benanchi *et al.*, 1988)).

Spiders of the genus *Latrodectus* (black widows) are distributed widely and about 2,500 bites are reported each year in the USA (Litovitz *et al.*, 1991-2000). The neurotoxic effects of its venom were studied on the isolated phrenic nerve diaphragm preparation. Low venom concentrations (1mg/L) were stimulatory while high venom concentrations (10mg/L) caused nerve blockade which was potentiated by increased calcium levels.

Although effective, the Merck black widow spider antivenom, which is unprocessed horse serum, causes unacceptable risks. The second purpose of this project was to prepare an improved *Latrodectus* spider antivenom using the new platform technology.

Different immunisation schedules were studied in sheep to optimise their humoral immune response. Animals immunised with 2mg *La.hesperus* venom produced the highest levels of specific antibodies as assessed by ELISA, using an isolated nerve diaphragm, and by *in vivo* studies in mice. A pure $F(ab')_2$ antivenom was produced in high yield from this antisera with the new manufacturing process which retained 78% of its original *in vivo* ED_{50} neutralising power, could neutralise all the non-lethal *in vivo* effects produced by the venom and was ~ twice as effective as the Merck antivenom.

Because a single toxin (α-latrotoxin) is known to cause the clinical effects of *Latrodectus* envenoming it is possible to produce an immunotherapeutic product comprising humanized monoclonal antibody (Pescatori & Grasso, 2000). However, the high production costs would make such a product prohibitively expensive in some countries. A partially effective antivenom was produced in the present studies by immunising sheep with just 10μg of α latrotoxin, since its high cost (~ £2,600 per mg) prevented immunisations with higher doses. The recently found DNA sequence to encode for the active toxin and its expression in a cell line (Ichtchenko *et al.*, 1998; Volynski *et al.*, 1999) may allow large quantities of toxin to be produced at relatively low cost. A new pioneering immunisation technique has also been developed recently in which DNA (3μg) encoding part of a snake venom toxin was fired intracellularly into a mouse with a gene gun and the expressed toxin fragment shown to produce an antibody response comparable to that produced by conventional immunisation of a rabbit with the intact toxin (Harrison *et al.*, 2000). Such a method may provide a new approach to developing a *Latrodectus* antivenom which avoids the need for large amounts of venom, but further studies are required to investigate its effectiveness (i) in larger species such as sheep and (ii) with more complex toxins such as α-latrotoxin which is ~ five times the size of the fragment used by Harrison and his group (2000).

To summarise, effective new antisera were raised in sheep against the black widow spider and bee venoms and a new, simple method of processing the sera developed which provided highly pure $F(ab')_2$ in high yield which should be suitable for the large scale production of these and other antivenoms.

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