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DNA immunisation to generate snake antivenom: cloning and characterisation of novel cDNAs encoding haemostasis-disruptive venom toxins of saw scaled viper, *Echis ocellatus.*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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DEDICATION

Dedicated to my sweet heart mother and my endless support father Mr. Sayed Anwer A. Hasson, without them I would not be able to finish my Ph.D. Also to my beloved wife and children Hamza, Yousuf and my little lovely boy Abdullah as they endured my absence while carrying out my lengthy experiments.

Contents

Abstract

Envenoming by the African saw scaled viper, *Echis ocellatus* causes 23,000 deaths each year in West Africa. Envenoming results in massive haemorrhagic and necrotic morbidity in the short term and often in permanent disfigurement and renal dysfunction in the long term. The only effective treatment is the administration of antivenom prepared from horses and sheep immunised with whole venom. Although life-saving, antivenoms contain numerous redundant antibodies that dilute the effectiveness of the toxin-specific antibodies. To address this deficiency, our group have initiated a project to develop a DNA immunisation strategy to generate a panel of toxin-specific antibodies to neutralise haemostasis-disruptive toxins in the venom of the most medically important vipers in Africa.

The objective of my research project was therefore to generate, using DNA immunisation, antibodies specific to the major haemostasis-disruptive toxins in venom of *E. ocellatus*. Because cDNAs encoding these haemostasis-disruptive toxins were not present in the genetic databases, it was necessary for me to create an *E. ocellatus* venom gland cDNA library to isolate cDNA sequences encoding these toxins, including snake venom metalloproteases [SVMPs], C-type lectins [CTLs], serine proteases and phospholipase A_2 [PLA_2s]. Utilising a PCR approach to isolate cDNAs encoding such toxins was highly successful and resulted in a significant contribution to the literature and genetic databases concerning SVMPs, PLA_2 CTL and serine proteases. The deduced amino acid sequences of the *E. ocellatus* cDNAs encoded proteins with high overall sequence similarity to the viper group II PLA_2 protein family, the α and β CTL subunits in venoms of related Asian and American vipers, kinin-releasing and fibrinogen-clotting serine proteases from venom of *B. jararaca* and a prothrombin-activating metalloproteinase from the venom of the East African viper, respectively. Moreover, the data obtained in this study showed that the *E. ocellatus* genome contains several, nearly identical, copies of these genes that differ only by a small number of amino acid residues.

The high degree of sequence conservation of these *E. ocellatus* toxins with analogous molecules from related vipers extended to the conservation of structural domains, predicted to have high immunogenic potential. Thus, my next task was to prepare DNA immunisation constructs from the cDNAs that encoded *E. ocellatus* SVMP, CTL and serine protease toxins. To achieve this, one of the cDNA sequences encoding the carboxyl-disintegrin cystine-rich domain [EoDC] of the prothrombin activator, was incorporated within the mammalian expression plasmid pSecTag-B. Mice immunised with the EoDC DNA construct responded with high titres of IgG. The results showed that the epidermal route of DNA delivery by Gene Gun was superior in inducing high IgG titres over intradermal or intramuscular injection. The cellular responses of the DNA immunised mice was not examined in this study because the ultimate intent was to generate antibodies for passive serotherapy and not for vaccine purposes. Furthermore, the results showed that the EoDC-specific antibody reacted with analogous molecules in venoms of a variety of *Echis* species. This result was predicted from analysis of the antigenic index profile. Thus, results suggest that the structural conservation of venom toxins from phylogenetically-distinct vipers can be exploited by this toxin-specific antivenom production approach to generate antivenoms with remarkable potential.

Acknowledgments

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Declaration

The work presented in this thesis, entitled "DNA immunisation to generate snake antivenom: cloning and characterisation of novel cDNAs encoding haemostasis-disruptive venom toxins of saw scaled viper, *Echis ocellatus"* was performed entirely by myself, except for the DNA sequencing, which performed by the DNA sequencing department Liverpool School of Tropical Medicine.

Communications and publications arising from this work:

1. Bharati, K., S. S. Hasson, et al. (2003). "Molecular cloning of phospholipases A(2) from venom glands of Echis carpet vipers." Toxicon **41**(8): 941-7.
2. Harrison, R. A., Oliver, J., Hasson, S. S., Bharati, K., Theakston, R. D. (2003a). "Novel sequences encoding venom C-type lectins are conserved in phylogenetically and geographically distinct Echis and Bitis viper species." Gene **315**: 95-102.
3. Hasson, S. S., R. D. Theakston, et al. (2003). "Cloning of a prothrombin activator-like metalloproteinase from the West African saw-scaled viper, Echis ocellatus." Toxicon **42**(6): 629-34.
4. Hasson, S.S., Theakston, R.D.G., and Harrison, R.A., (2004). "Antibody zymography: a novel adaptation of zymography to determine the protease-neutralizing potential of specific antibodies and snake antivenoms." A manuscript of this paper is currently under review for publication in the journal of Immunological Methods.

Abbreviations

APC	Antigen Presenting Cell
BLAST	Basic Local Alignments Search Tool
bp	base pairs
µl	Microlitre
µg	Microgram
C^o	Degree Celsius
CpG	Cytidine-phosphate-guanosine motifis
cDNA	Complementary DNA
cGTP	Deoxyguanisine 5'-triphosphate
CTL	C-type lectin
dNTP	Deoxyribonucleotide
dATP	Deoxyadenosine 5'-triphosphate
dCTP	Deoxycytidine 5'-triphosphate
dTTP	Deoxythymidine 5'-triphosphate
DNA	Deoxyribonucleic Acid
DMSO	Dimethylsulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic acid
Eo	*Echis ocellatus*
$EoPLA_2$	*Echis ocellatus* Phospholipase A_2
EoCTL	*Echis ocellatus* C-type lectin
EoMP	*Echis ocellatus* Metalloprotease
EoSer	*Echis ocellatus* serine protease
FCS	Fetal claf serum
GG	GeneGun
ID	Intradermal
IM	Intramuscular
IPTG	Isopropyl-B-D-thiogalactopyranoside
kb	kilobase

kDa	Kilo-Dalton
LC	Langerhans cells
LB	Luria Betani
MOPS	3-N-Morpholinopropanesulphonic acid
MND	Minimum necrotizing dose
MHD	Minimum haemorrhagic dose
M	Molar
MCS	Multiple cloning site
ml	Milliliter
mg	Milligram
MP	Metalloprotease
NMS	Normal mouse serum
N-terminal	Amino-terminal
ng	Nanogram
OD_{406}	Optical Dennsity at 406nm wavelength
ORF	Open Reading Frame
PAM250	2.5 mutations per residue
PLA_2	Phospholipase A_2
pI	Isoelectric point
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RE	Restriction Enzyme
RNA	Ribonucleic Acid
rRNA	ribosomal RNA
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl Sulphate-polyacrylamide gel electrophoresis
TAE	Tris-acetate and EDTA
TE	Tris-HCl, EDTA
TEMED	N, N, N, N- (Tetramethylethylenediamine)
S.I.M.A.R	South African Institute of Medical Research
SVMP	Snake venom metalloproteinase

TBS	Tris buffered saline
TCA	Trichloroacetic acid
Tris	Trishydroxymethylaminomethane
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organisation
X-Gal	5-bromo-4choro-3indoyl-β-D-galactopyranoside

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Chapter 1

General introduction

1.1 Introduction

The objective of this thesis was to generate toxin-specific antibodies by immunisation with novel cDNA sequences encoding pathologically important toxic molecules isolated from a cDNA library constructed from venom gland RNA of West African saw-scaled viper *Echis ocellatus* (*E.o*). The cDNA sequences involved in the DNA immunisation encoded C-type lectins (CTLs), a disintegrin-like (DC) domain of the snake venom metalloproteinases (SVMPs) and serine proteases. These molecules have been shown to be responsible for the most prominent effects of envenoming. The study also describes the isolation and characterisation of other cDNA sequences encoding *E. ocellatus* phospholipases A_2 (PLA_2) enzymes that may contribute to the haemostatic-disruption which results from systemic *E. ocellatus* envenoming.

The African saw-scaled viper *E. ocellatus,* was identified a long time ago by Warrell and Arnett, (1976) and Pugh et al., (1979) as one of the most dangerous snakes to mankind. It was found to be the most abundant (Trape et al., 2001) and medically important viper species in many rural areas of West Africa (Wüster et al., 1997; Habib et al., 2001; Molesworth et al., 2003). This species is responsible for 95% of severe cases of envenoming and very high mortality rates in the rural savannah region of West Africa (Warrell et al., 1977; Meyer et al., 1997; Edgar et al., 1980).

The clinical manifestations of envenoming by *E. ocellatus* show many parallels with venoms from related African vipers as well as from those of the Asian and American pit vipers (Warrell, 1996). Bites by *Echis* species result in clinical symptoms which can be divided into two different categories; (1) localised and (2) systemic effects (Bjarnason and

Fox, 1994). The local effects at the bite site, which may appear within minutes after the bite are pain, swelling, echymosis, local haemorrhage and local necrosis (Hite et al., 1992; Warrell et al., 1977). These local effects, while being seriously debilitating, are not usually life-threatening. Systemic viper venom effects are life-threatening and are due to spontaneous bleeding and coagulopathic complications which are mainly due to degradation of vascular epithelium by metalloproteinases, a decrease in coagulation factors (e.g., factor II, V, VIII and XIII) and an increase in the fibrin/fibrinogen degradation products and incoagulable blood (Edgar et al., 1980; Pugh and Theakston, 1987; Bjarnason and Tu, 1978). The vast majority of deaths due to snake bite in West Africa are due to the systemic envenoming by the saw-scaled viper, *E. ocellatus,* as opposed to any other snake.

Apart of the small peptide disintegrin the venom toxins responsible for these pharmacological affects following *E. ocellatus* envenoming have not previously been identified at the molecular level. Despite their medical importance, there has been surprisingly little research performed on the toxin composition and constituent functionality of venom from *E. ocellatus* (Bahrati et al., 2003; Hasson et al., 2003; Howes et al., 2003). A more comprehensive description of the venom proteases and toxin proteins is required to fully understand their systemic and local affects and to guide improved therapy. The extensive literature on the related American and Asian vipers indicates that of the one hundred or more constituents of viper venoms, only a relatively small number of venom toxins are responsible for the life-threatening pathological effects of envenoming (Markland, 1998). Confirmation of the presence of this group of toxins (i.e.,

SVMPs, CTLs, PLA_2, Serine proteases) in the venom of *E. ocellatus* was therefore my first priority.

Antivenom is the only effective treatment for systemic snake envenoming (Lalloo and Theakston, 2003). However, conventional antivenoms have many drawbacks which reduce their potential effectiveness to fully neutralise all the toxin molecules in snake venom (Theakston and Smith, 1995).

The research described in this thesis was performed to identify and isolate cDNAs encoding the most important *E. ocellatus* venom toxins for use as immunizing reagents to generate specific antibodies. The following sections of this chapter review the literature on the medical importance of *E. ocellatus*. Because so little is known about the venom composition of this species, this review also describes the well characterized haemostasis-disruptive venom toxins of the American and Asian vipers that are thought to be responsible for the systemic effects of *E. ocellatus* envenoming. The chapter concludes with a section describing the rational and strategies of using immunisation with DNA encoding these toxins to generate a panel of toxin-specific antibodies.

1.2. The Venomous Snakes

There are approximately 3200 species of snakes, of which 41% are venomous (Emmett and Shaw, 1995). The broad classification of the latter is based on fang morphology (Okuda et al., 2001). Although there are over 400 different species of front-fanged snakes

native to Africa, only a few (23%) are considered to be of medical importance (Warrell, 1983; Spawls and Branch, 1995).

Glands of all venomous snakes are situated laterally at the back of their head. The ducts from the glands connect directly to the canal or groove in the fangs. The contraction of the muscles surrounding the venom glands during the process of striking causes venom to be ejected through the duct down the fangs and into the wound. Some venomous snakes such as the spitting cobras are capable of projecting their venom several feet (Swaroop and Grab, 1954). Vipers on the other hand have two hinged mobile fangs, which become erect during biting, but which otherwise remain folded back against the upper jaw (Tan et al., 1992). However, not all bites in humans result in venom being injected (McNamee, 2001).

Taxonomically, snakes belong to the Class Reptilia, Order Squamata and Suborder Serpentes. Venomous snakes are distributed in five families within the sub-order serpentes; I) Family *Viperidae*, which include the Old World vipers and New World pit vipers, II) Family *Elapidae* to which cobras, kraits and coral snakes belong, III) Family *Hydrophiidae* which is entirely represented by sea snakes, IV) Family Atractaspididae and V) Family *Colubridae*, to which nearly two-thirds of the known species of snakes belong and which are mainly non-venomous.

The Viperidae comprise two sub-families, the *Viperinae* and the *Crotalinae*. Viperinae are sometimes called true vipers and are distributed in Africa, Europe, and Asia. This sub-family is further divided into genera: *Echis, Vipera, Bitis, Adenorphinus, Atheris, Causus, Cerastes, Eristicophis and Pseudocerastes Daboia, Macrovipera* (Warrell, 1983; Tan et al., 1992).

The *Echis* genus of carpet vipers is one of the most medically-important groups of snakes and is responsible for the majority of snakebite deaths and morbidity across Africa and is very important on the Indian subcontinent (Warrell and Arnett, 1976). Although the systemic taxonomy of the *Echis* complex remains in despite (lenk et al., 2001), the classification by Drewes and Sacherer, (1974) is largely accepted in the filed. *E. ocellatus* is the subject of this project.

1.3 Echis ocellatus

E. ocellatus is a small, rather unimpressive snake, usually measuring between 35-40 cm (Fig.1.1) but rarely exceeding a length of 85 cm (Warrell and Arnett, 1976). Pugh et al., (1979) described *E. ocellatus* as an irritable snake which when aroused starts rubbing its rough, heavily keeled scales against each other by making undulating movements with its body which produces a rasping sound that can mistakenly be described as a hissing sound.

The fangs are long (~5mm based on observation) and mobile and it is often in a defensive position and quick to attack. The usual habitat ranges from savanha and semi-arid rocky terrain as found in Kaltungo, Nigeria to frank desert (Warrell and Arnett, 1976). Warrell and Arnett (1976) described residents of the Benue valley border region of Nigeria (Fig.1.2a) as being particularly at risk to *E. ocellatus* envenoming. Trape and colleagues, (2001) reported that *E. ocellatus* was the most abundant of the 1280 different snake species collected in south-eastern Senegal. *E. ocellatus* is also spread widely through large

areas of the West African savannah south of the Sahara, from Senegal in the West to Chad in the east and possibly further east to the Central African Republic and possibly Sudan (Fig.1.2b), (Wűster et al., 1997).

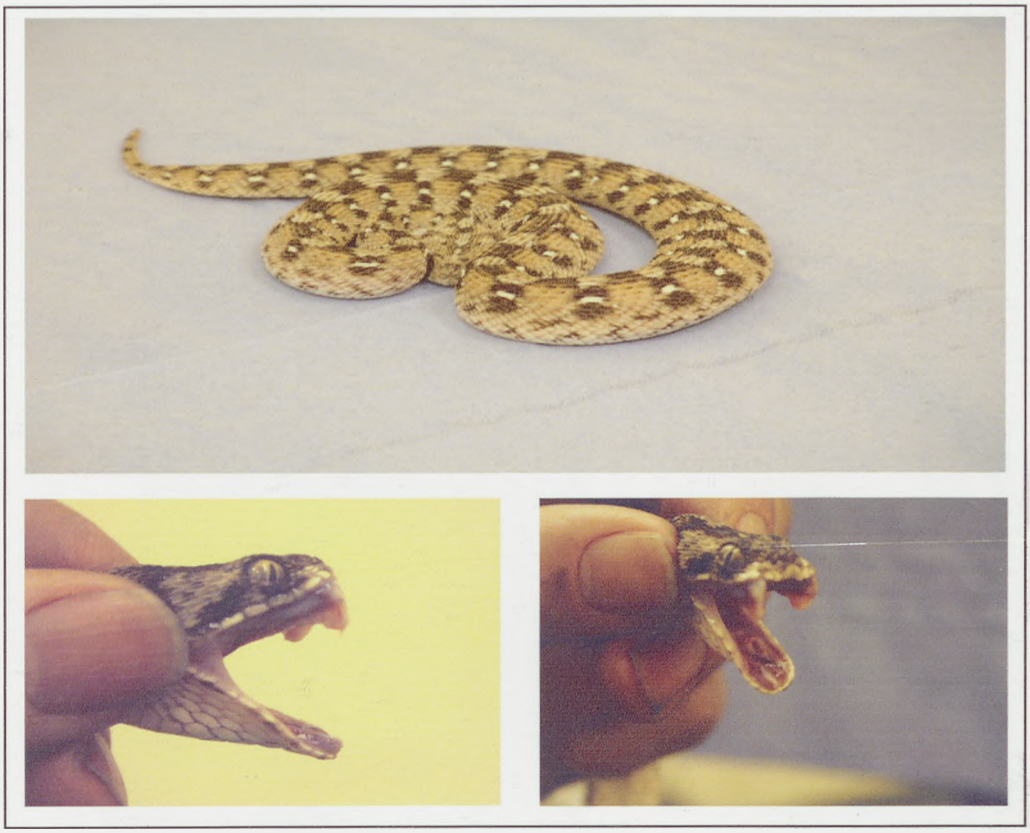


Fig.1.1. ***Echis ocellatus*** from Kaltungo-Nigeria. *Photographs courtesy of R. Harrison.*

1.2b

Fig. 1.2a

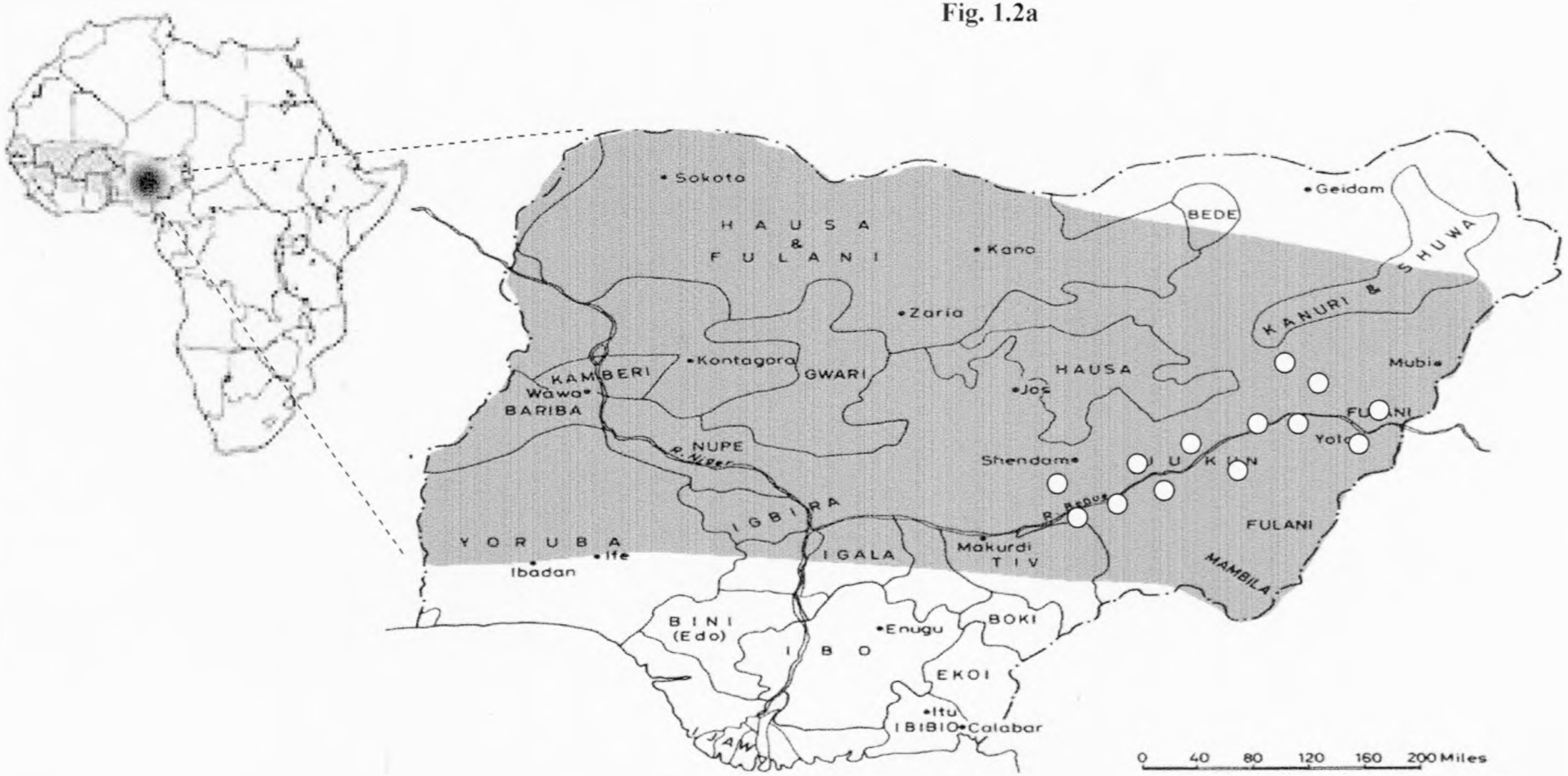


Fig. 1.2. (**a**) ○ Illustrates the areas where ***E. ocellatus*** is commonly present in Nigeria as defined by Warrell and Arnett (1976). Map was taken from (Udo, 1970). (**b**) The shaded areas illustrates the distribution of ***E. ocellatus*** from the southeast Guinea and savannah to northwest of the Ivory Coast and the Sahel in West Africa (Spawls and Branch, 1995).

1.4. Incidence rate of envenoming

Snakebite continues to be a significant cause of morbidity and mortality in many parts of the world (Warrell, 1996), especially in tropical countries and particularly among rural communities of the West and Central Africa (Laing et al., 1995; Theakston et al., 1995; Chippaux et al., 1998). In 1998, the worldwide mortality rate caused by snakebite was estimated at 125,000 deaths per year (Lalloo and Theakston, 2002). However, these figures are likely to be an "under-estimate" because snakebites tend to be neglected in many parts of the world (Lalloo and Theakston, 2002). This is mainly due to the lack of reliable epidemiological data, but also because ministry of health in the developing countries have other more important diseases to deal with such as malaria and HIV AIDS.

E. ocellatus is an important health problem in Nigeria, Ghana and through large areas surrounding the rural tropics of West Africa and is responsible for significant rates of the morbidity and mortality (Molesworth et al., 2003; Edgar et al., 1980; Laing et al., 1995; Rugman et al., 1990). The medical importance of this African viper has been extensively documented (Warrell et al., 1976; Pugh 1979; 1980; Theakston, 1976; Chippaux et al., 1997; 1998; Trape, 2001; Enwere et al., 2000). It accounts for 85 to 95% of the average annual incidence of all envenomings by snakes in Nigeria and Ghana respectively causing several hundred of deaths annually (Meyer et al., 1997; Chippaux et al., 2002). In some rural areas, such as in Ouagadougou in Niger, hospital records indicate that most of the *E. ocellatus* victims were young males that occupied 74% of the hospital bed capacity at the times of greatest snake-bite incidence (Revault, 1996).

Epidemiological studies carried out in Nigeria and elsewhere within the region clearly established that the groups at greatest risk of being bitten by this viper include subsistence farmers, children, hunters, herdsmen and others who have a high level of contact with the snake (Pugh et al., 1980; Warrell et al., 1976; Lalloo et al., 2002; Laing et al., 1995; Molesworth et al., 2003). The view that snakebite is an occupational hazard is further strengthened by observations that the frequency of bites shows seasonal fluctuations (Pugh, 1979; Chippaux et al., 2002; Laing et al., 1995; Molesworth et al., 2003); more than 40% of envenomings by *E. ocellatus* took place when human activity is highest on the farms, particularly during the planting and harvesting seasons (Fig.1.3) (Warrell and Arnett, 1976; Chippaux et al., 2002). Most victims are bitten on the ankle or foot whilst walking through their farms during daylight hours, or during hoeing, weeding, collecting grass, firewood, or when they walking along a path with bare feet at night (Theakston and Reid, 1979; Revault, 1996; Pugh et al., 1979).

An epidemiological survey of snakebite was carried out between 1971 and 1973 at Kaltungo hospital which demonstrated that 76% of the 256 annual incidents were due to *E. ocellatus* (Warrell and Arnett, 1976). The annual incidence rate of snakebite among the farming communities of the Benue and Niger valleys (Fig.1.2) was estimated to be 602 per 100,000 populations with a mortality rate of 12.3% (Pugh et al., 1979; Pugh and Theakston, 1987). Recent studies carried out in Bauchi State hospital in Nigeria demonstrated that 400 cases of snakebites are admitted each year with 4% mortality (Meyer et al., 1997). In general without serotherapy or an efficient treatment, the mortality rate among hospitalised victims of *Echis* bites can exceed 10% (Kabore and

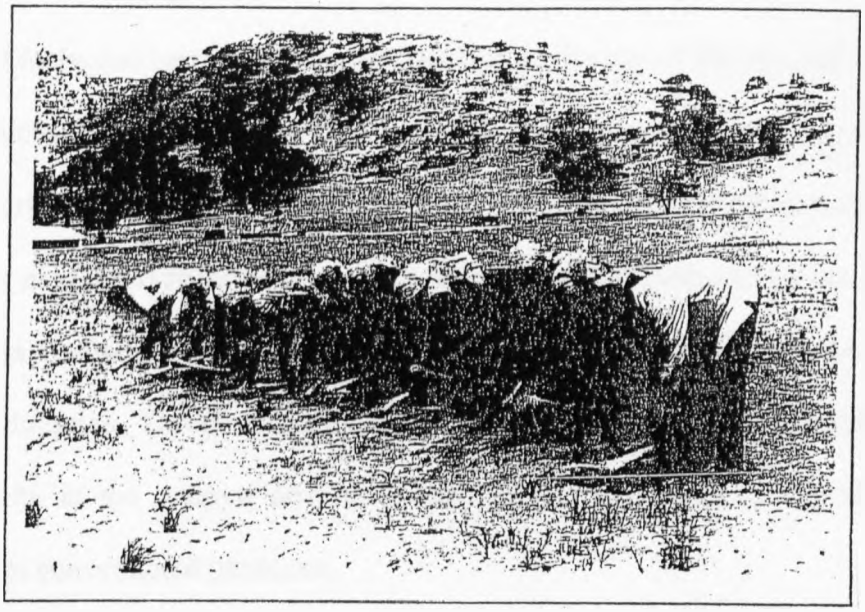


Fig.1.3. Farmers during the planting season. *Photograph courtesy of Warrell and Arnett 1976*

Revault, 1996; Warrell and Arnett, 1976). Although the snakebite incidence varies from one region to another (Enwere et al., 2000) these figures are likely to be an under-estimation and may increase over time in some countries due to improved reporting of the epidemiological data (Warrell, 1992; Molesworth et al., 2003). Thus in many cases, the available information relates only to cases treated in hospitals and dispensaries; it is therefore usually not possible to judge accurately the extent of the snakebite problem.

A major problem in many rural areas is that approximately 50% of snakebite victims seek traditional remedies (Pugh et al., 1979; Newman et al., 1997) because of cultural practices, difficulties in obtaining transport to hospital or medical centre (Theakston and Reid, 1979), lack of health personnel or the non-availability of antivenoms. For instance Newman and colleagues (1997) reported that 90% of the 147 patients bitten by snakes had used some form of traditional remedy prior to hospital admission. An early report by Pugh et al., (1979) also illustrated that 40% of snake bite victims drink an elixir of

crushed *aloe* leaves (a local herbal mixture) to induce vomiting. Sixteen percent applied a mixture of herbs and carried out scarification around the site of the bite and 12% received local incisions (Pugh et al., 1979; Newman et al., 1997). Furthermore, some patients were found to drink urine (mostly their own) as a traditional remedy (Newman et al., 1997). Godwin et al., (2000) reported on cases where envenomed patients had used black stone (Fig.1.4) [a common remedy in rural areas] as a source of traditional remedy prior to hospital admission. The therapeutic value of these practices is at best questionable and often results in the delayed arrived of the patient in a health center which greatly complicates conventional treatment.

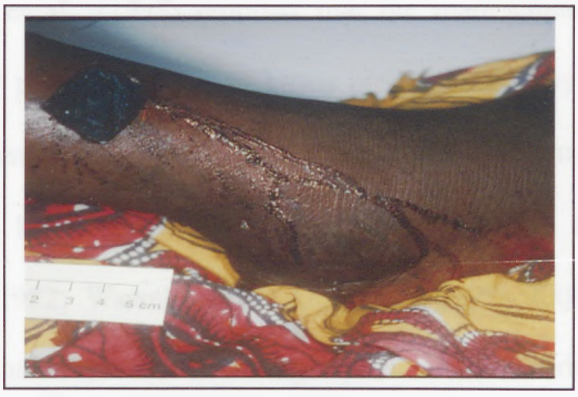


Fig.1. 4. The use of Black stone as traditional remedy. *Photography courtesy of D A Warrell.*

1.5 Clinical features of E. ocellatus envenoming

Snake venoms in general can be characterised into two categories based on their diverse pharmacological effects. Neurotoxicity is the most pronounced effect of venomous of the *Elapidae* (Leonardi et al., 2002) while envenoming by the *Viperidae* is normally characterised by local and systemic haemostasis-disruptive effects (Warrell, 1983; Laing et al., 1995; Leonardi et al., 2002).

Envenoming by *E. ocellatus* causes a series of clinical complications characterised by local and spontaneous systemic haemorrhage which is attributed to the prominent destruction of tissues and blood vessels, from sites such as fang puncture wounds (Fig. 1.4), the gingival sulci (Fig.1.5C) and old wounds (Pugh et al., 1979; Warrell, 1983; Chippaux et al., 1997). Pain and swelling at the site of the bite appear to be the most immediate indications of venom absorption and activity; this may appear as early as 15 minutes after the bite but sometimes later (Warrell, 1983); swelling may spread rapidly and may involve the whole limb or even extend beyond it (Fig.1.5B). A recent study performed by Chippaux et al., (1998) reported that the main clinical and prominent disorder observed in 223 patients who been bitten predominantly by *E ocellatus* were oedema (93.7%) and haemorrhage (48.9%). In severe cases, local necrosis can lead to (Fig.1.5E) (Laing et al., 1995) permanent disfigurement and even amputation of the affected limb (Ownby, 1982; Warrell, 1995; Gutierrez, 1995; 2000). Other features commonly observed included shock, blistering, spontaneous bleeding, renal failure, cardiotoxicity (Habib et al., 2001), hypotension, echymosis and intercranial haemorrhage (Fig.1.5D) (Weiss et al., 1973; Warrell, 1983). Warrell et al., (1977) reported that results of coagulation assays revealed that 65% of 136 patients showed a prolonged clotting time of over 30 minutes, indicating that blood incoagulability is the most sensitive and reliable diagnostic indicator of *E. ocellatus* envenoming. Hypovolaemic shock has also been reported as a result of *E. ocellatus* envenoming; this is caused by extravasation of fluid into the bitten limb (Warrell, 1983). Shock (Fig.1.5A), vomiting, regional lymphadenopathy and headache have also been associated with such envenoming (Warrell, 1977; 1983).

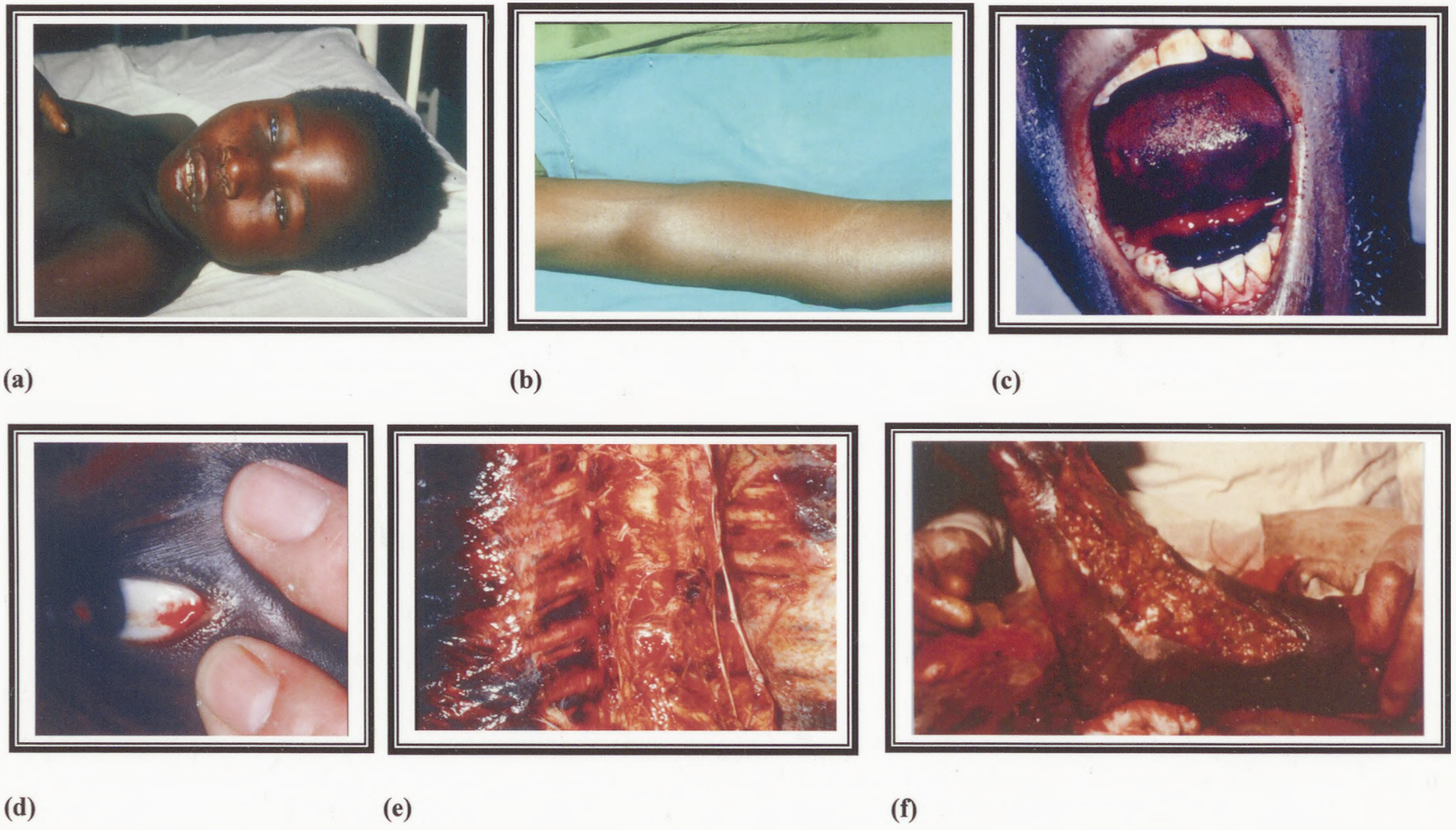


Fig.1.5. Snake bite by ***E. ocellatus*** causes a series of clinical manifestations including; **(a)** shock, **(b)** local swelling, **(c)** spontaneous bleeding, **(d)** haemorrhage, **(e)** severe haemorrhage and **(f)** local necrosis. *Photographs curtsey of D.A. Warrell*

Early treatment and first aid remedies include codeine phosphate tablets to treat pain. If the patient develops necrosis, anti-tetanus serum (ATS) may be given in parallel with appropriate antibiotics to prevent secondary infection (Theakston et al., 1990; Habib et al., 2003) depending on both the patient's sensitivity to antibiotic and/or the degree of the affected area (Warrell et al., 1977; 1983; Habib et al., 2003). Patients with haemorrhagic shock may require transfusion of blood and/or fresh frozen plasma; depending on the availability of antivenom and health deterioration of the patient (Warrell, 1983). Appropriate antivenom should be given when available and as soon as possible.

Deaths, when they occur, are directly related to the systemic effects and/or hypovolaemic shock (Fig.1.5F) caused by loss of blood into the tissues or bleeding from the brain, and may occur one to twelve days after the bite (Warrell and Arnett, 1976). The cytolytic enzymes in the venom and/or to the secondary bacterial infections, such as tetanus are complicating factors which may result in death if left untreated (Warrell and Arnett, 1976; Warrell, 1983).

1.6 Immunotherapy and its limitations

The administration of conventional antivenoms is the most effective and only available treatment for systemic envenoming. The first antivenoms were developed over a century ago by Henry Sewall who immunised pigeons with pygmy rattlesnake (Sistrirus species) venom and demonstrated that they were more resistant to subsequent venom injections (Sewall, 1887; Chippaux and Goyffon, 1998; Lalloo and Theakston, 2003). Calmette in

1887 was the first to prepare commercial antivenom for medical use against envenoming by the Indian cobra (Chippaux and Goyffon, 1998).

Antivenoms are currently prepared by purifying the sera of large animals, usually horses or sheep, hyperimmunised with venoms from individual snake species (*monospecific antivenom*) or from a range of different species venom of medical importance (*polyspecific antivenom*). Animals should be immunized with pools of venom from a large number of snakes to avoid intraspecific variation. The earliest preparations of antivenom consisted of whole serum and these were later followed by whole molecule IgG precipitated from serum using ammonium sulphate (Pope, 1939). These antivenoms often led to a series of clinical complications, including anaphylactoid reactions such as bronchospasm and late serum sickness reactions (Malasit et al., 1986; Chippaux and Goyffon, 1998; Lalloo and Theakston, 2003). To overcome these problems, current antivenoms are prepared by IgG digestion with pepsin or papain to cleave the IgG molecule to produce refined $F(ab')_2$ and Fab fractions, respectively (Lalloo and Theakston, 2003; Miller et al., 2002). Although the use of Fab and $F(ab')_2$ fragment antivenoms were found to produce fewer adverse reactions, both of these antivenom types have advantages and disadvantages.

The Fab fragment has a relatively low MW (50kDa) compared with $F(ab')_2$ (100kDa). Therefore, Fab has a theoretical advantage over $F(ab')_2$ because of its rapid tissue penetration and larger apparent volume of distribution (Meyer et al., 1997). However, it is rapidly eliminated from the patient's circulation via the kidney with a half life of about 4 hours (Lalloo and Theakston, 2003). In contrast, $F(ab')_2$ fragments are eliminated via cellular immunity such as macrophage interaction with a half-life of 18 hours, thus

avoiding renal complications (Chippaux and Goyffon, 1998; León et al., 2001) and being available for longer periods in the systemic circulation. Theoretically therefore, the longer the half life of the $F(ab')_2$ fragment, the smaller the volume of antivenom required for administration (Meyer et al., 1997; Chippaux, 1998).

Apart from the poor availability of $F(ab')_2$ or Fab antivenoms for treating the local effects of envenoming, antivenom also often suffers from deficiencies encountered during production. Venoms consist of numerous molecules, many of which are non-toxic or only marginally toxic. Because there is no correlation between the toxicity of a venom component molecule and its ability to stimulate antibody, the most potent antibodies in antivenom are not necessarily targeted to the most toxic molecules (Mandelbaum and Assakura, 1998; Schöttler, 1951). Consequently, antivenoms contain numerous redundant antibodies that dilute the toxin-specific antibodies (Theakston and Reid, 1983). As a result of these problems it is often necessary to administrate a large volume of antivenom to the patient.

In some parts of the world (e.g., Africa) antivenom production has decreased to critically low levels since 1980s due to several factors. The unit cost of the available commercial antivenom is often high making it expensive for the health services of developing countries and individual patients. For instance an antivenom ampoule in South Africa costs US $35. This is expensive for Africa. The problem is exacerbated by the need to produce individual antivenoms for different snake species for several geographical areas. Overall, antivenoms are relatively expensive drugs which are needed for treating poor people; there is therefore very little profit in antivenoms. As a result over the past 15 years

major pharmaceutical companies have decreased or cessed production (Theakston and Warrell, 2000; Lalloo and Theakston, 2003).

Because of these problems production of Aventis Pasteur and SAIMR antivenoms for Africa has declined. Behringwerkwe stopped production in the early 1990s. As a result of this critical shortage of available antivenom in West Africa patients were often given relatively ineffective antivenoms prepared in Iran or India; these resulted in frequent treatment failures in patient in rural communities, leading in a fall in hospital attendance and a concomitant increase in snakebite morbidity and mortality (Pugh et al., 1979; Laing et al., 2003). In 2000, Theakston and Warrell reported that fake antivenoms had been imported into Nigeria. The current state in Africa is the choice to import either non-specific and/or ineffective antivenoms manufactured in Asia and unproven and frequently dangerous traditional treatments (Laing et al., 2003). To overcome this problem, a monospecific ovine *Echis* Fab fragment antivenom called (EchiTab™) was developed (Laing et al., 1995). This was found to be four times more potent than the *Echis* SAIMR - $F(ab')_2$ fragment antivenom (Meyer et al., 1997). However, as stated earlier such Fab fragment have the major disadvantage of being rapidly eliminated through the patient's renal system (Theakston, 1997; Lalloo and Theakston, 2003).

In conclusion the lack of adequate and regular supplies and the high cost of antivenom play a pivotal role in the provision effective treatment. Such problems affect attitude of communities to hospital treatment, and therefore influence them to seeking traditional remedies (Pugh et al., 1979; Lalloo et al., 2002; Laing et al., 1995; Lalloo and Theakston,

2003). Consequently such factors lead to a concomitant increase in snakebite morbidity and mortality (Laing et al., 2003).

1.7. Disruption of haemostasis by venom toxins

1.7.1 Normal haemostasis in mammalian systems.

Haemostasis is the mechanism by which both blood clotting and clot dissolution is kept in equilibrium by ensuring that blood fluidity in the circulation is sustained (Braud et al., 2000) (Fig.1.6). The normal haemostatic response to vascular damage depends on closely linked interactions between the blood vessel wall, circulating platelets and blood coagulation factors. Injuries to the endothelial lining induce changes in the vascular endothelial surface causing collagen exposure and thrombin production at the site of the reduced blood flow as well as adherence and activation of platelets in the presence of von Willebrand factor (vWf) and the local release of procoagulant factors. Exposed collagen and thrombin activate prostaglandin synthesis by platelets leading to the formation of thromboxane A2 that triggers a series of events leading to aggregation and adhesion of platelets and a change in platelet morphology from discs to spheres. These changes stimulate platelets to release their granular contents including, ADP, serotonin, fibrinogen, lysosomal enzymes and heparin-neutralizing factor (McNicol and Israels, 1999; Kini and Evans, 1990). Activation of platelets and their secretions also have powerful vasoconstrictive ability. Release of ADP causes platelets to swell and aggregate. This attracts and binds other circulatory platelets to the area of injury. The aggregation of platelets also triggers a series of cellular interactions that promote specific binding of

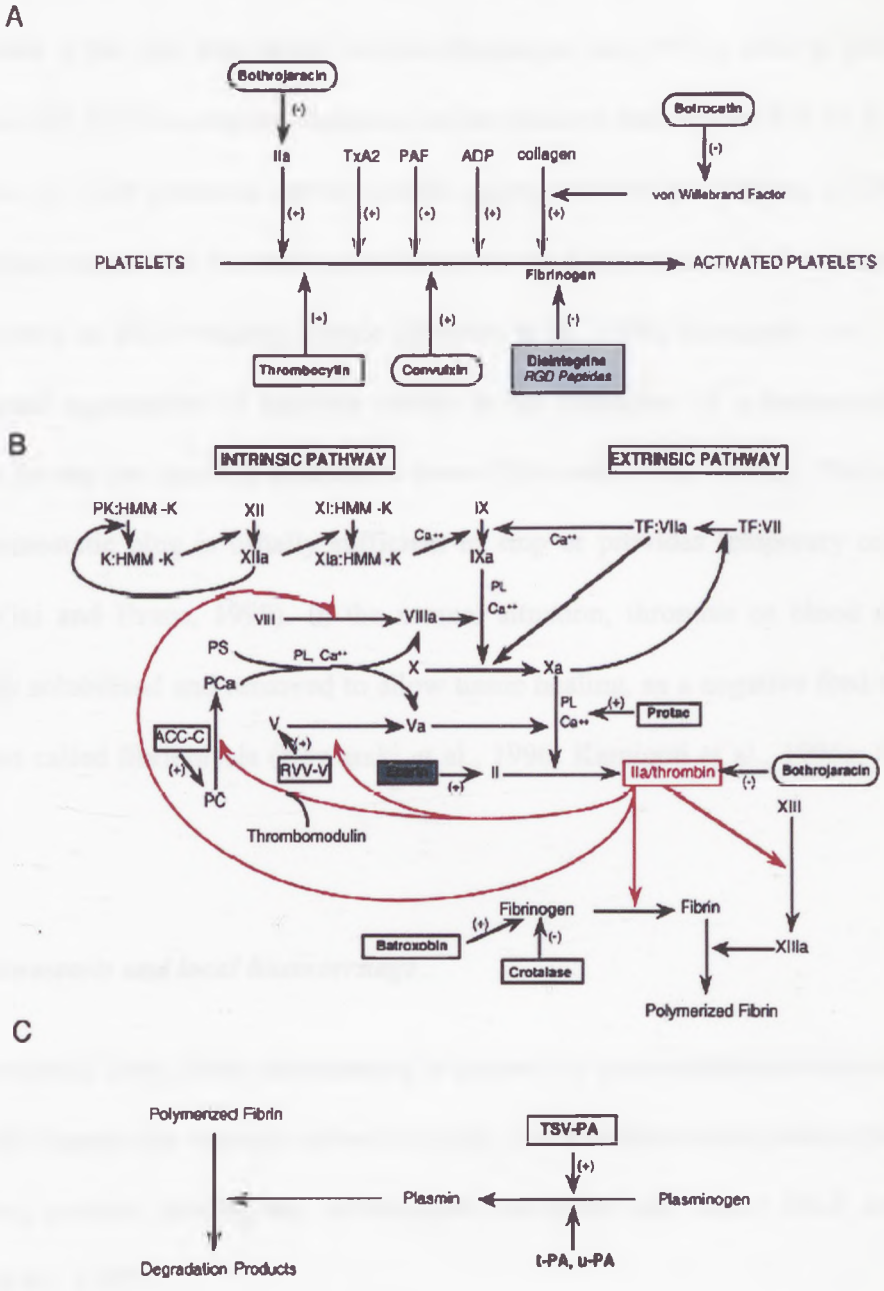


Fig.1.6. Physiological processes of homeostasis. Action of various snake venom protein families on the three pathways of homeostasis. **A.** Platelet aggregation. **B.** Coagulation. **C.** Fibrinolysis. Snake venom serine proteinases are in right-angled boxes, C-type lectins are in rounded boxes, disintegrins are in shaded boxes and ecarin is a metalloproteinase. Diagram adapted from Braud et al., (2000).

adhesive proteins to cell surface integrins (Scarborough et al., 1993). The change in shape of the platelets is the first step which enables fibrinogen and vWf to bind to fibrinogen-glycoprotein(GP) IIb/IIIa complex receptors on the platelets membrane (Yeh et al., 1998). The secretion of ADP promotes further platelet aggregation by the binding of GPIIb/IIIa [$\alpha_{II}\beta_3$] complex receptor on the activated platelets to the fibrinogen as well as to the vWF; these are known as RGD-binding ligands (Suehiro et al., 1996; Kamiguti et al., 1996a). This continued aggregation of platelets results in the formation of a haemostatic plug, which soon covers the exposed connective tissue (Kawasak et al., 1996a). This unstable primary haemostatic plug is usually sufficient to stop or provides temporary control of bleeding (Kini and Evans, 1990). In the normal situation, thrombis or blood clots are subsequently solubilised and removed to allow tissue healing, as a negative feed back, by a mechanism called fibrinolysis (Kawasaki et al., 1996; Kamiguti et al., 1996a; Braud et al., 2000).

1.7.2. Haemostasis and local haemorrhage

Bleeding resulting from *Echis* envenoming is caused by zinc metalloproteinases which enzymaticaly degrade the vascular subendothelium. Other factors which prevent the blood from clotting include disintegrins, prothrombin activators and factor IX/X activators (Kamiguti et al., 1998).

Several studies have demonstrated that purified *Crotalinae* and *Viperinae* venom metalloproteinases (SVMPs) cause local haemorrhage (Kamiguti et al., 1996b) by degradation of the major components of the extracellular matrix ECM (Matsui et al.,

2000) and walls of small blood vessels. It is believed that this is caused by proteolysis of the components of the basal lamina of the microvasculature such as type IV collagen, fibronectin, and laminin (Rucavado et al., 1995) of small blood vessels (Kamiguti et al., 1996b; Maruyama et al., 1992; Matsui et al., 2000; Shannon et al., 1989). Moreover, when the toxins are distributed through the systemic circulation those effects spread to sites distant from the point of venom injection (Marsh et al., 1995). SVMPs specifically cleave the key peptide bonds of the basement membrane and endothelial cells (Takeya et al., 1992; Gutiérrez et al., 2000) leading to a series of morphological and functional alterations of the endothelial cells *in-vivo.* In more advanced degenerative stages such as in the early stages of necrosis, lysis of the vessel wall lead to gaps in the endothelial cells through which blood escapes into the extravascular space (Marsh et al., 1995; Kamiguti et al., 1996a: 1996b). Although the mechanism of action of these haemorrhagic toxins is not entirely clear, Ownby et al., (1993) suggested that haemorrhagic toxins can exert their effects by (i) lysis of vessel wall, creating large breaches between capillary and endothelial cells leading to large intracellular gaps (*per rhexis*) and/or (ii) by slow oozing of blood through small gaps in capillaries (*per diapedesis*) leading to blood escaping into the connective tissue space. In addition, SVMPs have been demonstrated to inhibit platelet aggregation by blocking collagen binding to the $\alpha_2\beta_1$ integrin on the platelet surface (Kamaguti et al., 2001; Zigrino et al., 2002), thereby preventing platelet-assisted repair to injured blood vessel walls.

1.7.3. Haemostasis and systemic haemorrhage

The most characteristic effects of envenoming by *Echis* species are systemic haemorrhage and uncontrolled bleeding brought about by interference with the thrombotic and haemeostatic mechanisms of the victims (Kini et al., 2000). The entry of SVMPs into the systemic circulation is thought to be responsible for the spontaneous bleeding that occurs in many areas of the vasculature of the envenomed victim. Apart from the effect of SVMP; uncontrolled bleeding is also thought to be influenced by other venom toxins that interfere with the normal functioning of blood platelets and coagulation factors. Some venom toxins act as agonists and behave in a similar way to a natural ligand or activator of a specific step, whereas others act as antagonists and interfere in the function of a natural ligand or activator (Kamiguti et al., 1996a; Kawasaki et al., 1996).

Venom molecules that interfere with blood coagulation include disintegrins, serine proteinases and C-type lectins (Markland et al., 1998; Kamiguti et al., 1996b). Small molecular weight bradykinin potentiating peptides (BPPs) also cause hypotension, a common symptom in some victims of *E. ocellatus* envenoming (Weiss et al., 1973; Warrell, 1983). These peptides inhibit angiotensin-converting enzyme and enhance bradykinin action. SVMPs also contribute significantly to the systemic effects by their ability to hydrolyse the α chain of fibrinogen (α-fibrinogenases) which is a key factor in platelet aggregation (Huang et al.,, 2000).

In addition to the haemostatic mechanism described above the adhesion of platelets to the exposed subendothelium at the site of vascular injury involves the binding of plasma vWF to subendothelial tissue, followed by its binding to platelet GPIb (Sakariassen et al.,

1987). To date several GPIb binding proteins have been purified and characterized from snake venom including: alboaggregin-B from *Trimeresurus albolabris* venom (Peng et al., 1991), echicetin from *Echis carinatus* venom (Peng et al., 1993) and agkicetin from *Agkistrodon acutus* venom (Chen et al., 1995). Because these toxins competitively inhibit the binding of vWF to GPIb, they prevent the binding of platelets to the collagen receptor integrin $\alpha_2\beta_1$ (Moura-da-Silva et al., 2001; Yeh et al., 1996; Huang, 2000). Moreover, the small peptide disintegrins and the disintegrin-like domain of the P-III SVMPs inhibit collagen-induced platelet aggregation (Zhou et al., 1996; Gan et al., 1988; Hong et al., 2002) and, also inhibit fibrinogen binding to $\alpha_{IIb}\beta_3$ receptor of activated platelets (Moura-da-Silva et al., 2001).

As stated above, fibrinolysis is a negative feedback system essential to dissolve thrombin and allow tissue healing. Some snake venoms, not *Echis*, contain thrombin-like enzymes that interrupt this system (Jin et al., 2002). *In vivo*, fibrin produced by the venom proteases may be quickly hydrolyzed by the natural fibrinolytic system, resulting in a state of defibrinogenation (Markland and Pirkle, 1977). Venoms from many vipers also contain anticoagulant proteases with fibrinogenolytic or fibrinolytic activities capable of directly rendering fibrinogen incoagulable. These include the venom of *Crotalus atrox* (Bajwa et al., 1980; Pandya & Budzynski, 1984; Komori et al., 1985; Nikai et al., 1984), *A. contortix mokasen* (Moran & Geren, 1981), *A.contortrix contortrix* (Bajwa et al., 1982) and *C. horridus horridus* (Civello et al., 1983).

1.8. Composition of snake venom

In general, the main target of the snake venom proteins is either the normal haemostatic mechanism and/or the neuromuscular system of the prey (Vishwanath et al, 1987).

Snake venoms are complex mixtures of protein and non-protein components, secreted by specialised glands (Al-Saleh, 2002; Leonardi et al., 2001; Alape-Giron et al., 1999). Venom is at least 90% protein by dry weight, and most of these proteins are enzymes (Warrell, 1983), toxins or nerve growth factors (Al-Saleh, 2002). The non-protein fractions of the venom are mainly represented by sodium, potassium, phosphorus, chloride, calcium, zinc, magnesium, copper, manganese, riboflavin, nucleosides, peptides, lipids and carbohydrates (Al-Saleh, 2002).

1.8.1. Metalloproteinases

Generally, venoms from *Viperinae* and *Crotalinae* species exert similar local and systemic effects. Analysis of their venoms indicates that the snake venom zinc-metalloproteinases (SVMPs) are arguably the most clinically significant group of viper toxins (Bjarnason and Fox, 1994; 1995; Gutierrez and Rucavado, 2000), causing major microvascular damage and blood loss (Borkow et al., 1993). The presence of metalloproteinases in venom of *Echis* species has been confirmed (Nishida et al., 1995; Paine et al., 1994: 1992; Howes et al., 2003) and it would be expected that *E. ocellatus* would also posses hemorrhagic SVMPs.

The SVMPs are members of the reprolysin family (M13) of Zinc-metalloproteinases (Zigrino et al., 2002) and form, together with the ADAM ('A *D*isintegrin And

Metalloprotease'), the subfamily of metzincins because they share a common overall molecular organisation (Gutierrez and Rucavado, 2000; Omari-Satoh et al., 1995; Zigrino et al., 2002). The SVMPs range from 20 to 100 kDa proteins with strong and weak haemorrhagic effects (Gutierrez and Rucavado 2000; Toshiaki et al., 2000; Ito et al., 2001). All SVMPs, irrespective of their size contain a conserved zinc-binding motif [**HEXXHXXGXXHD**] in the catalytic domain, (Moura-de Silva et al., 1996; Selistre de Arujo and Ownby, 1995). The SVMPs have been classified into four classes (PI-PIV) (Fig. 1.7).

Class P-I SVMPs consist of only the zymogen and MP domain without a carboxy-terminal disintegrin domain, and are usually about 22-30 kDa express varying degrees of haemorrhagic activities (Gutierrez and Rucavado 2000). The P-II class SVMPs have either a disintegrin (Dis) or disintegrin-like domain carboxyl to the catalytic domain (Jia et al., 1997). The P-III SVMPs contain a cysteine-rich domain extending from the MP and Dis domain. Class III SVMPs include some of the most potent haemorrhagic and haemostasis-disruptive snake venom toxins with pathological activities exerted by each of the MP, Dis and Cys-rich domains, as discussed in the next sections (1.8.1.3-1.8.1.6). The class P-IV SVMPs possess all these domains and have an additional lectin-like polypeptide [e.g., epidermal growth factor-like (Primakoff et al., 1987), transmembrane, or cytoplasmic domains (Wolfsberg et al., 1993)] linked by a disulphide bridge to the metalloproteinase-containing polypeptide chain (Jia et al., 1996; Rodrigues et al., 2000; Gutiérrez and Rucavado, 2000). SVMPs are structurally related to the mammalian matrix metalloproteinase (MMP) family (Fox and Long, 1998). MMPs differ from the reprolysins or ADAMs in that some of them do not possess proteolytic activity (Jia et al., 1996).

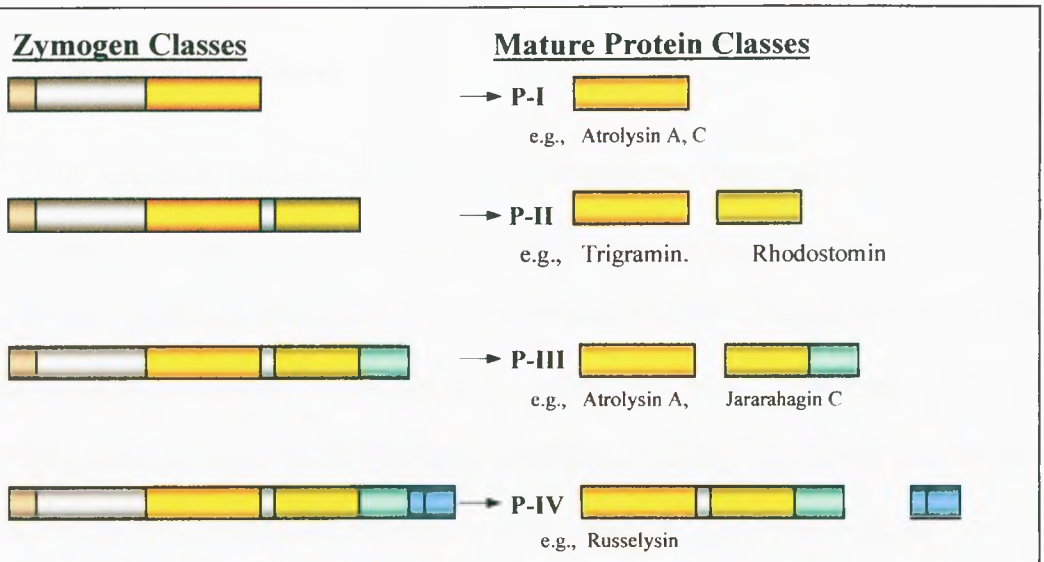


Fig. 1.7. Schematic representation of the SVMP classes. Adapted from Jia et al., 1996.

Key. Signal peptide: Propeptide: Proteinase domain: Spacer region: Disintegrin-like domain: Cystine rich domain: Lectin binding domain.

Since the amino acid sequence data of SVMPs in related New and Old World vipers show a high degree of sequence conservation, particularly of residues encoding functional domains and the propeptide domain (Paine et al., 1994, Bjarnason and Fox, 1995), it is useful to describe briefly the structure–function relationship of SVMPs.

1.8.1.1. The prepeptide domain

This domain is referred to as a signal peptide, which is highly conserved among SVMPs and comprises 18 amino acid residues with the putative cleavage site between Gly-18 and Ser-19 (Jia et al., 1996; Bjarnason and Fox, 1995).

1.8.1.2. The propeptide domain

The cDNA sequence structure of all SVMPs indicates that they are synthesised as pre-pro- zymogens (Gutiérrez and Rucavado, 2000; Jia et al., 1996). The nascent zymogen is proteolytically processed prior to secretion into the lumen of the venom gland via a cysteine switch mechanism to yield the active venom enzymes (Grams et al., 1993; Jia et al., 1996). The sequence of the propeptide domain is highly conserved and comprises approximately 190 amino acid residues and contains the consensus sequence cysteine-switch motif **PKMCGVTQ** 23 residues upstream of the MP domain (Matsui et al., 2000; Grams et al., 1993; Jia et al., 1996). It has been hypothesised that the cysteine switch motif may serve an analogous function to that seen in MMPs which prevents zymogen processing (Bjarnason and Fox, 1994; Grams et al., 1993; Springman et al., 1990; Jia et al., 1996).

1.8.1.3. The metalloproteinase domain

The MP domain of SVMPs ranges from 202 to 206 residues in length (Jia et al., 1996; Hasson et al., 2003) and differ from other member of the reprolysin family such as the astacin family (Nishida et al., 1995), by the retention of the consensus sequence **X-His-Glu-X-X-His-X-X-Glu-X-X-His** involved in zinc binding (Bode et al., 1992; Gomis-Rüth et al., 1993). The consensus Met-turn (CI/VM) region 258-260 in the MP domain (Hasson et al., 2003) is thought to ensure hydrophobic interactions within protein structure and may be involve in the zinc-binding site (Selistre de Arujo and Ownby, 1995).

1.8.1.4. The spacer Region

The spacer region is represented by a short residue stretch of 18 to 21 amino acids between the MP and Dis/Cys domains. It was Jia et al., (1996), suggested that the function of this motif may act as a linker to connect the proteinase and the disintegrin-like domains. Several studies based on the functional characterisation showed that this peptide region does not contribute significantly to the toxicity of the SVMP (Kini et al., 1997a).

1.8.1.5. The disintegrin-like domain

The disintegrin domain of SVMPs is identified by an RGD sequence that is characteristic of the group III SVMPs. The disintegrin domain of the SVMPs differs from that of the nascent disintegrin proteins by the possession of two additional cysteinyl residues in the structure (Jia et al., 1996). The group III SVMPs, such as jararhagin (Paine et al, 1992a), ecarin (Nishida et al., 1995), HR1a (Kishimoto, and Takahashi, 2002), Ca: Cat (Zhou et al, 1995), ECH-I and ECH-II (Paine et al., 1995) contain disintegrin-like domains where the RGD sequences are substituted by either ECD or DCD at the same position (Moura-da-Silva 1996). The functional role of this non-RGD disintegrin domain appears to be homologous with the small peptide disintegrin in several aspects; for instance this structure has been shown to inhibit platelet aggregation (Kamiguti et al., 1997; Jia et al., 2000; 1996). Evidence for this is strengthened by the discovery of a non-RGD motif in the disintegrin domain of mammalian sperm proteinases, including PH30-α and PH30-β, which binds to the integrins located on the egg surface during fertilization (Kratzschmar et al., 1996, Blobel et al., 1992).

1.8.1.6. The cysteine-rich domain

The C-terminal cysteine-rich domain of the SvMPs has a high percentage of cysteine residues (Jia et al., 1996). Kamiguti et al., (2003) reported that the cysteine-rich domain of SVMPs inhibits both platelet aggregation by binding to $\alpha_2\beta_1$ integrin and adhesion of α2-expressing K562 cells to this protein. Early studies by Kini and Evans (1990) and Calvete et al., (1992) suggested that this domain may be responsible for their protein structural and biological characterisation as well as the cleavage of the putative domains.

1.8.1.7. Metalloproteinases and E. ocellatus

Despite the medical importance of (i) *E. ocellatus* and (ii) metalloproteinases, it was only very recently that a haemorrhagic PIII metalloproteinase was purified from *E. ocellatus* venom (Howes et al., 2003). This report also described a non-haemorrhagic PIISVMP in *E. ocellatus* venom. This study did not however, describe the complete amino acid sequence of either proteins. Prior to my work the genetic databases contained no DNA sequences encoding *E. ocellatus* SVMPs

1.8.2. C-type lectins

Snake venom C-type lectins (CTLs) are a group of proteins which show structural similarities to the classic C-type lectins such as mannose-binding protein and the selectins (Navdaev et al., 2001a) and contain consensus cysteine and other amino acid residues that define the carbohydrate recognition domain (CRD) of the animal C-type lectins

(Drickamer, 1999; Braud et al., 2000; Castro et al., 2003; Wang, 2001a; Sarray et al., 2003). Snake venom CTLs consist only of a C-terminal carbohydrate-recognition domain (CRD) in comparison to these of the animal membrane lectins which contain additional domains (Giga et al., 1987).

Most of the 30 kDa snake venom CTL proteins exist as αβ-heterodimers linked by a single interchain disulphide bond and possess striking sequence similarity (Harrison et al., 2003a; Wang et al., 2001a). Despite this high degree of sequence similarity, venom CTLs have distinct effects on factors in the coagulation cascade or platelet components. For example, some snake venom CTLs exhibit anticoagulation activities and bind to γ-craboxyglutamic acid (Gla)-containing domains of the coagulation factors IX and X in the presence of calcium ions (Wang, 2001a; Atoda et al., 2002). Other venom CTLs inhibit or activate platelets by modulating their binding interactions to the platelet receptors such as glycoprotein (GP) Ib, $\alpha_2\beta_1$, GPVI and vWF (Navdaev et al., 2001b; Polgar 1997; Shin and Morita, 1998; Wang, 2001a). An example of such an agonist affect is represented by the heterodimeric protein echicetin from *E. carinatus* venom (Peng et al., 1993). Echicetin binds specifically to platelet GPIb to inhibit platelet aggregation by blocking its interactions with vWF or with thrombin (Peng et al., 1993; Polgar et al., 1997). *In vivo* experiments showed that echicetin induces thrombocytopenia when injected into small animals (Navdaev et al., 2001a). In contrast alboaggregrin-B isolated from *Trimeresurus albolabris* venom, binds to GPIb and induces platelet agglutination in the absence of vWF (Peng et al., 1991).

C-type lectins such as bothrojaracin from *Bothrops jararaca* venom demonstrate another variant of snake venom CTLs; these are based on its ability to interact with both thrombin anion-binding exosites I and II. Bothrojaracin also induces allosteric changes in the thrombin active site to inhibit (i) the activity of thrombin toward fibrinogen clotting, (ii) aggregation of platelets and (iii) protein C (Matsuzaki et al., 1996; Tani et al., 2002).

1.8.2.1. C-type lectins and E. ocellatus venom

Despite the medical importance of *E. ocellatus,* comparatively nothing is known about the contribution of CTL activity to the pathology of *E. ocellatus* envenoming.

1.8.3. Serine proteases

Venom serine proteases also exhibit homologous structures with different biological activities such as fibrinogen-clotting (Itoh et al., 1987), kinin-releasing, (Serrano et al., 1998), plasminogen-activating (Zhang et al., 1998), increasing of blood capillary permeability (Hahn et al., 1998) and β-fibrinogenolytic (Hung et al., 1994). The multifunctional ability of this enzyme family strongly suggests that serine proteases cleave their substrates or plasma proteins in a protein-specific manner resulting in activational or inhibitory effects on a variety of mammalian physiological systems (Hutton et al., 1993; Matsui et al., 2000; Felicori et al., 2003).

Although no native serine proteases have been purified from *E. ocellatus* venom, or from other *Echis* species in general, proteolytic serine proteinases have been identified and

characterised in venoms of other viperid species such as that of *Bothrops jararaca* (Serrano et al., 1998), *Trimeresaurus jerdonii* (Samel et al., 2002), *Vipera lebetina* (Siigur et al., 2001) and *Deinagkistrodon acutus* (Wang et al., 2001b). Serine proteases isolated from the *B. jararaca* for example has been shown to induce diverse effects such as their ability to release bradykinin from kininogen-like mammalian kallikrein ['kallikrein-like' proteases] (Matsui et al., 2000), specific activities depending on specific substrates and induce blood clotting (Serrano et al., 1998).

1.8.4. Phospholipase A_2s

Phospholipases are Ca^{2+}-dependent esterolytic enzymes, which are separated into various classes, namely A_1, A_2, B, C, and D, based on the site of hydrolysis (Kini, 1997). Phospholipases A_2 are a superfamily of enzymes which occur ubiquitously in nature and are usually divided into either intracellular or secreted enzymes (Kini, 1997). Secreted PLA_2s ($sPLA_2$s) are small, soluble proteins that have been isolated from a large number of biological sources, especially from the mammalian pancreatic juice, snake, scorpions and bee venoms (Lambeau et al., 1994; Kini et al., 1986; Nicolas et al., 1995). The (13-18 kDa) group I and II secreted venom PLA_2s are the most extensively studied PLA_2 enzymes and share high sequence similarity and similar kinetic behaviour (Hendrickson and Dennis, 1984). Group I PLA_2s are represented in the mammalian exocrine pancreas, in venoms of *Elapidae* and *Hydrophidae* snakes and in human spermatozoa (Langlais et al., 1992). Group II PLA_2s are abundant in *Viperidae* venoms (Scott, 1997). Group II PLA_2s are

subdivided further into six subgroups based on the number and location of disulphide bridges and the sequence of the C-terminus (Kini, 1997).

In contrast to the toxicity of snake venom PLA_2s, mammalian PLA_2s mediate many physiological processes but appear to posses relatively weak pharmacological effects.

The general function of PLA_2 enzymes is the hydrolysis of acyl ester bond at the *sn*-2 position of the glycerophospholipid vesicles to liberate free non-esterified fatty acids and lysophospholipids (Hanasaki and Arita, 1992). The term phospholipase A_2 (PLA_2) is used to denote the 2-acyl specificity of the enzyme (Uthe and Magee, 1971). However, this function is not necessary applicable to all PLA_2s because some snake venom PLA_2s exhibit pharmacological effects independent of the hydrolysis of phospholipid (Petan et al., 2002; Nicolas et al., 1995; Machado et al, 1993). To date no native PLA_2s have been purified from the venom of the saw-scaled viper, *E. ocellatus*. However, several toxic PLA_2 have been identified in venoms of related *Viperinae* species [including *Echis*] and have been shown to possess oedema-inducing, neurotoxic, lipolytic and platelet aggregating activities (Kemparaju et al., 1994; 1999a; Polgár et al., 1996). It is well established that snake venom PLA_2s of related vipers in Asia, Europe, the Middle East and the Americas contribute to venom-induced pathology through a broad spectrum of toxic activities as illustrated Table 1.1 (Kini, 1997).

Table 1.1.Pharmacological effects of snake venom PLA_2 enzymes

- Neurotoxicity;
    - Presynaptic neurotoxicity
    - Postsynaptic neurotoxicity
- Myotoxicity;
    - Local myonecrosis
    - Systemic myotoxicity
- Cardiotoxicity
- Anticoagulant effects
- Platelet aggregation initiation
- Platelet aggregation inhibition
- Hemolytic activity
- Internal hemorrhage
- Antihaemorrhagic activity
- Convulsant activity
- Hypotensive activity
- Oedema-inducing activity
- Organ and/or tissue damage;
    - Liver, Kidney, Lungs, testis, pituitary damage

1.8.5. Other venom components

There are other toxins which also play a pivotal role in the localized and systemic effects as a result of snake envenoming. These include, hyaluronidase which is present in most snake venoms (Khoo, 2002); it cleaves hyaluronic acid (hyaluronan), a high molecular weight glycosaminoglycan and a major connective tissue constituent in animals (Cerna et al., 2002). This results in a decrease in the viscosity of connective tissues (Russell, 1980; Pukrittayakamee et al., 1988). Hyaluronidases are therefore known as 'spreading factors' (Pessini et al., 2001) because they facilitate toxin diffusion into the tissues of envenomed prey (Khoo, 2002), thus contributing to local and systemic envenoming (Pessini et al., 2001). L-amino acid oxidases (LAOs) are widely found in most snake venoms (Sakurai et

al., 2001; Tempone et al., 2001), particularly in venoms of *Echis* species (Nathan et al., 1982) and are thought to contribute to toxicity upon envenoming by producing α-ketoacids, hydrogen peroxide (H_2O_2) and ammonia from L-amino acids (Du and Clemetson, 2002; Ali et al., 2000) resulting in prolonged bleeding from blood vessel walls at the bite site (Takatsuka et al., 2001; Du and Clemeston, 2002). Other enzymes include acidic and basic phosphatases, ribonucleases, lactate dehydrogenases, deoxyribonucleases, nucleotidases which are thought to initiate cell apoptosis (Warrell, 1997; Russell, 1980).

1.9. DNA immunisation

DNA vaccination or genetic immunisation, a rapidly developing technology which has been described as a third generation of vaccines (Dixon, 1995), offers new approaches for the prevention and therapy of several diseases of both bacterial and viral origin (Gurunathan et al., 2000; Koide et al., 2000). DNA immunisation has also emerged more recently as a strikingly novel approach to immunoprophylaxis (Ramsay et al., 1997) and has been widely used in laboratory animals and non-human primates over the last decade to induce antibody and cellular immune responses (Donnelly et al., 2003).

Successful *in-vivo* transfection of mammalian cells following injection of purified DNA was first reported over 30 years ago (Atanasiu, 1962). However, its potential went largely unrealised until 1990 when Wolff and colleagues demonstrated that a reporter gene encoding an enzyme protein could be expressed in murine skeletal muscle *in-vivo* and the tissue retained its transgenic biological activity for up to 60 days after inoculation. These

observations were extended by several studies such as those of Tang et al., (1992) who demonstrated that mice injected with plasmid DNA encoding human growth hormone (hGH) elicit antigen-specific antibody responses. Based on these findings, it is concluded that this technology is promising as it can enhance both cellular and humoral immunity.

1.9.2. Advantages of DNA vaccines

The ability of plasmid DNA to induce both cellular and humoral immune responses after inoculation has been demonstrated in several animal models and hopes have been raised that its applications will led to new therapies for a range of human diseases. Studies to evaluate the safety and immunogenecity of DNA vaccination in humans began within 2 years of the first published reports of protective immune responses against infectious diseases in animals and many studies are still ongoing.

DNA-based immunisation exhibits several important advantages over conventional immunisation strategies that involved live-attenuated or killed pathogens, proteins, or synthetic peptides; it appears to incorporate many of the most attractive features of each approach. One of the important advantages of the DNA immunisation (Babiuk et al., 2000a), is that the immune response to immunisation can be directed to elicit either humoral or cellular immune responses or both without the need for live vectors or complex biochemical production techniques.

Other advantages of DNA vaccines are that they are highly specific and that the expressed immunizing antigen is subjected to the same glycosylation and post-translational

modifications as natural viral infection. Logistic advantages of DNA vaccines include the relative ease and low cost of production and transportation making them more suited to production in the developing world than other systems. A summary of these perceived advantages of DNA vaccines is described in Table 1.2.

1.9.2. Disadvantages of DNA vaccines.

The disadvantages of DNA vaccines are based mainly on health and safety issues. Most of the safety issues concerning the system are based on the activation of oncogenes as a

Table 1.2. Summary of relative advantages of DNA vaccines over conventional vaccines. ***Table adapted from Gurnathan et al., 2000***

		DNA vaccine	Live attenuated	killed/protein subunit
Immune response				
Humoral →	B cells →	+++	+++	+++
Cellular →	CD4+ →	Th2	Th1	Th1
	CD8+ →	++	+++	-
		MHC class	MHC class	MHC class
Antigen presentation →		I&II	I&II	II
Memory →	- Humoral	+++	+++	+++
	- Cellular	++	+++	+/-
Manufacturing				
	- Ease of development and production	++++	+	++
	- Cost	+++	+	+
	- Transport & Storage	+++	+	+

result of genomic incorporation of immunising DNA (Robertson, 1994) as well as eliciting anti-DNA antibodies; however, this has rarely been detected in experimental studies (Ramsay et al., 1997). While these issues are of concern and require careful monitoring, it would not apply to DNA immunisation of captive animals to produce antibodies, particularly if gene gun is used. This is because the likelihood of eliciting anti-DNA antibodies when using the gene gun is minimised because it requires 100-fold less DNA than intramuscular injection to achieve equivalent seroconversion efficiencies (Harrison and Bianco, 2000).

1.9.3. Principles of DNA immunisation.

DNA vaccination involves the introduction of nucleic acid into host cells where it directs the synthesis of its encoded polypeptide(s) and stimulates an immune response (Shedlock and Weiner, 2000). Unlike gene therapy, genetic integration is not intended. Indeed, the construction of a DNA vaccine is designed to permit localized, short-term expression of the target antigen.

Although several attempts have been made to study the cellular pathways for the processing of antigens and their presentation to T lymphocytes, the precise mechanism involved in the induction of an immune response following DNA immunisation is not yet fully understood (van Drunen Littel-van den Hurk et al., 2001). However, it is well documented that the magnitude and type of immune response induced after DNA immunisation is influenced by a number of different parameters, some of which are represented by the type and components of the expression plasmid.

1.9.3.1 Essential components of a DNA Plasmid

A typical "first generation" DNA vaccine plasmid requires (i) the incorporation of a strong viral promoter to achieve optimal expression in mammalian cells (Liu, 2003), such as cytomegalovirus (CMV) or simian virus 40 [which provide the greatest gene expression]; (ii) an origin of replication (ori) allowing plasmid propagation in *E.coli*; (iii) a bacterial antibiotic resistance gene [this allows plasmid selection during bacterial culture]; and (iv) a transcription-stop sequence such as bovine growth hormone 3'-untranslated region (BGH 3'-UTR) (Fig.1.8).

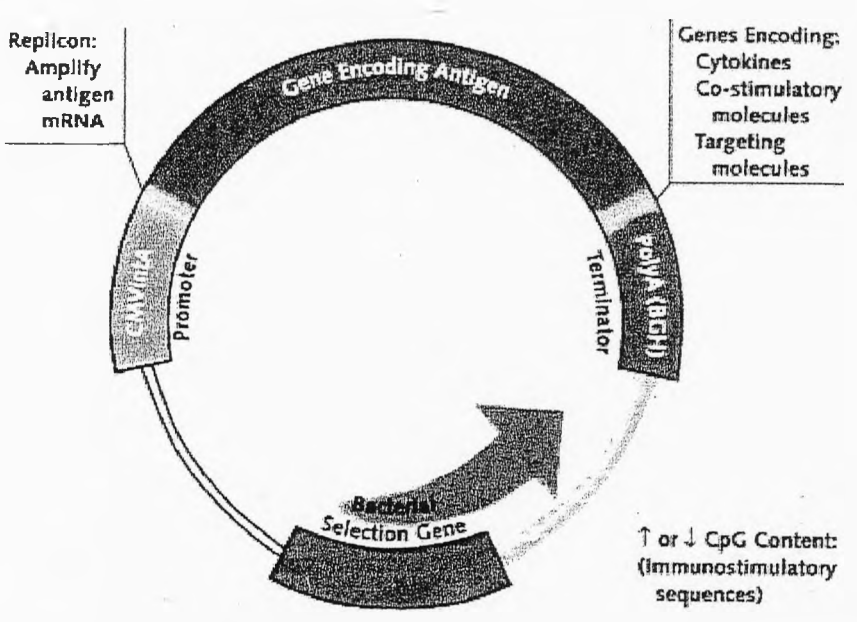


Fig.1.8. A schematic representation of a simple DNA plasmid. Diagram adapted from McDonnel and Askari (1996)

1.9.3.2. The influence of mode and site of gene delivery

Several studies have shown that the type of immune responses induced by plasmid immunisation is significantly affected by (i) the mode and site of gene delivery, (ii) the

dose of plasmid and (iii) the administration of booster injections and the interval between immunisations (van Drunen Littel-van den Hurk et al., 2000). In general, immunisation with DNA can be accomplished in two fundamentally different ways. One approach is the use of needle injection into different tissues, the most effective route being intramuscular injection (i.m) into the hind leg quadriceps or tibialis anterior (Seddegah et al., 1994; Wang et al., 1993) followed by intrademal (i.d) routes (Raz et al., 1994; Sato et al., 1996). These routes usually provoke strong, antigen-specific Th1-biased, humoral and cellular immune responses (Feltquate et al., 1997; Babiuk et al., 2000b). An improvement in efficacy of plasmid transfection was achieved by injection of DNA into regenerating skeletal muscle, achieved by prior injection of either cardiotoxin or local anaesthetic such as bupicaine (Donnelly et al., 2003). Several methods have been investigated to improve delivery of DNA vaccines including (1) mechanical delivery consisting of microinjection by various types of needles including pressure injection, (2) electrical [electroporation, ionophoresis] and (3) chemical [liposomes and various polymers] (Felgner et al., 1987; Ramshaw et al., 1997; Ando et al., 1999; Aggarwal et al., 1999; Zhang et al., 1996) and mucosal delivery. Each one of these methods of delivery introduces plasmid DNA into distinct areas of immune surveillance and therefore primes the immune system in distinct ways.

Gene gun delivery of DNA which propels the DNA-coated gold particles into the epidermis (Tang et al., 1992; Ulmer et al., 1993) resulted in a more Th2 biased antibody isotype response and efficient humoral and cellular responses (Feltquate et al., 1997; Babiuk et al., 2000b). The distinct Th1- or Th2-biased immune responses elicited by i.m or gene gun delivery, respectively, is not fully understood. Bacterial DNA contains CpG

motifs that induce non-specific Th1-dominant responses. Gene gun delivery requires 100-1000 fold less DNA to stimulate immune responses to that achieved by i.m injection. The reduced number of Th1-promoting CpG motifs involved in gene gun immunisation may therefore explain the Th2-bias response to gene gun DNA vaccination.

1.9.3.3. Antigen presentation following DNA immunisation.

An important step in the design of DNA immunisation constructs is to understand the immune correlates of protection. Antigen peptides expressed after DNA immunisation are usually presented by antigen-presenting cells [APCs] in the context of either MHC class II or class I molecules to CD4+ and CD8+ T cells, respectively. There are at least three means by which MHC class I–restricted CTL might be elicited following administration of plasmid DNA: (i) *transfection of professional* APCs, (ii) *antigen presentation mediated directly by transfected myocytes* or, (iii) *cross priming*, (Shedlock et al., 2000) as illustrated in Fig. 1.9.

(i) Transfection of professional APCs

The immune response following DNA immunisation was found to be dependent upon professional APCs, specifically bone marrow-derived dendritic cells [DC] (Iwasaki et al., 1997). In 1993, Fu and colleagues demonstrated that when parental bone marrow chimeras were immunised with plasmid DNA encoding influenza nucleoprotein [NP] by i.m. injection and gene gun, CTL responses were specific to the peptide presented by the MHC class I molecules found on the donor bone marrow (Condon et al., 1996) (Fig.1.9b).

Furthermore, the same authors reported that, although only a small proportion of the DCs were transfected with plasmid DNA, it was noticeable that there was general activation (maturation) and migration of large number of DCs that had not been transfected. However, whether this generalised maturation of untransfected DCs could also present antigen via additional mechanisms remains uncertain.

(ii) Antigen presentation mediated directly by transfected myocytes

Ulmer et al., (1993), by demonstrating that direct intramuscular inoculation of plasmid DNA induced a strong CD8+ CTL to influenza nucleoprotein, provided the first evidence that cellular responses could be induced *in vivo* by DNA immunisation and that the induced immune responses had a potentially important protective role. Subsequent experiments were then undertaken to directly test whether DNA-transcribed muscle cells alone are sufficient to prime immune response (Fig.1.9a). However, Iwasaki et al., (1997), reported that muscle cells failed to prime CTLs responses when injected with DNA plasmid encoding CD86 or GM-CSF only (i.e., without antigen). To examine the contribution of both bone marrow- and non-bone marrow-derived cells to CTL priming, Agadjanyan et al., (1999) found that antigen-specific CTL responses could be induced by non-bone marrow-derived (muscle) cells only when mice were immunised with DNA encoding either the antigen and CD86.

Surgical ablation experiments have been used to identify the contribution of antigen expression in tissues subjected by DNA immunisation by distinct routes. Torres et al., (1997) demonstrated that removing the DNA-injected muscle bundle within 10 minutes of

DNA injection had no affect on the longevity and magnitude of humoral and CTL responses, suggesting a rapid migration of transfected cells or plasmid DNA from the site of injection. These authors also found that excision of the epidermal site 24 hours after gene gun bombardment abrogated the induction of CTL responses, suggesting that the immune response was dependent on the transfected epidermal cells. This finding indicates that i.m injected plasmid DNA is likely to gain rapid access to the lymphatic or circulatory system, thus obviating the need for transfection of muscle cells at the site of injection. In conclusion, these data indicate that DC cells such as Langerhans and myocytes play a crucial role in the primary response triggered by DNA vaccines.

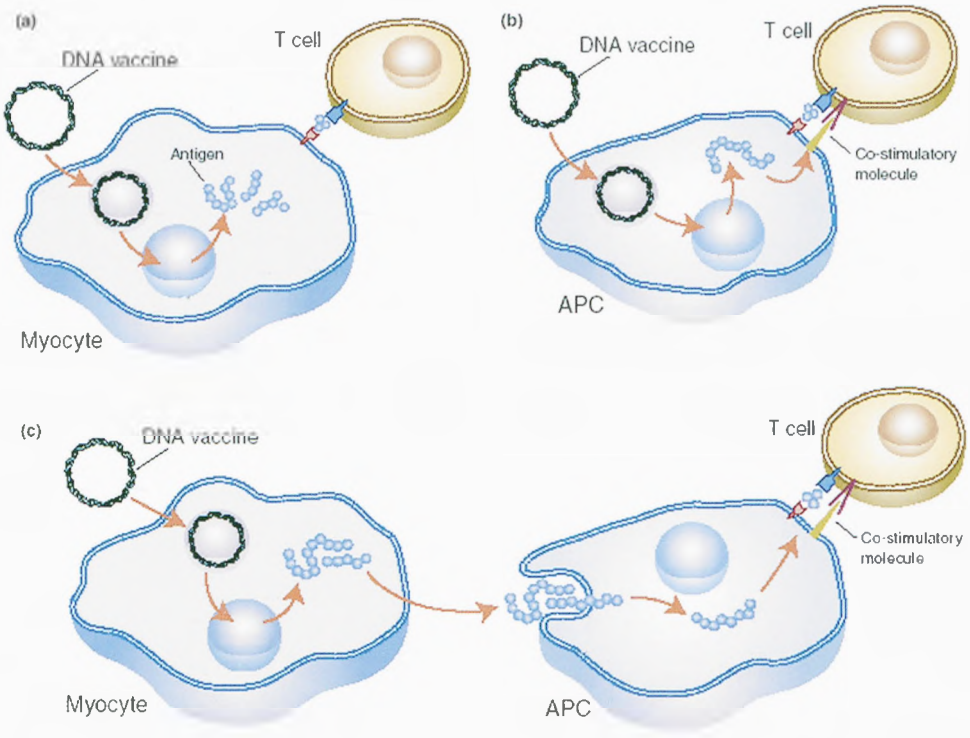


Fig.1. 9. Mechanisms of antigen presentation following DNA immunisation. *Diagrams from Liu* (2003).

(iii) *Cross-Priming*

Cross priming has been suggested as a mechanism to explain antigenic transfer from DNA-transfected somatic cells to professional APCs (Fig.1.9c). The concept of cross-priming, in which triggering of CD8+ T-cells responses can occur without *de novo* antigen synthesis within the APCs, was first described by Ulmer (1996) and Fu (1997) and provides an additional mechanism by which DNA immunisation can enhance immune responses (Shedlock and Weiner, 2000; Donnelly et al., 1997). During cross priming, antigens or peptides expressed by DNA-transfected myocytes or DCs presented in context of either MHC I or II can be taken up by professional APCs to prime T-cell responses. Thus, DNA-transfected myocytes or DCs may serve as antigen-producing "factories" which magnify and maintain the immune response via cross priming (Donnelly et al., 1997; Shedlock and Weiner, 2000).

1.10. Strategy of DNA immunisation in the development of toxin–specific antivenoms

The remarkable advance and diverse applications of DNA immunisation attracted the attention of many researchers as an alternative procedure for analysing the structure and expression of genes in general (Chattergoon et al., 1997) and studies for improving the treatment of systemic envenoming by snakes in particular (Harrison et al., 2000b; 2002a; 2003b; 2004).

This section of my thesis describes the rational of using DNA immunisation as an alternative means of developing toxin-specific antibodies for improving the treatment of systemic envenoming by the saw-scaled viper, *E. ocellatus*.

Conventional venom immunisation protocols have remained unchanged over the past century with no attempts being made to direct the immune response to specific, clinically important toxins. Currently, antivenoms are generated by conventional immunisation protocols. Although very good at inducing immune responses to highly immunogenic proteins or epitopes, however, these protocols do not result in the induction of clones secreting antibodies to weakly reactive or to minor proteins present in snake venom as stated previously. This means that antivenoms contain redundant antibodies to many non toxic venom components which dilute the toxin specific antibodies (Theakston and Reid, 1983). In addition, the most toxic venom components are not necessarily the most immunogenic; for example toxins of low molecular weight may be toxic but poorly immunogenic (Mandelbaum and Assakura, 1998; Theakston, 1983b). Furthermore, the efficacy of antivenom is to an extent controlled by the intraspecific variation in the venom batches used for immunisation. Therefore, an immunising protocol that generates toxin-specific antibodies is essential for increasing the dose-efficiency of antivenoms thereby reducing the risk of early anaphylactoid and late serum sickness reactions to equine and ovine antivenoms that have to be administered in large volumes.

Using an expression library encoding these toxic proteins combined with DNA immunisation protocols such as that developed by Harrison et al., (2000b) should (i) permit the expression of *E. ocellatus*-derived cDNAs in a eukaryotic environment so that

the protein presented to the immune system should faithfully represent the glycosalated tertiary structure of the native toxins, (ii) permit the production of antibodies to the weakly immunogenic, but highly toxic, low molecular weight venom proteins. The later are often poorly represented in conventional antivenoms, (iii) induce a potent antibody response to be rapidly generated, minimal amounts of protein expressed by DNA-transfected cells, (iv) allow construction of a series of plasmids that contain epitopes from a variety of medically- important toxins in the venom of *E. ocellatus,* thereby inducing the broadest range of responses, (v) result in a higher level of safety because snake maintenance, costs and the hazards of venom extraction to obtain immunising material are avoided, (vi) be better suited than protein-based systems to antivenom production in countries with poorly developed technical resources, because plasmid DNA is readily renewable and batch-batch standardised (vii) induce high titres of venom toxin-specific IgG (Harrison et al., 2000b; 2002a; 2003b; 2004).

1.11. Aim and objectives

My research project is part of a research effort to generate, using DNA immunisation, antibodies specific to the major haemostasis-disruptive toxins in venoms of the most medically important vipers in Africa (Harrison et al., 2002; 2003a; 2003b; Bahrati et al., 2003; Hasson et al., 2003). The focus of my research is the *E. ocellatus*. Before my work remarkably little research had been performed to characterise the venom components of this dangerous viper. It was therefore necessary to construct a cDNA library from venom gland mRNA and to isolate cDNA sequences encoding the major haemostasis-disruptive

molecules, including SVMPs, CTLs, serine proteases and PLA_2s. The sequences of these *E. ocellatus* toxins were compared with the literature and their deduced structure examined to predict the potential of antibodies generated by DNA immunisation. The extent to which these objectives were achieved is described in the following chapters.

Chapter 2

Materials and Methods

This chapter outlines the materials and methods used throughout the work described in this thesis and is divided into two major parts. The first part (2.1) describes the various techniques involved in the construction of the venom gland cDNA library of the saw-scaled viper *E. ocellatus* and the second (2.2) describes techniques frequently used in different stages of the project. Where techniques were modified to suit the stage of the work, those modifications are described in the individual chapter. All solutions and their source of purchase, including equipment are described in appendix A.

2.1: Construction of cDNA library

2.1.1 Extraction of E. ocellatus venom glands

Two adult carpet vipers *E. ocellatus* (source: Kaltungo, Nigeria) used in this study were maintained in the herpetarium, Liverpool School of Tropical Medicine, Liverpool, U.K.

Venom glands dissected from *E. ocellatus* [sacrificed three days after venom extraction when toxin gene transcription rates are at a peak, Paine et al., (1994)] were placed into liquid-nitrogen and homogenized using a pestle and mortar until they were a fine powder.

2.1.2. Isolation of total RNA

Total RNA was then extracted using guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987) using a commercial kit (Trizol, Life Technologies). Trizol (1ml) was added to the homogenized gland powder in sterile glass homogenizer and homogenization continued for 5 minutes on ice. The homogenized sample was transferred into a 50 ml Falcon tube and kept for 5 minutes at room temperature (RT). Chloroform (400µl) per 2ml Trizol reagent was added, the tube mixed vigorously for 15 seconds,

incubated at RT for 3 minutes and centrifuged at 13,000 rpm for 16 minutes at 4°C. The RNA-containing aqueous phase was recovered, taking care not to disturb the interface, and transferred into a sterile Eppendorf tube. RNA from the aqueous phase was precipitated by thorough mixing with 1ml isopropyl alcohol [0.5ml/1ml Trizol] and incubated at RT for 10 minutes. Precipitated RNA was pelleted by centrifugation at 12,000 rpm for 10 minutes at 4°C. The RNA pellet was washed with 1ml 75% ethanol (1ml for every 1ml Trizol) and either stored at -80°C for future application or centrifuged at 7,500 rpm for 5 minutes at 4°C. The ethanol was discarded and the RNA pellet allows to air dry for 10 minutes. The remaining trace of the ethanol was removed by using nitrogen gas and the RNA pellet was then resuspended in 25µl RNase-free deionised water. The size and quality of the RNA extracted was estimated by agarose electrophoresis (Fig.2.1). The size of RNAs ranged from > 0.2 to over 1.0. The presence of large RNA fragments, ribosomal bands and the absence of low molecular weight degraded products indicated that the RNA was intact or not significantly degraded. The total RNAs extracted from the snake venom glands of *E. ocellatus* have the 28s and the 18s (Fig.2.1).

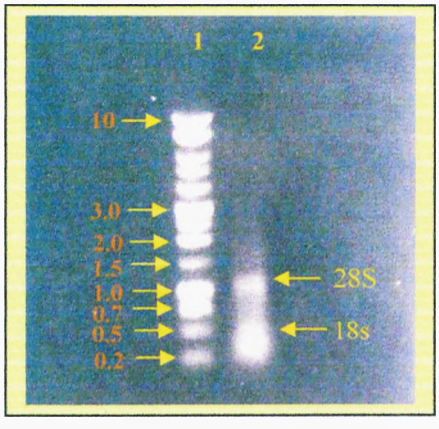


Fig.2.1. *Analysis of total RNA from E. ocellatus venom gland*

Analysis of *E. ocellatus* venom gland total RNA was subjected to 1% agarose gel electrophoresis with 10µl [1kb ladder, Lane 1], 1µl [total RNA, *E. ocellatus*] + 4µl (RNA free water) + 1µl (6x SLOB), Lane 2]. The position of the DNA markers and the identities of the 28s and 18s bands of rRNA of *E. ocellatus* venom gland are indicated.

2.1.3. Isolation of mRNA

Messenger RNA was isolated from all other nucleic acid contaminants using Oligo dT magnetic beads isolation system (PolyATract mRNA, Promega) as outlined in Fig. 2.2.

The volume of the RNA sample (24µl) was made up to 500µl using RNase-free water and incubated at 65°C for 10 minutes. Biotinylated-Oligo (dT) (3µl) and 13µl

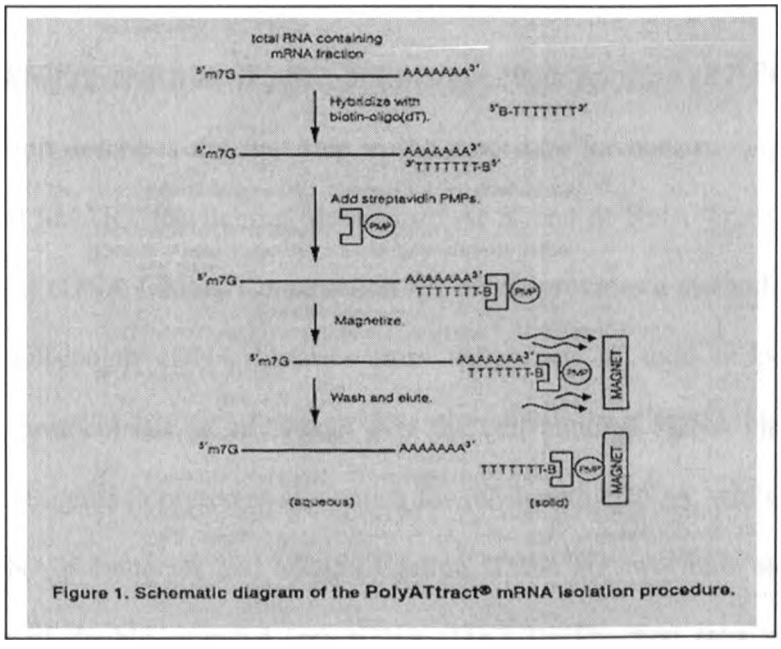


Fig.2.2*. Flow chart of the PolyATract mRNA isolation procedure*

of 20xSodium Chloride Sodium citrate (SSc) (supplied) were added, mixed gently and incubated at RT for 10 minutes. In the meantime the Streptvidin-Paramagnatic Particles (SA-PMPs) were prepared and added to the entire content of the annealing reaction and the mixture incubated at RT for 10 minutes. The SA-PMPs were captured using the magnetic stand and the supernatant was carefully removed. The SA-PMPs were then washed four times with 0.1x SSC (0.3ml). The mRNA was concentrated by precipitation in 25µl of 3M sodium acetate and 250µl of iso-propanol and incubated at –20°C overnight. The precipitated RNA was centrifuged for 30 minutes at 13,000rpm at 4°C, washed, resuspended in 75% ethanol treated with DEPC and stored at –80°C or used immediately for the next step.

2.1.4. mRNA reverse transcription-polymerase chain reaction (RT-PCR)

This section describes the first step in the procedure for constructing a cDNA library. I have used SMART (**S**witching **M**echanism **A**t 5' end of **R**NA **T**ranscript, CLONTECH, CA U.S.A) cDNA Library Construction Kit which provides a method for producing high-quality, full-length cDNA libraries from nanograms of total or poly A+ RNA. The SMART protocol has an advantage over the conventional cDNA cloning procedures in that it is designed to preferentially enrich for full-length cDNAs, while eliminating the use of T4 DNA polymerase and adapter ligation. There are two main steps involved in the synthesis of double stranded (ss) cDNA (Fig.2.3). The first step is based on using a modified oligo (dT) primer (CDS III/3' PCR Primer, appendix A) to prime the first-strand [mRNA → ss cDNA] synthesis reaction (Fig.2.3). When the reverse transcriptase reaches the 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides,

primarily deoxycytidine, to the 3' end of the cDNA, resulting in a full-length ss cDNA containing the complete 5' end of the mRNA, as well as the sequence complementary to the SMART IV Oligo. This then serves as a universal priming site (SMART anchor) for the subsequent amplification by LD-PCR (Long-distance PCR) as described in the next section.

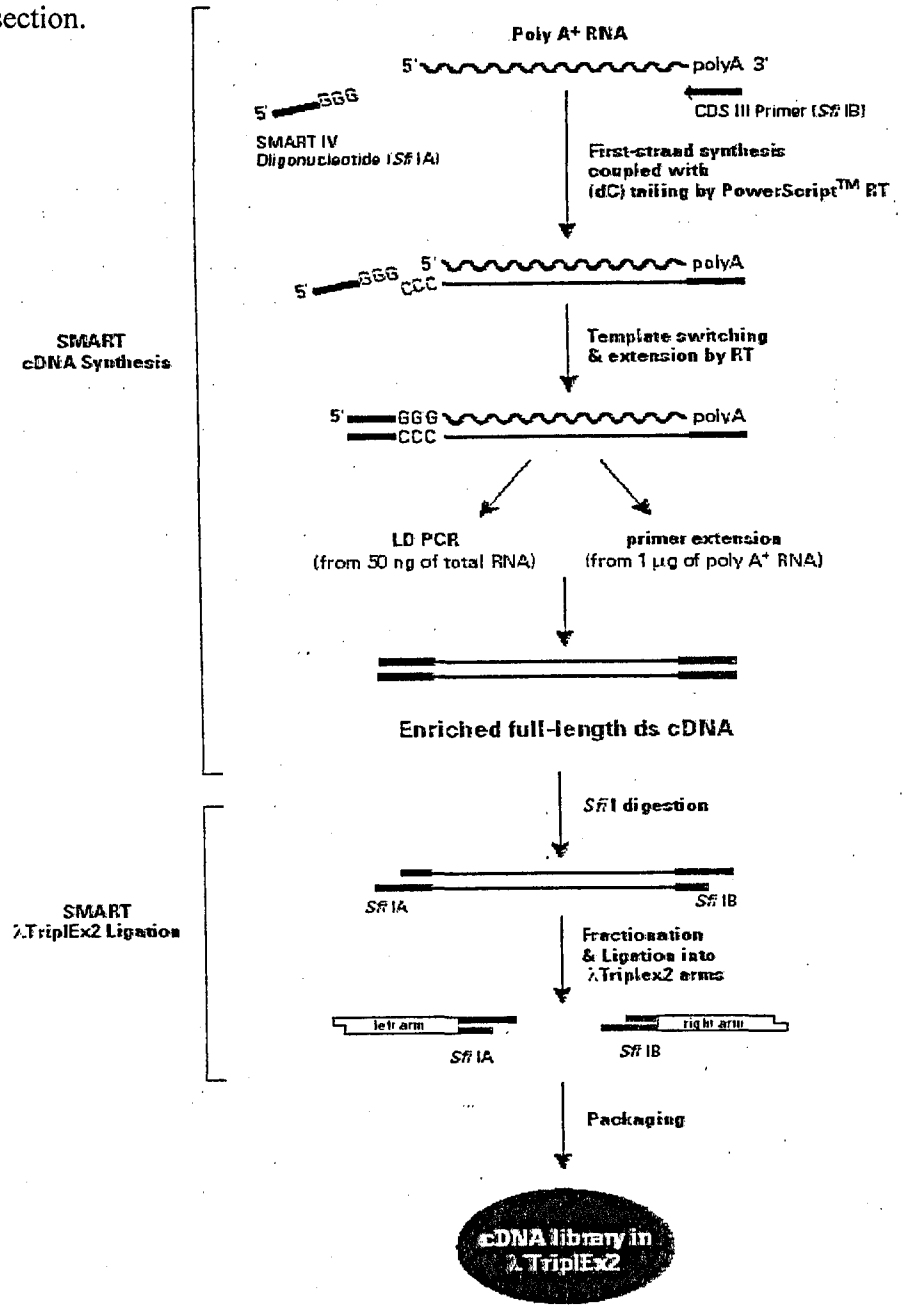


Fig.2.3. Flow chart of the SMART cDNA Library Construct kit protocols.

Isolated mRNA prepared as described above was centrifuged for 30 minutes at 13,000 rpm, the pellet was air dried and resuspended in 6µl RNase free water. Only 3µl was used in the following procedure and the remaining 3µl was resuspended in

200µl of 75% ethanol treated with DEPC and stored at –80°C. The following master mix was prepared;

- 3µl mRNA
- 1µl SMART IV oligonucleotide
- 1µl CDS III/3' PCR primer

- 5µl Total volume

The mixture was centrifuged briefly and incubated at 72°C on a heating block for 2 minutes. The tube was cooled on ice for 2 minutes and then spun briefly to collect the contents at the bottom of the tube and added to the reverse transcription mixture as follows:

- 5 µl the above mixture
- 2µl 5x first strand buffer
- 1µl DDT (20mM)
- 1µl dNTP mix (10mM)
- 1µl PowerScript Reverse Transcriptase

- 10µl Total volume

The contents were then mixed by gentle pipetting, centrifuged at 1300rpm for 5-10 seconds and overlaid with drop of mineral oil. The tube was then incubated at 42°C for one hour in a sterile water bath. The tube was then incubated on ice to terminate first-strand synthesis. The sample was then stored at –20°C until used.

2.1.5. cDNA Amplification by LD PCR

LD-PCR is the next step where only those ss cDNAs having a SMART anchor sequence at the 5' end can serve as a template and can be exponentially amplified. Incomplete cDNAs and cDNA transcribed from poly A^- RNA will lack the SMART anchor and will not be amplified. Thus, contamination by genomic DNA and Poly A^- RNA is eliminated. The following components were combined as follows;

- 2µl First strand cDNA (from section 2.1.4)
- 80µl Deionized water.
- 2µl 50X dNTP Mix.
- 2µl 5' PCR Primer
- 2µl CDC III/3' PCR Primer
- 2µl 50X Advantage 2 Polymerase Mix.

- 100µl Total volume

The contents were mixed by gently flicking the tube and followed by brief centrifugation (5-10 seconds at 13000rpm) to collect the contents at the bottom of the tube. This was then over-laid with two drops of mineral oil. Thermal cycling was performed using the following program;

GeneAmp 2400/9600

95°C 20 seconds

95°C 5 seconds
68°C 6 minutes. ⟵ 22 cycles

Five microlitre of the dscDNA was analysed using 1.0% agarose gel electrophoresis (Fig.2.4).

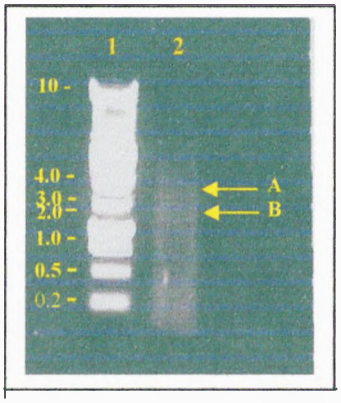


Fig. 2.4. Analysis of *E. ocellatus* dscDNA construction.

It can thus be seen that LD-PCR generates two distinctive bands [A: >3.0 and B:≥2.0]. This may corresponded to an abundant transcript. However, both bands are weaker than the 1-kb DNA size marker; this may be due to a higher complexity of the Poly A+ starting material. Moreover, LD-PCR generates some low-molecular-weight material (< 0.4 kb) which could consist of some degraded RNA fragments.

2.1.6. Proteinase K Digestion

To isolate the dscDNA from the mixture it was subjected to proteinase K digestion followed by phenol/chloroform/isopropanol purification according to the manufacturer's instructions.

Two µl proteinase K was added to the tube containing 45µl PCR product incubated at 45°C for 20 minutes and then spun briefly. RNase free water (45µl) and 100µl phenol: chloroform: isoamyl alcohol (pH 8, Sigma) was added, respectively. The solution was mixed thoroughly by gentle inversion for 2 minutes and then centrifuged at 13,000 rpm for 5 minutes. The dscDNA was precipitated from the final aqueous phase by addition of 10µl

sodium acetate (3M), 1.3µl glycogen (20µg/ml supplied-CLONTECH) and 260µl ethanol (100%-sterile) at RT and immediately centrifuged at 13,000 rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended with 100µl (80%) ethanol and stored at –20°C overnight. The sample was then centrifuged for 20 minutes at 13,000 rpm, the supernatant discarded and the pellet allowed to air dry to evaporate off any residual ethanol. The pellet was resuspended with 79µl RNase-free deionised water.

2.1.7. Sfi I digestion

Another feature of the SMART Kit is the incorporation of asymmetrical *Sfi*I restriction enzyme sites at the 5' and 3' cDNA ends, respectively. After digestion with *Sfi*I and size fractionation using CHROMA SPIN-400 Column, the SMART cDNA is ready for ligation into the *Sfi*I-digested λTriplEX2 vector. λ TriplEX2, which contains the asymmetrical *Sfi*I sites in the MAC, eliminates adaptor ligation and facilitates directional cloning. Furthermore, *Sfi*I sites are extremely rare in mammalian DNA; therefore, all SMART cDNA remains intact after *Sfi*I digestion methylation steps are eliminated and valuable internal restriction sites are preserved.

The following components were combined in a fresh 0.5ml tube:

- 79µl cDNA (from previous section)
- 10µl 10x *Sfi*I buffer
- 10µl Sfi enzyme
- 1µl 100x BSA

100µl Total volume

The solution was mixed thoroughly and incubated at 50°C for 2 hours. Xylene (2µl, 1%) cyanol dye was added to the tube and mixed well.

2.1.8. cDNA size fractionation

The size fractionation of the *E. ocellatus* dscDNA was performed to remove low-molecular-weight cDNA fragments, small DNA contaminants, and unincorporated nucleotides from the cDNA. Failure to remove low-molecular weight contaminants results in a library having a preponderance of very small inserts and/or apparently non-recombinant clones.

CHROMA SPIN-400 Column (supplied with the kit) was prepared before used by resuspending the matrix with its preserved solution and allowing to drain naturally. Once the preserved solution had drained from the column, 700µl of column buffer was gently added without disturbing the matrix. The 102µl mixture of the *Sfi*-digested cDNA and xylene cyano dye was added carefully and evenly to the top-centre surface of the matrix and allowed to be absorbed fully into the surface of the matrix. Another 100µl of the column buffer was used to wash the tube that had contained the cDNA and this was then added to the CHROMA SPIN-400 column. The buffer was allowed to drain out of the column until no liquid was left above the resin. Column buffer (600µl) was added and immediately collection of single drop fractions (approximately 40µl per tube) into 16 tubes was started. The profile of each fraction was checked using 3µl of each fraction loaded onto a 1.1% agarose electrophoresis gel (Fig.2.5). This was run at 150v for 5 minutes. Only four [5-8] fractions were found to contain the ds cDNA above 500bp.

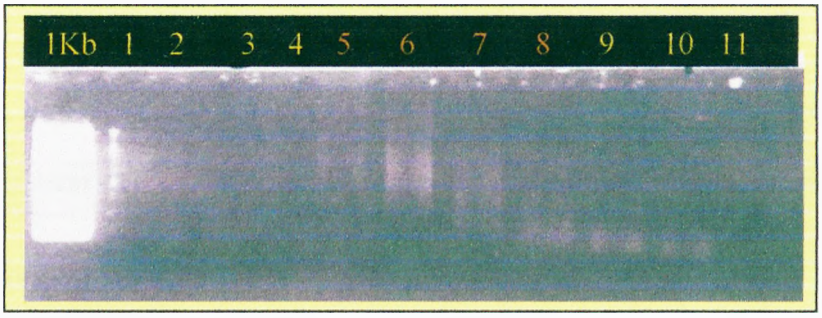


Fig.2.5. Analysis of size fractionation of *E. ocellatus* ds cDNA.

Fractions 5-8 were pooled and precipitated using the following components

- 1/10 vol. sodium acetate (3M; pH 4.8)
- 1.3 µl glycogen (20mg/lm)
- 2.5 vol. 95% ethanol (-20°C)

The sample was then incubated overnight at –20°C for better recovery and centrifuged at 13,000 rpm for 30 minutes at RT. The pellet was then air dried and resuspended gently with 7µl deionised water.

2.1.9. Ligation of cDNA with dephosphorylated λTriplEx2 vector (Stratagene, California, U.S.A)

Because the ratio of cDNA to vector in the ligation reaction is a critical factor for determining the transformation efficiency, three different concentrations of *E. ocellatus* cDNA were used (Table 2.1) and incubated at 16°C overnight. A control ligation was performed in parallel (1µl of vector, 1µl of insert [Human placenta, supplied with kit], 1.5µl of deionised water and other reagents).

Table 2.1. Ligation reaction of cDNA with dephosphorylated λTriplEx2 vector

Component	1st ligation	2nd ligation	3rd ligation
CDNA	0.5	1.0	1.5
Vector (500ng/ µl)	1.0	1.0	1.0
10x Ligation Buffer	0.5	0.5	0.5
ATP (10mM)	0.5	0.5	0.5
T4 DNA Ligase	0.5	0.5	0.5
Deionized water	2.0	1.5	1.0
Total volume (µl)	**5.0**	**5.0**	**5.0**

2.1.10. Packing reaction

A commercial kit (Gigapack III Gold Packaging Extract, Stratagene) was used to package the ligation reaction into lambda phage with high efficiency. Four packing extract vials [three for those illustrated in Table 2.1 and the fourth as a control] were thawed on ice. Once the extract had begun to thaw, 1µl of the each ligation reaction was added into tube containing the packing extract; this was then mixed gently with a sterile pipette tip, briefly centrifuged and incubated at RT for two hours. Subsequently, 500µl of SM buffer [Appendix A] was added to each reaction tube followed by 20µl chloroform. It was then mixed gently without generating bubbles and incubated at 4°C.

2.1.11. Bacterial culture plating

An early preparation of *Escherichia coli* (*E. coli*) XL1-Blue cells streaked on an agar plate containing tetracycline (15mg/ml), was performed as described in section 2.2.4. A single colony was selected and used to inoculate 30ml LB (Luria-Bertani)/$MgSO_4$/maltose with tetracycline (15 mg/ml) and incubated at 37°C overnight while shaking at 200 rpm. Cells

were then centrifuged at 5,000 rpm for 5 minutes at 4°C, the supernatant was discarded and the pellet was resuspended in 15 ml $MgSO_4$ and kept on ice.

2.1.12. Infection of E. coli with recombinant λ phage

These were 1:5 [1µl sample + 4µl SM buffer], 1:20 [1µl sample + 19µl SM buffer] and 1:100 [1µl sample + 99µl SM buffer]. One µl of each λ phage sample was added to 200µl of the XL1-Blue [section 2.1.11] *E. coli* suspension and incubated at 37°C for 15 minutes. This allows the phage to infect the XL1-Blue cells. Subsequently, 2ml of melted LB/$MgSO_4$ top-agar was added to the *E. coli* / λ phage and the mixture was immediately poured evenly onto 90-mm LB/$MgSO_4$ plates pre-warmed to 37°C. The plates were allowed to cool to RT for 20 minutes to allow the top agar to harden. They were then incubated at 37°C overnight. The number of plaques on each plate was counted (Table 2.2) and the phage titre [pfu/ml] calculated using the following formula:

pfu/ml = [No. of plaques x dilution factor x 10^3µl/ml]/ µl of diluted phage Plated

Table 2.2. Titration of λ phage titre

Tubes	1:5	1:20	1:100	Phage titre [pfu/ml]
1st Ligation	4	0	1	$2.0x10^4$
2nd Ligation	177	28	11	$9.0x10^5$
3rd Ligation	247	65	13	$1.2x10^6$
Control	337	111	24	$1.7x10^6$

2.1.13. Determination of the percentage of recombinant clones

A blue/white [IPTG/X-gal] procedure was used to determine the ratio of recombinant against non-recombinant *E. coli*. The results demonstrated that the dilution factor 1:5 of the 3rd ligation reaction (2.1.12) induced a 100% transformation efficiency. The remaining dscDNA was packaged into λ phage and transformed *E. coli*, using the optimal ligation and dilution factors identified above to produce the library.

2.1.14. Amplification of the E. ocellatus cDNA library.

In order to optimise the amplification process, a test titration of the amplified library and *E. coli* was performed to determine the concentration of λ phage required to produce confluent *E. coli* lysis on a large plate. As a result of this assessment, 100µl of λ cDNA was added to 600µl XL1-Blue cells and the mixture incubated at 37°C for 15 minutes. This was performed for 20 agar plates until all the unamplified library had been utilized. The infected cells were then mixed with 12 ml soft-top agar $MgSO_4$ and immediately poured onto a pre-warmed large LB MgSO4 agar [containing tetracycline, 15mg/ml] plate and incubated overnight at 37°C.

The next procedure was to harvest the amplified λ phage. This was performed as follows: 12 ml of 1X lambda dilution (SM) buffer was added to each of the twenty plates and incubated with gentle shaking at RT for one hour. The plates were subsequently incubated at 4°C on a rocking platform overnight. The amplified packed cDNA (12ml) was harvested next day from each plate and pooled into a sterile glass bottle. 30ml chloroform was added to the total volume (186ml) of the *E. ocellatus* cDNA library to lyse any *E. coli*.

2.1.15. Titering the amplified library

Several dilutions of the amplified λ phage library were used to infect *E. coli* and the number of lysed *E. coli* (plaques) used to estimate the pfu of the amplified library. An XL1-Blue overnight cell culture was prepared as previously described [without added antibiotic], except that the cell pellet was resuspended in pre-chilled 7.5ml (10mM) $MgSO_4$. Dilution of phage lysate was prepared as follows: the library lysate (10µl) was pipetted into 1ml SM buffer, [dilution 1 = 1:100]. Ten microlitres this dilution was transferred into a 1.5ml Eppendorf tube containing 1ml fresh SM buffer [dilution 2 = 1:10,000]. Four 1.5ml Eppendorf tubes were prepared as shown in Table 2.3a;

Table 2.3a. Titration reaction the amplified library

Tube	SM Buffer	XL1-Blue [overnight cell culture]	Phage Dilution "2"
A	100	200	5
B	100	200	10
C	100	200	20
Control	100	200	0

Tubes were incubated at 37°C for 15 minutes. 3ml of melted (45°C) LB/$MgSO_4$ soft-top agar was added to each of the four tubes, mixed quickly and poured onto four x 90 mm LB/$MgSO_4$-agar plates without antibiotic. Plates were quickly swirled for even distribution of the soft-top agar.

Plaques were subsequentlycounted for each dilution and phage titration was obtained using the same formula that was used in section 2.1.12 (Table. 3.3b).

Table.2.3b. Titration results of amplified library

Sample	Plaques count	Pfu/ml
A	4326	**$8.6x10^9$**
B	10800	**$11.0x10^{11}$**
C	Confluent	
Control	2656	

2.2.: Miscellaneous techniques

2.2.1. Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

A 15% gel (unless otherwise stated) was prepared (Appendix A). A Bio-RAD mini-protein II dual slab cell was assembled according to the manufacturer's instructions. The two glass plates (long and short) between which the gel was to be caste were cleaned with detergent, washed thoroughly with distilled water and then with methanol for in order to further clean and remove any additional grease or debris etc.

The separating (resolving) gel solution was prepared (Appendix A) and then poured between the glass plates and immediately over-laid with water saturated-iso-butanol. The gel was then allowed to polymerise for about 30-40 minutes. The stacking gel was then prepared (appendix A), the water saturated-iso-butanol drained out, the stacking gel poured on top of the separating gel and the comb placed in the gel sandwich and tithed, so that the teeth were at slight angle to prevent air from being trapped under the comb teeth. The stacking gel was then allowed to polymerise for about 30-40 minutes. Running buffer (800ml) was prepared (Appendix A) and the electrophoresis tank filled. The samples were then loaded using multi flex 0.44 flat pipette tips (Bio-RAD). Samples were loaded with 10µl of low molecular weight markers 97–14 kDa (Bio-RAD). The lid was placed on top

of the electrophoresis tank and electrophoresis performed at 150 volts for 1 hour and 45 minutes or until the dye front had just migrated from the gel. The protein bands were then visualised by Coomassie blue staining. SDS-PAGE gels were submerged for 15 minutes to 1 hour [depending on the freshness of the staining solution] in 0.5% Coomassie blue (appendix A). The gels were then de-stained by covering them with de-staining solution (appendix A) to remove the background (usually 1 to 3 hours).

2.2.2. Western blot

2.2.2.1. Transfer of protein bands to nitrocellulose membrane

A Mini Trans-blot Electrophoresis Transfer cell (Bio-RAD Labotatories, USA) was used. The electrophoresis buffer salts and SDS were removed from the SDS-PAGE gel by equilibrating it in transfer buffer (appendix A) for 20 minutes prior to blotting. A nitrocellulose membrane was cut to the dimensions of the gel and labeled with a soft pencil to identify the gel and the orientation of the membrane. The membrane was allowed to soak in transfer buffer for several minutes. Two pieces of filter (3mm Whatman) paper and fibre pads were soaked in transfer buffer taking care not to trap air bubbles in the fibre pads or thick filter papers. The Mini-Trans-Buffer electrodes in the buffer chamber were installed. The buffer tank was half-filled with approximately 750ml of transfer buffer and a one-inch stirrer magnetic-bar was placed at the bottom of the tank. A frozen Bio-Ice cooling block was installed in the buffer chamber next to the electrodes, a few minutes before starting the transfer. With gloves on, the gel holder cassette was assembled. This consists of red and black panels, which were opened by sliding the lifting latch. The opened gel holder was then placed in a shallow vessel so that the red panel was left in the

bottom of the vessel and the black panel was rested at an angle against the wall. The pre-soaked fibre pad was placed on the red panel of the cassette making sure to center all components. The soaked filter paper was placed on top of the fibre pad and the surface of the filter paper was saturated with transfer buffer. The equilibrated gel was placed on top of the filter paper and aligned in the center of the cassette making sure not to trap bubbles between the gel and the filter paper. The pre-soaked nitrocellulose membrane was then lowered on top of the gel taking care not to trap bubbles in the area between the membrane and the gel. The surface of the membrane was flooded with transfer buffer. A pre-soaked filter paper was placed on top of the membrane and finally a soaked fibre pad was placed on top of the filter paper. The cassette was closed and held firmly so that the components could not move. The holder cassette was fitted in the cathode electrode so that the red panel of the cassette faced the red panel of the cathode electrode and placed in the buffer tank. The transfer was carried out at constant voltage (100v) for one hour. Following this, the buffer and the nitrocellulose membrane were removed and to check the efficiency of protein transfer, the membrane was stained briefly with 0.5% Ponceau S (Appendix A) for 2 minutes to visualize the bands at which from the migration of the protein molecular weight marker were recorded in pencil on the membrane.

2.2.2.2. Probing with specific antibodies

The immunoblot was transferred into 5% blocking solution (appendix A) overnight with shaking at 4°C to block non-specific sites. The membrane was then washed three times (10 minutes each) with PBS-tween (Appendix A) and then incubated with the appropriate primary antibody diluted 1/1000 [unless specified otherwise] in blocking solution for 3

hours at RT (22°C). The membrane was then washed three times (10 minutes each) with PBS-Tween to remove excess antibody. Subsequently the membrane was incubated for one hour with the appropriate secondary conjugated-antibody (e.g., Horseradish peroxidase conjugated goat anti-mouse IgG (H&L), Nudic, poole, UK) usually diluted at 1:2000. The membrane was then washed six times as above and submerged in the peroxidase substrate solution (Appendix A) until the band were visualized. The membrane was then washed in distilled water to prevent over-development of the peroxidase/substrate reaction.

2.2.3. Preparation of glycerol stock culture.

Selected colonies from transformation plates were inoculated into 5ml LB medium broth containing the appropriate antibiotics (usually ampicillin 15mg/ml) in 50ml conical tubes and incubated at 37°C with shaking at 200rpm overnight (16 hours). A 0.5ml aliquot of each culture was removed and added into fresh autoclaved Eppendorf tube containing 0.5ml sterile glycerol. The stock cultures were then frozen at –80°C until required.

2.2.4. Preparation of bacterial cultures from –80°C stock cultures.

Glycerol stock cultures of recombinant *E. coli* colonies were streaked onto LB agar plates containing the ampicillin [15mg/ml) antibiotic and incubated overnight at 37°C. A well-separated colony was removed from the LB agar plate and used to inoculate LB broth containing the ampicillin antibiotic [15mg/ml] and incubated overnight at 37°C with shaking at 200rpm.

2.2.5. Northern blot

The Northern blotting technique was used in this project to analyse venom toxin RNA transcripts. While working with RNA, all working surfaces, gloves, pipettes etc., were made sterile and sprayed with 75% ethanol at every step. Gloves were worn at all times while working with RNA, as hands are a major source of RNase. Gloves must be changed frequently if they come into contact with any external source of contamination. All glassware and solutions were treated with DEPC was removed from the solution by autoclaving.

2.2.5.1. Isolation of total RNA

Venom glands were dissected from two adult *E. ocellatus* [three days after venom extraction], pooled and weighed (144mg) and immediately transferred into labelled sterile cryopreservation tubes (Nunc) and stored in liquid nitrogen. To extract RNA the glands were ground under liquid nitrogen with a mortar and pestle following standard protocols (Sambrook et al., 1989). According to the manufacturer's instructions (Aqua Pure Genomic RNA kits, Bio-RAD) a volume of 4.32ml (30µl/mg) RNA Lysis Solution was added to the autoclaved glass homogeniser containing the ground glands. The tissue was homogenized with the RNA lysis solution for a further 5 minutes.

2.2.5.2. Removal of protein and DNA and isolation of total RNA precipitate.

Protein-DNA Precipitation solution (1.44ml) was added to the cell lysate (10µl/mg). The tube was gently inverted (10 times), incubated on ice for 5 minutes and centrifuged at 13,000 rpm at 4°C in a microcentrifuge for 3 minutes. A tight white pellet was formed

consisting of protein and DNA. The supernatant was transferred into two sterile 1.5ml Eppendorf tubes containing 2.11 ml (100%) Isopropanol (2 volumes isopropanol: 1 volume RNA solution). The tubes were thoroughly mixed and centrifuged at 13,000 rpm in a microcentrifuge for 3 minutes. The small, translucent RNA pellet was washed with 3.0ml 70% ethanol and then centrifuged at 13,000 rpm in a microcentrifuge for 1 minute and the ethanol discarded carefully. Tubes were inverted and drained on clean paper and allowed to air dried for 10-15 minutes. 50µl RNA hydration buffer was then added to each tube. RNA samples were allowed to rehydrate for at least 30 minutes on ice and then used immediately or stored under 100% ethanol at –80°C.

2.2.5.3. Agarose-formaldehyde Gel Electrophoresis

A large 1.1% formaldehyde-agarose gel was prepared (500ml, Appendix A) as follows. When the melted agarose had cooled to 60°C, 10ml of 10x MOPS (Appendix A) running buffer and 18ml (12.3M) formaldehyde (2.2 M final concentration) were added. The gel was poured gently, making sure no bubbles are formed, and allowed to set. The gel was placed in the electrophoresis tank and 1x MOPS running buffer (Appendix A) was added to cover the gel. An equal volume of RNA-SLB (Appendix A) was added to the RNA sample (84µl) and 16µl of mixture sample was loaded into each well (10 wells in all, excluding the RNA ladder well). The gel was run at constant voltage (34v) until the orange dye reached the bottom of the gel (18 hours). The gel was soaked twice in 1.0l 0.5M ammonium acetate solution for 20 minutes to remove the formaldehyde followed by 0.5M amonium acetate containing ethidium bromide (10mg/ml) for 45 minutes. The gel was then rinsed in 1.0l (0.5M) ammonium acetate [without ethidium bromide] for one

hour to remove excess ethidium bromide and an image of the fractionated RNA taken under UV light with a ruler laid alongside the gel.

2.2.5.4. Transfer of fractionated RNA to nylon membrane

The gel was equilibrated in 10 gel volumes of 20X SSC (Appendix A) for 45 minutes. The northern "transfer stack" was assembled with care as shown (Fig.2.6) to remove air bubbles and the 20xSSC allowed to transfer the RNA to the nylon membrane by capillary action for 24 hours.

Subsequently the stack was disassembled, the membrane recovered and the positions and orientation of the wells on the membrane were marked by pencil. The membrane was rinsed with 2x SSC (Appendix A), allowed to air dry and the fractionated RNA cross linked to the membrane completed by exposure to UV radiation for two minutes each side of the membrane in a dedicated instruments. The membrane was then cut into strips and carefully sandwiched between two clean sheets of 3mm Whatman paper and stored at 4°C until used.

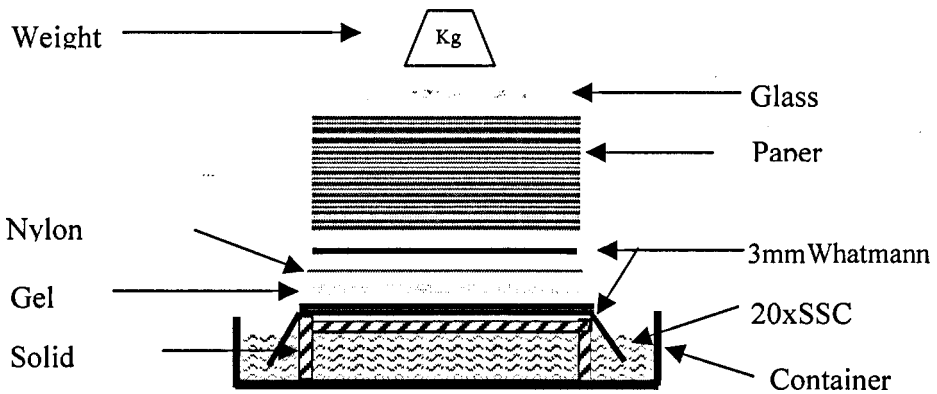


Fig.2.6. Northern blot

2.2.5.5. Pre-hybridization of Northern blot membrane

Membrane was incubated at 42°C overnight in a hybridisation oven in 10ml formamide pre-hybridisation (FPH) solution (Appendix A) containing 100µl (1000µg) of denatured salmon sperm ssDNA to block non-specific sites.

2.2.5.6. Probe labelling

To detect which of the many RNA molecules on the agarose gel hybridize to a particular probe, the probe DNA molecule usually labelled, strand–specifically, with radioactive isotope such as ^{32}P. Strand-specific DNA probes usually carried out by using single-stranded DNA obtained after cloning in plasmid vector that has had part of one strand removed by denaturising or by a 3'-5' exonuclease. DNA polymerases can then be used to re-synthesise the missing strand by incorporating radioactive NTPs (in this study project a γ-^{32}P dATP was used) nucleotides. So that the antisense (non-labelled) strand will hybridise to cellular RNA while the sense probe will act as a control.

E. ocellatus venom toxin DNA (25ng) was used as a probe using a dATP ^{32}P isotope (Promega, Bedford, UK). A master mix of dNTP's except dATP was made [10µl of each of dCTP, dGTP and dTTP]. Components of the mixture reaction were set up as described in (Appendix A). The sample was then mixed gently and incubated for one hour at RT. The reaction was then terminated by heating at 100°C for 5 minutes and immediately kept on ice followed by the addition of 2µl of (0.5M) EDTA to the reaction tube.

2.2.5.7. Removal of unincorporated label

Unincorporated radioactive label was removed by size exclusion chromatography (pre-prepared Sephadex G50 column, Pharmacia). The column storage buffer was drained and flushed three times with TE buffer (400µl) and the dATP-^{32}P label mixture added and immediately followed by 700µl of TE buffer. Fractions (400µl) were collected into 1.5ml Eppendorf tubes and those which showed the intermediate radiation levels [measured by Geiger-Muller counter] were pooled in a 1.5ml Eppendorf tube. The labelled DNA probe was then heated at 100°C for 5 minutes to separate the DNA strands. It was then incubated immediately on ice for several minutes before adding it to the Northern blot membrane [that previously incubated with the FPH solution] and incubated at 42°C overnight.

2.2.5.8. Stringency washes

Hybridisation solution was removed and the membrane washed as shown in Table.2.4.

Table 2.4. Stringency wash strategy

Stringency buffer	Temperature (°C)	Number of repeat	Period (minute)
2.0xSSC/0.1%SDS	RT	2	5
0.2xSSC/0.1%SDS	RT	2	5
0.2xSSC/0.1%SDS	42	2	15
0.2xSSC/0.1%SDS	68	2	15
0.1xSSC/0.1%SDS	68	2	15

During each stringency wash, the membrane was checked for residual radiation activity using Geiger-Muller counter. After the final stringency wash, the membrane was removed

and allowed to air dry. it was then placed on 3mm Whatman paper in parallel with a fluorescent ruler and exposed to X-ray film at –20°C for two weeks.

2.2.6. Isolation of cDNA from λ phage

As a target of PCR amplification, the cDNA was isolated from the λ phage.

λ phage lysate (20µl) was boiled for 5 minutes, centrifuged immediately and then incubated on ice. RNase-free water (80µl) was added with 100µl phenol: chloroform: isoamyl alcohol [1:24:1], mixed vigorously and centrifuged for 5 minutes at 13000 rpm. The supernatant was transferred into a fresh 1.5ml Eppendorf and 100µl chloroform added; this was mixed and centrifuged for 5 minutes at 4°C. The supernatant was transferred into a fresh 1.5ml Eppendorf containing 1/10 vol. of 3M sodium acetate and 2.5 vol. 100% ethanol; this was incubated for one hour at –80°C to precipitate the cDNA. The tube was then centrifuged at 13000 rpm for 30 minutes at 4°C and the pellet washed in 70% ethanol, air dried for 10-15 minutes and then resuspended in RNase free water and stored at –20°C until used.

2.2.7. Polymerase Chain Reaction (PCR)

PCR is a method which essentially isolates a specific segment of DNA by amplification. PCR uses specific primers which allow *Taq* DNA polymerase to make copies of only a specific segment. *Taq* DNA polymerase is used because it is able to withstand the high heat (95°C) necessary for the many cycles of PCR. A unique aspect of *Taq* DNA polymerase is that it adds a single deoxyadenosine (A) to the 3' ends of PCR

products. The resulting products of PCR are many copies of a specific DNA sequence with 3' A overhangs.

A PCR strategy [Israel, 1993] was used to screen the cDNA library for DNA encoding the *E. ocellatus* toxin molecules. PCR was performed using a thermal cycler (Gene Cycler, BioRad Hercules CA, U.S.A.) programmed as shown in Table 2.5. The 50µl PCR reaction mixture on ice contained approximately 10ng of *E. ocellatus* cDNA [extracted from λ-phage by boiling and phenol chloroform treatment], 50mM KCl, 1.5mM $MgCl_2$, 50 µM each dNTP, 2.5µl (10µM) each primer, 5 units of Taq polymerase. RNase free water was then added to make a total volume of 50 µl. Before starting the PCR cycles, samples were over-laid with two drops of mineral oil to prevent evaporation during the PCR reaction.

The PCR- amplified product was detected using 0.7% agarose unless specified otherwise. 1µl 6x SLOB (Appendix A) and 5µl of the PCR product were pipetted into 0.7% agarose gel, containing 0.5µg/ml ethidium bromide, along with 1Kb DNA ladder (Bio-RAD). This was electrophoresed for 1 hour at 70 volts. DNA bands were examined under ultraviolet trans-illumination. The size of the amplicons was determined by comparison with the molecular-weight markers.

Table 2.5. Programmes for PCR-cycles used throughout this study work

Programme 1	**Programme 3**
95°C 6 minutes [An initial denaturation] 55°C 1 minutes [Annealing] 74°C 1 minute [extension] 94°C 1 minute [denaturation] | 35x Cycles 55°C 1 minute [annealing] 72°C 7 minutes [A final extension step]	95°C 6 minutes 63°C 2 minutes 74°C 3 minute 95°C 1 minute | 35x Cycles 63°C 2 minutes 74°C 7 minutes
Programme 2	**Programme 4**
95°C 6 minutes 54°C 2 minutes 74°C 2 minutes 94°C 1 minute 35x Cycles 55°C 1 minute 72°C 7 minutes	95°C 6 minutes 70°C 2 minutes 74°C 3 minutes 95°C 1 minute 35x Cycles 63°C 2 minutes 74°C 7 minutes

2.2.8. Isolation of DNA from agarose gel

The selected PCR band or restriction enzyme digestion product was excised from the agarose gel, cut into small pieces and transferred into a fresh sterile 5ml tube and purified using commercial kit (A Prep A–Gene DNA Purification System, Bio-RAD, USA).

The x volume [depending on the concentration of DNA band] of the binding buffer was added to the gel and incubated at 40°C until the slice gel dissolved completely. A predetermined amount of the Prep-A-Gene Matrix silica [the amount is based on the concentration of the DNA shown on the agarose gel; generally 0.2µg DNA per 1µl of completely resuspended matrix] was added, mixed and incubated for 30 minutes (RT) using end-over-end rocking to bind the DNA to the silica matrix. The matrix was pelleted

by centrifugation for 30 seconds, the supernatant discarded and pellet was resuspended with DNA binding buffer equivalent to 25 times the amount of added matrix. The later step was repeated twice but with ethanol containing wash buffer. After the second wash the pellet was centrifuged to remove residual wash buffer and bound DNA. The DNA was then eluted by adding at least 1 pellet volume of elution buffer and incubating at 40°C for 5 minutes. The tube was then centrifuged for 1 minute at 13,000 rpm and the supernatant containing the eluted DNA was transferred into a sterile 0.5ml Eppendorf tube.

2.2.9. Ligation of PCR product into TOPO-vector pCR2.1

TOPO cloning ligates the PCR product into the pCR 2.1-TOPO plasmid vector (vector) (Fig.2.7). This can be done quickly and efficiently because of the unique aspects of the vector. The vector has been engineered to be a linearized plasmid with 3' deoxythymidine (T) overhangs that is activated by being covalently bonded to topoisomerase I. The 3' A overhangs of the PCR product (section 2.2.7) complement the 3' T overhangs of the vector and allow for fast ligation with the topoisomerase I. The plasmid can then be transformed into competent bacterial cells as shown in Fig.2.7.

TOPO TA cloning provides a highly efficient, 5-minute, one-step cloning strategy (TOPO cloning) for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. The plasmid vector (pCR 2,1-TOPO) is supplied linearized with single 3'-thymidine (T) overhangs for TA cloning. *Taq* polymerase has a non template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

Ligation is the procedure where a desired DNA fragment is inserted into an appropriate vector. The chosen vector has been manufactured commercially and must carry markers that enable cells containing the vector to be identified and recombinant molecules to be distinguished from non-recombinant ones as described in section 2.2.11.

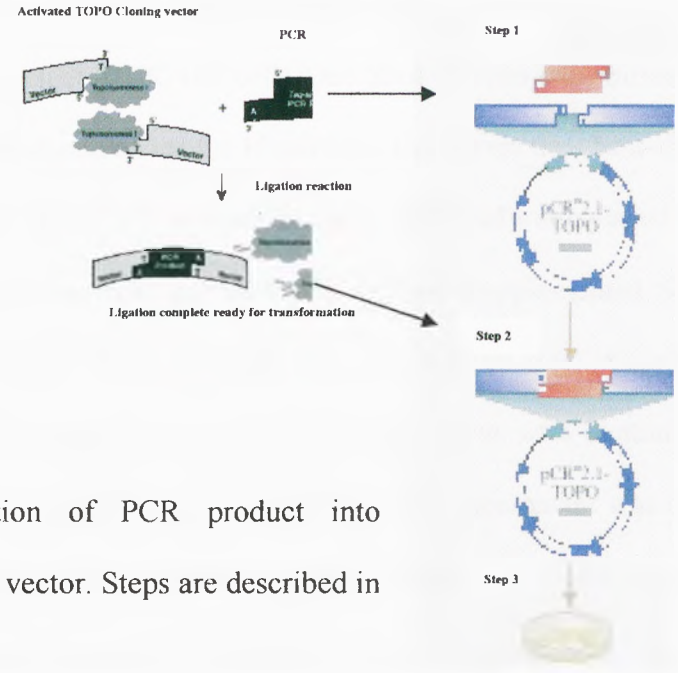


Fig.2.7. Ligation of PCR product into pCR2.1 TOPO vector. Steps are described in the text.

The desired PCR product was ligated into TOPO-vector pCR2.1 (Invitrogen) as shown in Table 2.6.

Table 2.6. Reaction of TOPO cloning.

Reagent	Quantity
Fresh PCR product	2.0µl
Salt Solution	1.0µl
Sterile Water	2.0µl
TOPO vector	1.0µl
Final volume	6.0 µl

2.2.10. Transformation of ligation reaction into chemically competent E. coli cells

Transformation is the process where the vector plus venom toxin DNA is inserted into a suitable bacterial cell. In this study, a chemically competent *E. coli* cell was used. For each transformation reaction, 6µl of the TOPO ligation reaction [section 2.2.9] was added to a vial of chemically competent TOPO10F'cells One Shot (Invitrogen), mixed very gently without pipetting and incubated on ice for 15 minutes. Cells were then heat-shocked for 30 seconds in a 42°C water-bath without shaking and immediately transferred onto ice and incubated for 2 minutes. Bacterial growth factor (250µl) (supplemented SOC medium, Appendix A) was added, incubated for two minutes on ice, transferred to an environmental shaker and then incubated at 37°C for 1 hour with continuous shaking. Cells were centrifuged at 13,000 rpm for 1 minute, 150µl supernatant was discarded and cells resuspended in the remaining 100µl medium. Resuspended cells were then plated onto pre-warmed ampicillin-plate(s) containing: 15g/l LB broth–agar, 50µl (50µg/ml-Calbiochem) X-gal and 50µl (100mM–Sigma Aldrich, UK) IPTG using a sterile glass spreader and incubated overnight at 37°C.

2.2.11. Identification of positive clones

If all the competent cells present in a transformation reaction were allowed to grow on an agar plate, then many thousands or millions of colonies would result. Therefore a method for the selection of clones containing a plasmid is required. This is almost always provided by the presence of (i) an antibiotic resistance gene(s) on the plasmid vector, for example the β-lactamase gene (*amp*^{r}) conferring resistance to ampicillin. Only those cells which are expressing β-lactamase due to the presence of a transformed plasmid will survive and

grow, and (ii) histochemical markers that result in colonies taking a distinctive colour (blue-white screening). Basically blue-white screening is a method involves the insertonal inactivation of a gene (*lacZ*), which encodes the enzyme β-galactosidae. Expression of a *lacZ* gene on the vector can be induced by using isopropyl-β-D-thiogalactopyranoside (IPTG) and the expressed enzyme can utilize the synthetic substrate 5-bromo-4chloro-3indolyl-β-D-galactopyranoside (X-gal) to yield a blue product. Therefore insertional inactivation of *lacZ* in the production of a recombinant plasmid would prevent the development of the blue color. The vector pCR 2.1-TOPO used in this study project consist both such factors as shown in (Fig.2.8).

As a result, only bacteria containing vector with PCR product will grow as white colonies. Therefore, only white colonies were selected, removed from the plate and inoculated into 4ml LB broth containing ampicillin and shaken at 37°C. The bacterial suspension was transferred into 1.5ml Eppendorf tube, centrifuged at 13,000 rpm for 1 minute and the pellet resuspended in 250µl of Qiagen cell re-suspension buffer (P1). According to manufacturer's instruction (Mini plasmid spin kit, Qiagen, Kit) 250µl of an alkali cell lysis buffer (P2) was added, the tube contents were mixed gently by inverting the tube 4 to 6 times and 350µl of neutralisation buffer (P3) was added. The tube was then centrifuged at 13,000 rpm for 15 minutes and the supernatant pipetted into a spin column attached to a 2 ml collection tube. The DNA solution was then passed through the column by centrifugation at 13,000 rpm for 1 minute. The spin column was subjected to three spin washes with 0.75 ml washing buffer (PE) and centrifuged at 13,000 rpm for 30 seconds.

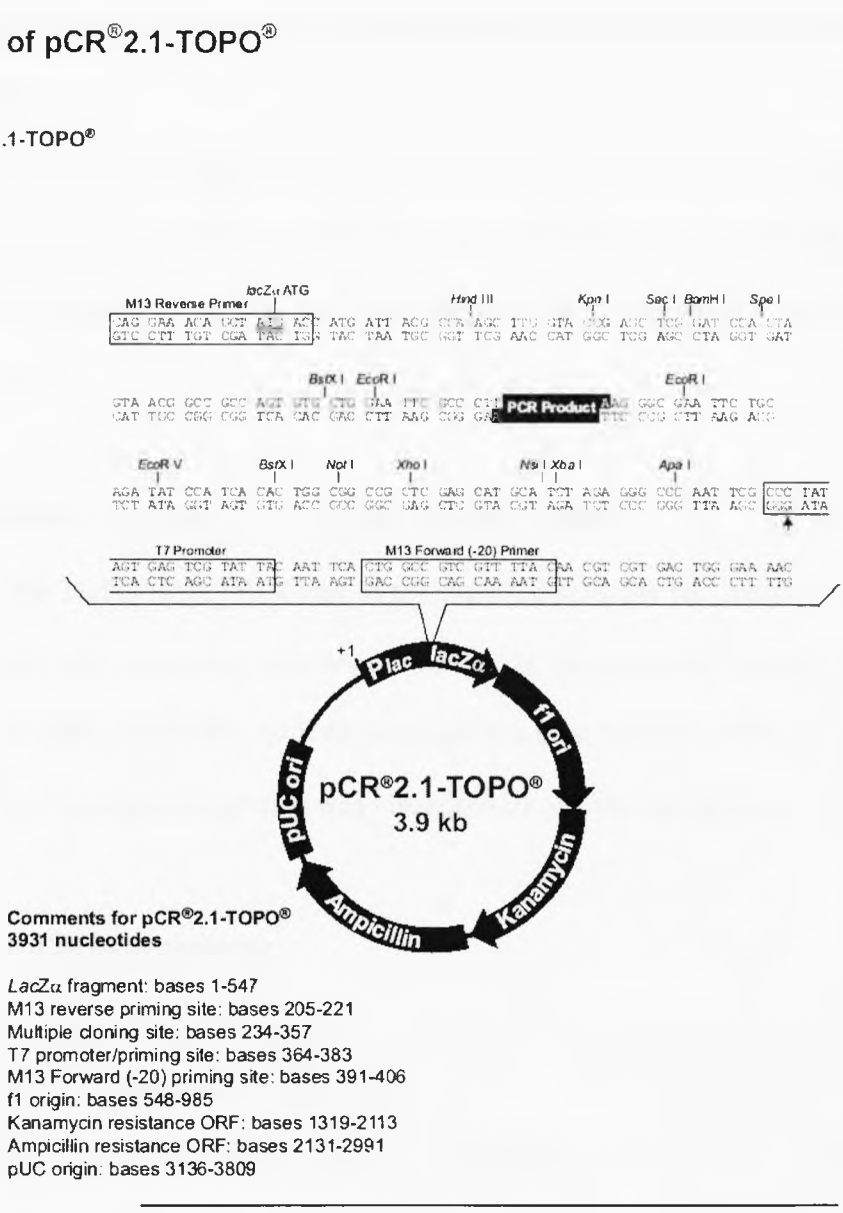


Fig.2.8. Schematic diagram of pCR2.1 TOPO vector map (Invitrogen).

The spin column was then placed in a 1.5 ml Eppendorf tube, and 50µl of sterile distilled water was added and allowed to stand for 1 minute to elute the DNA plasmid. The eluted DNA solution was then collected by centrifugation at 13,000 rpm for 1 minute. The DNA

yield was determined by 0.7% agarose gel electrophoresis and the DNA was stored at –20°C until required.

An up-scaled edition of the mini-plasmid DNA extraction kit (Maxi-prep, Qiagen) was used to isolate the larger quantities of DNA required for the future sub-cloning. The technique was similar with exception that the chromatography used was a stationary, not centrifuged, column.

2.2.12. Identification of clones by restriction enzyme digestion

To confirm that the TOPO/toxin DNA was of the expected size, the recombinant plasmid (1μg) was digested with restriction enzymes utilized in the primer design (usually BamHI and XhoI) with a buffer appropriate to both enzymes in a total volume of 20μl, overnight at 37°C. The size of the digested product was visualised by agarose gel electrophoresis.

2.2.13. Automated DNA sequencing

DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method [Sanger et al., 1977] in a Beckman Coulter CEQ™ 2000 XL DNA Analysis System. Each sequencing reaction was carried out according to manufacturer's instructions with at least 200ng of plasmid DNA and 10pM primers [M13 forward and reverse primers].

The principle of the automated DNA sequencing is based on the chemical method of DNA sequencing which has largely been superseded by the method of Sanger et al., (1977) which uses four specific dideoxynucleotides (ddNTPs) to terminate enzymatically synthesized copies of a template. Basically a sequencing primer is annealed to a ssDNA template molecule and a DNA polymerase extends the primer using ddNTPs. The

extension reaction is split into four and each quarter is terminated separately with one of the specific ddNTPs, and the four samples (labeled with different fluorescent dies) are analysed by PAGE. In the automated DNA sequencing the reaction products are mixed and run in the same lane in the gel. A fluorescent detector records the molecules as they pass a particular point in the gel and determines whether they are from A, G, C, or T reactions according to the fluorescence labeled nucleotides. The information is then fed directly into a computer.

2.2.14. BLAST search

Prior BLAST (Basic Local Alignment Search Tool) searches the amino acid sequences of each clone was predicted using the Clustal structure programme-MegAlign software (DNASTAR, USA). The predicted amino acid sequence was then compared to sequences in the GenBank, PDB, SwissProt, PIR and PRF databases using the BLAST program [Altschul et al., 1997] to determine clone identity. Basically, the BLAST search algorithm reports all significant regions of sequence identity between two sequences, using the highest scoring pair (HSP) of identical length segment. The approach to similarity searching taken by the BLAST program is first to look for similar segments (HSPs) between the query sequence and data base sequence, then to evaluate the statistical significance of any matches found and finally to report only those matches that satisfy a user-selectable threshold of significance. Finally, novel cDNA isolated in this project were assigned accession number using the NCBI (National Centre for Biotechnology Information) web site.

Chapter 3

Molecular cloning and characterization of novel cDNAs encoding Echis ocellatus phospholipase A_2

3.1. Introduction

Snake venom phospholipases are amongst the most toxic venom constituents with the sub-group A_2 (svPLA$_2$) having the potential to disrupt haemostasis at several distinct levels. In contrast to svPLA$_2$s, mammalian PLA$_2$ are non-toxic and act as mediators in various physiological processes without inducing potent pharmacological effects. The evolution of a toxic function for snake venom PLA$_2$ underscores the biological importance of this group of enzymes to snakes.

Besides their possible role in the hydrolysis of membrane phospholipids and/or plasma lipoproteins, viper PLA$_2$ induce a broad spectrum of toxic activities, including haemolysis (Kihara et al., 1992), neurotoxicity (Bon, 1997; Gubensek et al., 1997; Fletcher and Rosenber, 1997; Petan et al., 2002), cardiotoxicity, myotoxicity (Fletcher et al., 1981; Fuly et al., 1997) anticoagulant activity (Rosenberg et al., 1983; Evens and Kini, 1997), edema-inducing activity (Vishwanath et al., 1987; Wang and Teng; 1990; Liu et al., 1991; Tan et al., 1991) and initiation or inhibition of platelet aggregation (Kini and Evans, 1990; 1997). Several of these effects can be generated by venom PLA$_2$ of a single species such as in venom of *T. flavoviridis* (Kordis et al., 1998; Ohno et al., 2003). However, only some of these toxic activities are related to a direct hydrolytic activity on phospholipids (Machado et al, 1993).

Although svPLA$_2$s differ in their toxic effects (Rosenberg et al., 1983; Tsai et al., 2001), they exhibit a remarkable degree of sequence homology (≥50%). Since the isolation of the first svPLA$_2$ cDNA sequence (Guignery-Frelat et al., 1987), significant progress has been made to isolate and characterise numerous cDNA sequences encoding the sub-group I and

II PLA_2s [as described Chapter-1]. Valentin and Lambeau (2000), reported that a single snake species may contain up to 15 distinct PLA_2s in its venom with sequence identities ranging from 40% to 99%, each of which may exert a different effect. Kemparaju et al. (1994) isolated a basic (Ip 7.2-7.6) PLA_2 protein (EC-IV-PLA_2) of 14 kDa from the Indian saw-scaled viper (*Echis carinatus*) venom with neurotoxic and oedema-producing effects in mice but no direct haemolytic, myotoxic, cytotoxic or anticoagulant activities. In 1999 Kemparaju's group isolated a 16 kDa acidic (Ip 4.2-4.8) isoform of PLA_2 (EC-I- PLA_2) from the venom of the same species which was also non-lethal to mice and devoid of neurotoxicity, myotoxicity, anticoagulant activity and cytotoxicity but caused oedema. The existence of multiple molecular forms of PLA_2 enzymes within the same venom may be to increase the efficiency of its action on prey (Schiavo et al., 2000). Several studies have been performed to investigate and explain how a multiple isoforms of PLA_2 within the same venom can exert such diverse effects. Petan et al., (2002) in a study based on chemical modification, showed that replacement of Phe^{24} residue with Trp^{24} in the N-terminal region of ATXA PLA_2 isolated from venom of the western sand viper (*Vipera ammodytes ammodytes*), induced a considerable decline (40-80%) in enzymatic activity. The outcome of this study suggested that the PLA_2 isomer-specific effects are apparently linked to the existence of a diversity of very high affinity receptors for svPLA_2 (Lambeau et al., 1996).

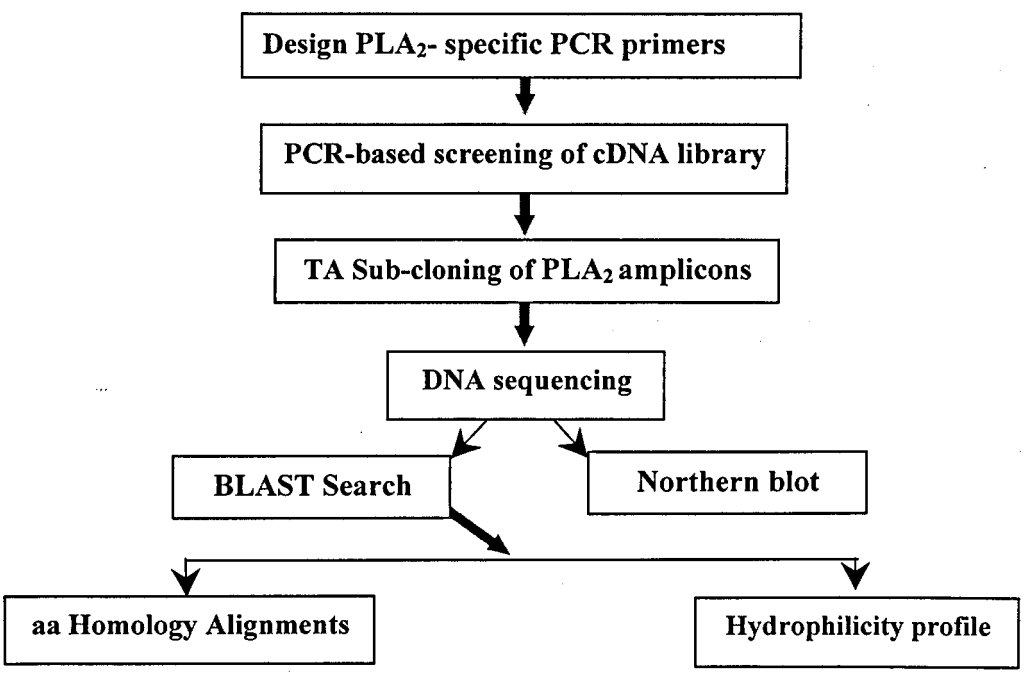
While several toxic PLA_2 in venoms of related *Viperinae* species have been identified and well charecterized [including *Echis*, as demonstrated above] (Kemparaju et al., 1994; 1999; Polgár et al., 1996; Lizano et al., 1997), either is known about the contribution of PLA_2 activity to the pathology of *E. ocellatus* envenoming.

The purpose of this chapter therefore was to isolate and characterise cDNAs encoding *E. ocellatus* PLA_2s. To achieve this, I have utilised the sequence similarity of viper group II PLA_2 enzymes to design PCR primers and amplify analogous cDNAs from a *E. ocellatus* venom gland cDNA library. The sequence characteristics of the *E. ocellatus* PLA_2 cDNAs isolated here were compared with analogous molecules in related vipers, particularly with those of *Echis* species of distinct residential boundaries in East Africa (*E. p. leakeyi*) and in the Indian subcontinent (*E. sochureki*). Many, but not all, of the results reported here have been published by Bahrati et al., (2003)

3.2. Strategy

The experimental strategy to achieve these objectives is show in Fig.3.1.

Fig.3.1. Showing the strategy plan



3.3. Designing primers

Sequences complimentary to highly conserved regions encoding the amino (**M-R-T-L-W-I**) and carboxyl (**E-S-E-K-C**) ends of the open reading frame of published group II svPLA$_2$ DNA sequences of related viper species were used to design the PLA$_2$ primers (Table 3.1). Where the nucleotide sequences were species-specific, the sequence of the most frequent codon was used. The following primers were used:

Sense primer PLA$_2$-5' GGA-TCC-ATG-AGG-ACT-CTC-TGG-ATA-3'**[24 bp].**

An antisense primer PLA$_2$- 3'-CTC-GAG-TCA-GCA-TTT-CTC-TGA-CTC-C TC-5'**[27bp]**.

Two stop codons (TCA) and restriction endonuclease sites for *Bam*H1 and *Xho*1 were included in the 5'- and 3'- primers respectively to facilitate future subcloning into mammalian expression plasmids. The two primers were custom-synthesized by Sigma-Genosys Ltd., U.K.

Table 3.1. Strategy for designing *E. ocellatus* PLA_2 primers

Sense primer (5')	Sense primer (5')	Antisense primer (3')	Accession No.
Echis coloratus ***(Ec)***		aa→ E - S - E - K - C nt→ GAG-TCA-GAG-AAA-TGT	**AF253050**
Viper palaestinae ***(Vp)***	aa→ M - R - T - L - W - I nt→ ATG-AGG-ACT-CTC-TGG-ATA	aa→ E - S - E - K - C nt→ GAG-TCA-GAG-AAA-TGC	**AF091855**
Viper ammodytes ***(Va)***		aa→ E - S - E - Q - C nt→ GAG-TCA-GAG-CAA-TGC	**AF253048**
Trimeresurus flavoviridis ***(Tf)***	aa→ M - R - T - L - W - I nt→ ATG-AGG-ACT-CTC-TGG-ATA	aa→ D - S - E - P - C nt→ GAT-TCA-GAA-CCA-TGC	**D10725**
Calloselasma rhodostoma ***(Cr)***	aa→ M - R - T - L - W - I nt→ ATG-AGG-ACT-CTC-TGG-ATA	aa→ E - S - D - P - C nt→ GAG-TCA-GAC-CCA-TGC	**AF104070**
Crotalus atrox ***(Ca)***	aa→ M - R - T - L - W - I nt→ ATG-AGG-ACT-CTC-TGG-ATA	aa→ E - P - E - P - C nt→ GAA-CCA-GAG-CCA-TGC	**AF269131**
Bothrops asper ***(Ba)***	aa→ M - R - T - L - W - I nt→ ATG-AGG-ACT-CTC-TGG-ATA		**AF109911**
Bothrops jararacussu ***(Bj)***	aa→ M - R - T - L - W - I nt→ ATG-AGG-ACT-CTC-TGG-ATA		**AY185201**
PLA_2-specific PCR primers	ATG-AGG-ACT-CTC-TGG-ATA / GAG-TCA-GAG-AAA-TGC		
Underline nucleotides (antisense)	indicate that they are not conserved among all viper species		

3.4. Results

3.4.1. PCR with primers complimentary to consensus sequences in viper PLA_2s

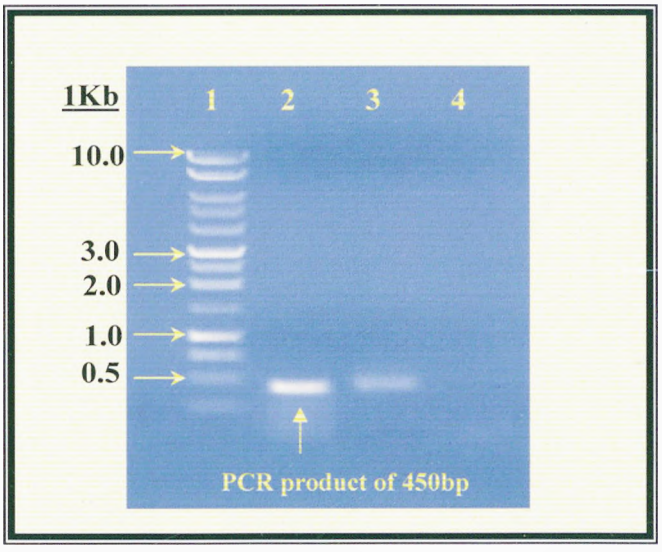
A band of about 450bp was obtained after the *E. ocellatus* cDNA was subjected to PCR with the PLA_2-specific primers (Fig.3.2). The ligation and transformation of the PCR product, using TOPO TA Cloning Kit with TOPO 10F' One Shot Competent Cell, generally resulted in a good yield of recombinant colonies. On average, the overall ratio between the blue and the white colonies was 2:1. Over 25 white transformant colonies were screened by (*Bam* HI and *Xho*I) restriction enzyme digestion prior to DNA sequencing.

3.4.2 Analysis of $EoPLA_2$ sequences

Twenty colonies [*$EoPLA_2$*-01 to *$EoPLA_2$*-20] contained restriction fragments of the correct size (450bp) and were therefore submitted for DNA sequencing. I submitted such a large number of cDNAs for sequencing in the expectation of amplifying a number of distinct isomers. All the *$EoPLA_2$* clones consisted of 415 nucleotides predicted to encode a protein of 138 amino acids with a molecular weight of 15.7 kDa (Fig.3.3), which were identified (BLAST) as belonging to the sub-group II of the phospholipase enzymes superfamily [The nucleotide and predicted amino acid sequences of all *$EoPLA_2$* clones are presented in Appendix B]. Clones were considered to be distinct if they contained nucleotide differences leading to amino acid substitutions. On this basis, six (*$EoPLA_2$*-04, *Eo*PLA_2-05, *Eo*PLA_2-09, *Eo*PLA_2-10, *Eo*PLA_2-11 and *Eo*PLA_2-12) of the twenty clones isolated represented distinct variants (Fig.3.3a and Fig.3.3b). The predicted amino acid sequences

of *EoPLA*$_2$*-04* and *Eo*PLA$_2$-05 show a single amino acid substitution of (Thr) to (Ala) at position 38, whereas the other variants revealed substitution of several amino acids restricted to the C-terminal region (Fig.3.3b).

Fig.3.2. PCR product of the two designed primers.



Analysis of PCR amplification products by 0.7% agarose gel electrophoresis. Bands were visualised using the ultraviolet trans-illumination. The amplified PCR product (425bp) from *E. ocellatus* venom glands cDNA showed in Lane 2 was compared with marker bands of known molecular weight (Lane 1). Lane 3 and 4, represents a positive control, using a SOD DNA template (10ng) as described in chapter 2, and a negative control where H_2O, instead of DNA template, was used respectively.

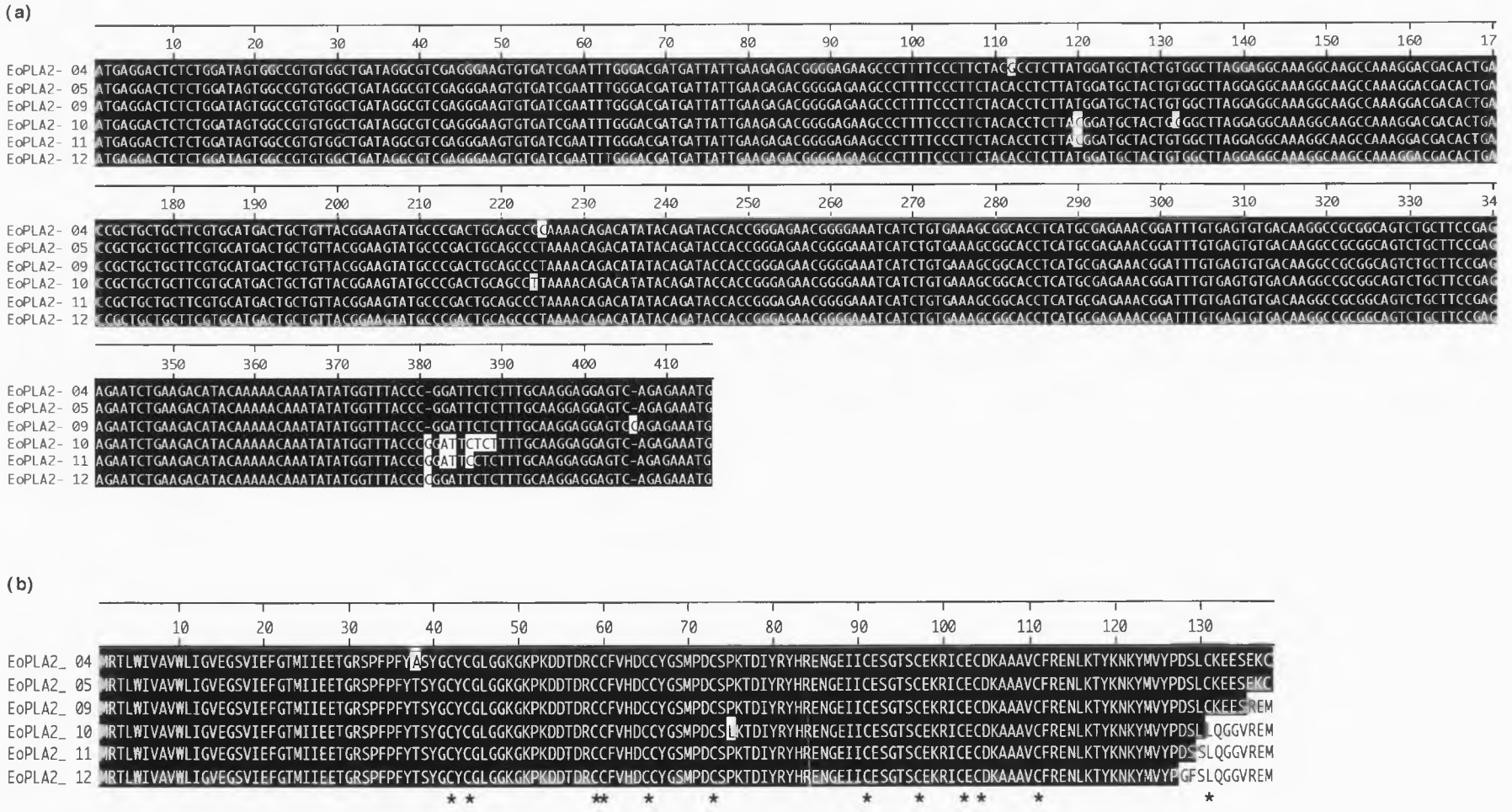


Fig.3.3. Nucllеotide **(a)** and deduced amino acid sequences **(b)** of the six $EoPLA_2$ cDNAs. Conserved sequences are shaded in black. Gaps were introduced to optimise alignment.

[identified at positions 42, 44, 59, 60, 65, 66, 73, 91, 97, 102, 131, 138 (Fig.3.2b)], *EoPLA$_2$*-10, 11 and 12 each showed a L for C^{131} and M for C^{138} substitution and *EoPLA$_2$*-09 possessed the latter Cys substitution. Because of the overall extensive sequence similarity between the EoPLA$_2$ 4-12, these cDNAs were considered to be variants of a single PLA$_2$ isomer.

3.4.3. PLA$_2$ sequence similarities between E. ocellatus and related viper

Because *EoPLA$_2$-05* showed the greatest sequence similarity (BLAST) to PLA$_2$s of related vipers, this variant was selected for comparison with the other viper PLA$_2$ in the remaining sections of this chapter. The predicted amino acid sequence of *EoPLA$_2$*-05 was aligned with PLA$_2$ sequences of related vipers that showed the highest degree of similarity during the BLAST search (Fig.3.4). The nearly identical EoPLA$_2$ variants showed a high percent sequence similarity with PLA$_2$ sequences from *Botrops jararacussu* (72%, X76289), *Crotalus* (61%, AF269131), *Vipera ammodytes* (57%, AF253048), *E. coloratus* (59%, AF253049), Macroviper *palaestinae* (59%, U60017), [*Echis pyramidum leakeyi* (*EplPLA$_2$-5,* 56%), *Echis sochureki* (*EsPLA$_2$-4,* 56%), Bahrati et al., 2003], *Trimeresurus flavoviridis* (53%, D10720), *Bothrops asper* (51%, AF109911), and *Calloselasma rhodostoma* (53%, AF104067). The *EoPLA$_2$-5* sequence showed (Fig.3.4) the requisite 14 cysteine residues that form the 7-disulphide bridges that define the characteristic tertiary structure of group II PLA$_2$ enzymes. The amino acid sequence numbering of Fig.3.3b and

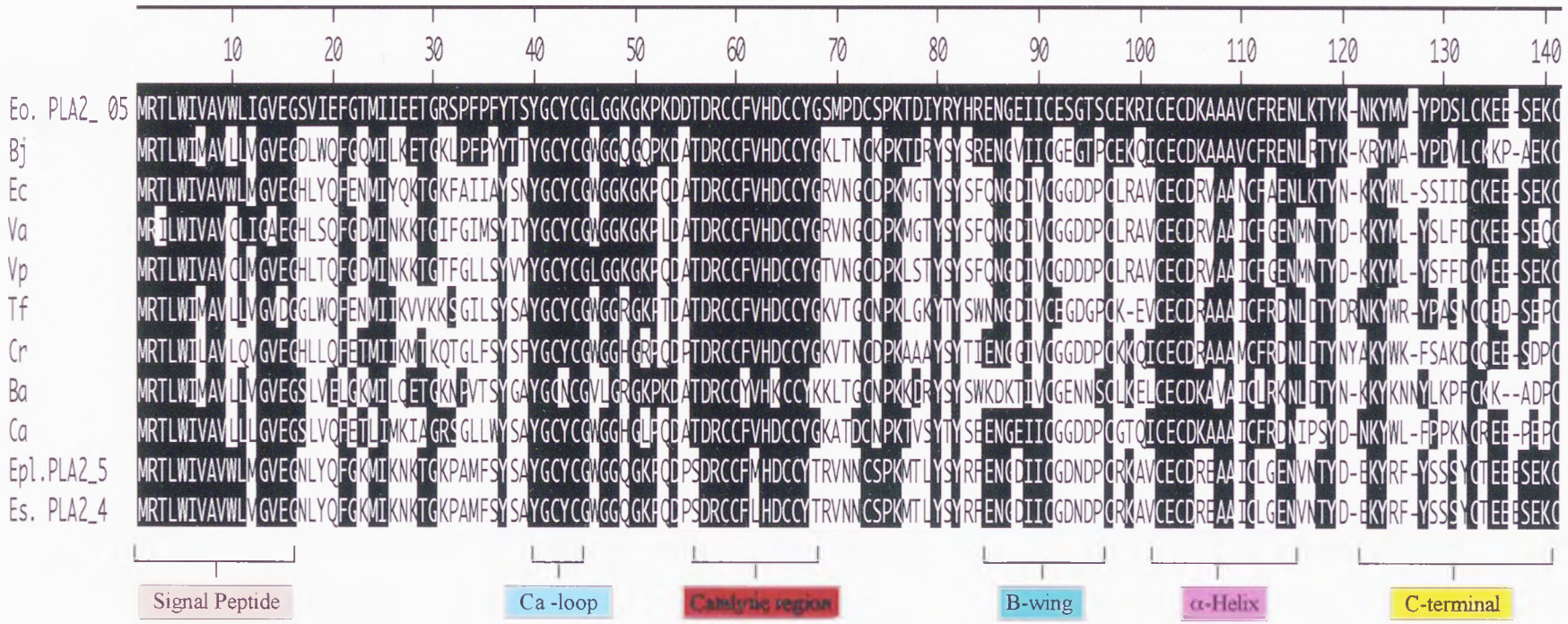


Fig.3.4. Analysis of the sequence similarity of the deduced amino acid sequences of EoPLA$_2$ isoforms with PLA$_2$s from related *Viperidae* snakes; Ec-*E. coloratus;* Va- *V. ammodytes*; Vp-*V. palaestinae*; Tf-*T. flavoviridis*; Cr- *C. rhodostoma*; Ba-*B asper;* Bj-*B. jararacussu*; Ca-*C. atrox;* EplPLA$_2$-5; EsPLA$_2$-4. Residues shaded in black were indetical to those of EoPLA$_2$-05. Gaps were introduced to optimise alignment. Segment known to be functionally important are labelled.

Fi.g.3.4 are different because of the gaps inserted into Fig.3.4 to optimize sequence alignment. The first 16 residues of the *$EoPLA_2$-5* matched the consensus signal peptides from other secreted PLA_2s (Blobel and Dobberstein, 1975; Bahrati et al., 2003). The signal peptide domain was followed by the amino terminal lipophilic residues of the substrate recognition domain. Although this PLA_2 domain has been reported by Jeyaseelan et al., (2000) to be highly conserved, containing: Leu^{18}, Phe^{21} and Ile^{25} (Fig.3.4), in *$EoPLA_2$*-05 Leu^{18} was substituted with Val^{18} with unknown functional implications.

With the exception of residues 46 and 48, a high degree of conservation within the overlapping Ca^{+2}-binding loop containing the glycine-rich sequence (Tyr^{40}-Gly^{41}-Cys^{42} - Tyr^{43}-Cys^{44}-Gly^{45}-X^{46}-Gly^{47}-Gly^{48}-X^{49}-Gly^{50}) was observed between *$EoPLA_2$-05* and analogous sequences in venoms of related vipers molecules (Fig.3.4).

Consistent with observations by Jeyaseelan et al., (2000), the catalytic-site of *$EoPLA_2$*-05 was very similar to that of related viper species (Fig.3.4). This homology included the presence of the His^{63} and Asn^{64}/Lys^{64} [often cited as His^{48} and Asn^{49} in the literature where the 16 amino acid signal peptide was excluded] which characterise PLA_2s. This Asn^{64} residue as well as the other conserved residues of His^{63}, Tyr^{67} and Tyr^{80}, were known to be important for the formation of the catalytic network (Jeyaseelan et al., 2000). His^{63} is highly conserved in PLA_2 enzymes and plays a significant role in phopholipid hydrolysis, participating in catalysis (Verheij et al., 1980; Scott et al., 1992). Moreover, alkylation of this residue leads to complete loss of enzymatic activity and reduction of the toxic and pharmacological effects of PLA_2s (Yang and King, 1980; Diaz-Oreiro and

Guteierrez, 1997). However, modification of His-48 did not disturb the metal ion-protein interaction (Soares and Giglio, 2003). Thus, His-modified PLA_2 enzymes are suitable for both *in vitro* and *in vivo* systems for studying the role of enzymatic activity.

Kini and Evans (1987) identified the location of an anticoagulant motif (residues 68-83) (Fig.3.4). Based on crystallography of a PLA_2 from *C. atrox* venom, Brunie, et al., (1985) reported that this segment was found to vary from one isomer to the next. Moreover, it has been suggested that this segment ultimately helps to fix the position and orientation of Tyr^{82} (Fig.3.4) which has a critical role linking the amino terminus with the catalytic region.

The locations of the β–wing and the α-helix regions were identified by Kini and Evans (1989) as illustrated in Fig.3.3. Although these regions are completely conserved in all *EoPLA₂* variants, they were less conserved among PLA_2 sequences of other viper species. Both motifs are known to play an important structure/function role in the PLA_2 molecule. The β–wing substructure can adopt a variety of orientations with respect to the enzymes proper and it may confer ancillary pharmacological properties such as anticoagulation (Kini and Evans, 1987). Scott, (1997) reported that the α-helix motif ensures a fixed active site geometry of the PLA_2 structure by (i) creating a rigid backbone brace for the substrate-binding pocket, (ii) assisting in the coordination of the primary Ca^{2+}, (iii) forming the deeper contours of the hydrophobic channel (Cys^{44}, Ala^{108}, Ala^{109}, Phe^{112}) and (iv) creating the catalytic network.

3.4.4. Phylogenetic Analysis

To determine the sequence evolutionary relationship of *$EoPLA_2$-05* with PLA_2 of related viper species, a phylogenetic tree was constructed using the CLUSTAL W program with PAM250 residue weight matrix (Thompson et al., 1994) (Fig.3.5). The amino acid sequence of *$EoPLA_2$-05* cDNA was very similar (68%) to the sequence of a native PLA_2 from *E. sochureki,* Ecarpholin S (Polgar et al., 1996). Such sequence similarity suggests a direct genomic relationship. In contrast, the predicted amino acid sequence of *$EoPLA_2$-05* showed only 35% identity to the peptide sequence of a native PLA_2 from *E. p.leakeyi.*

In general, this phylogenatic tree suggests that all the aligned PLA_2 sequences originated from a common ancestor, Ecarpholin S from *E. sochureki* (Polgar et al., 1996). It also shows that *$EoPLA_2$-05* diverged before the aligned venom PLA_2 species, particularly that of *Bothrops jararaca* and *Bothrops asper*, respectively.

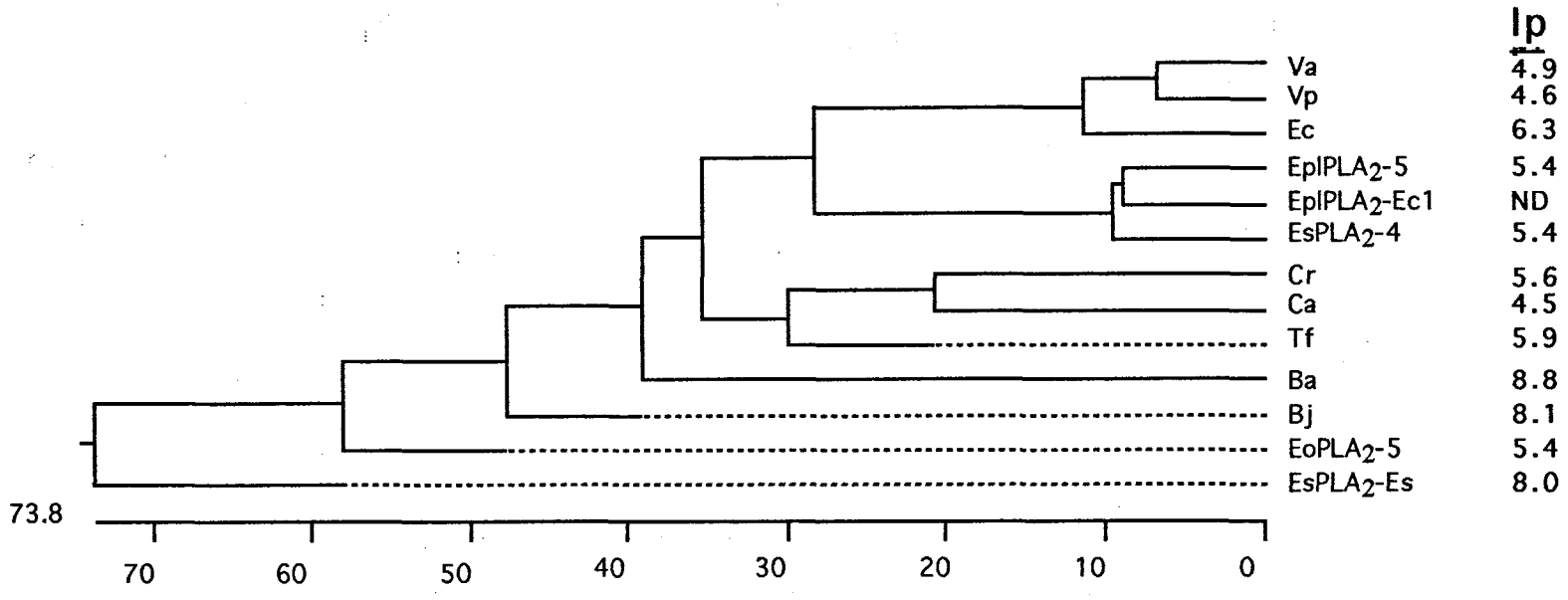


Fig.3.5. Phylogenetic tree and predicted isoelectric point [pI] of viper PLA2. The tree was constructed from the primary structure of deduced amino acid sequences of group II snake venom PLA2's shown in Fig.3.4, and sequences for native PLA2 from *E. sochureki* [Ecarpholin S (EsPLA2-Es)] and *E. p. leakeyi* [EplPLA2-Ec1]. The scale beneath the tree measures the distance divergence between sequences (in millions of years). Ec-*E. coloratus;* Va- *V. ammodytes*; Vp-M. *palaestinae*; Tf-*T. flavoviridis*; Cr- *C. rhodostoma*; Ba-*B asper;* Bj-*B. jararacussu*; Ca-*C. atrox;* EplPLA2-Ec1 (Desmond et al., 1991); EsPLA2-Es- (*E.sochureki,* Ecarpholin S); EsPLA2-4 and Epl PLA2-5 (Bahrati et al., 2003).

3.4.5. Hydrophilicity profile

To determine whether protein encoded by *EoPLA$_2$*-05 shared structural similarities with PLA$_2$s of other viper species, a hydrophilicity profile (Kyte and Doolittle, 1982) was constructed (Fig.3.6). The hydrophilicity profile generally outlines the linear surface profile of the molecule.

The hydrophilicity profile of all the PLA$_2$ molecules aligned in Fig.3.4. were remarkably similar, irrespective of their geographical distribution, Ip, taxonomic designation and toxicity (Bahrati et al., 2003) and suggests that these variants would have common functional properties.

The purpose of this chapter was to isolate *E. ocellatus* PLA$_2$ cDNAs as immunisaing material to generate specific antibody. Surface epitopes of proteins are highly likely to generate antibody and be site of antibody binding. The surface exposed epitopes of *EoPLA$_2$*-05 were identical with that of the other EoPLA$_2$ variants (data not shown) and very similar to that of other viper PLA$_2$s. Therefore, it is predicted that future generation of antibodies against *EoPLA$_2$*-05 immunogenic surface protein(s) will cross-react and neutralise PLA$_2$ of other viper species.

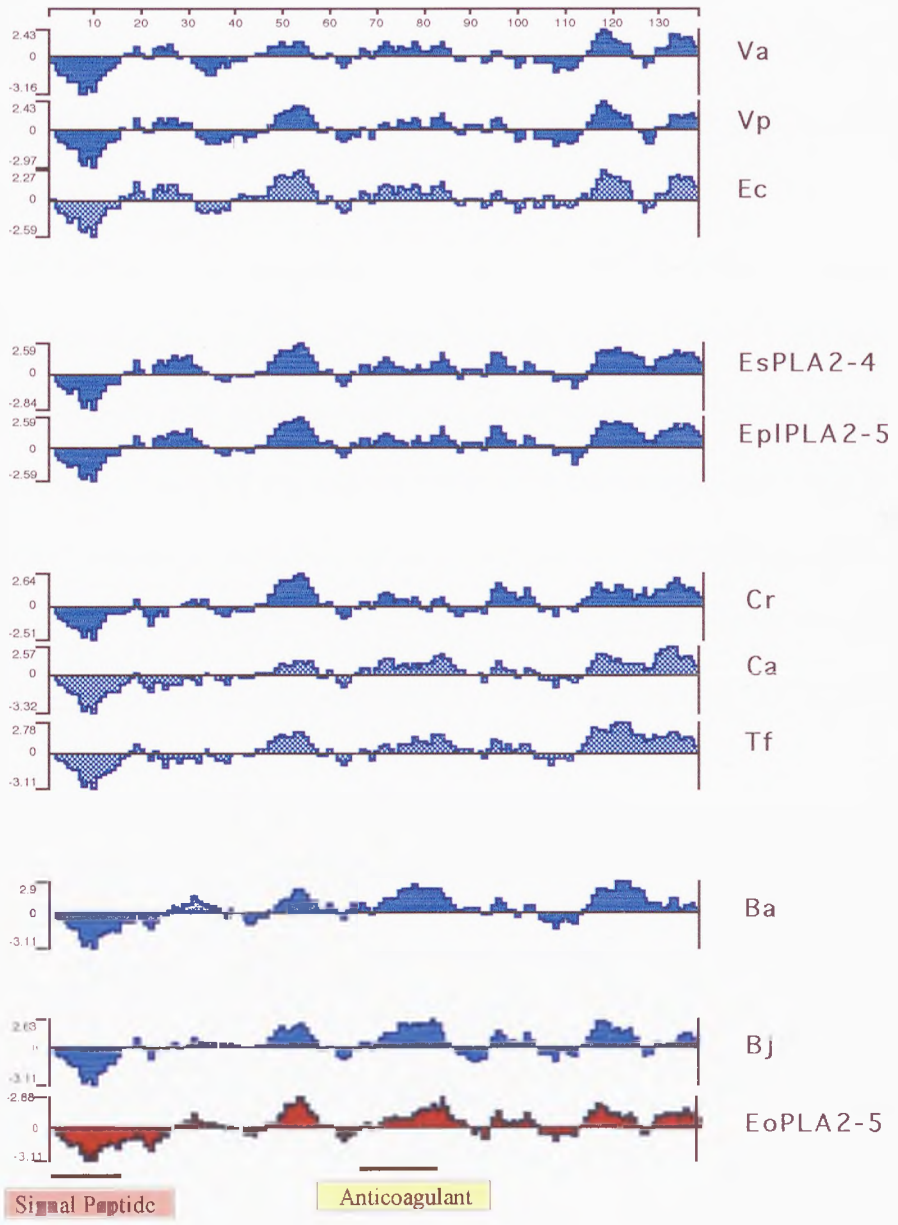


Fig.3.6. Comparison of the hydrophilicity profile of the *EoPLA2*-5 isoform with related viper species shown in Fig.3.5. Hydrophilicity analysis was carried out using the Kyte and Doolittle (1982) hydrophilicity scale using Lasergene software (Protean, DNASTAR). The top horizontal scale represents the number of amino acid residues. The vertical scale represents the hydrophilic (+) and hydrophobic (-) domains respectively. The conserved signal peptide and anticoagulant domains are indicated.

3.4.6. Analysis of $EoPLA_2$ transcript

The sequences of *$EoPLA_2$* 1-20 were derived from *E. ocellatus* venom gland cDNA amplified by PCR with primers complementary to consensus 5' and 3' regions of the open reading frame of viper venom group II PLA_2.

In order to establish the true RNA transcript size, *$EoPLA_2$*-5 TOPO construct was digested with both *Bam* H1 and *Xho* I restriction enzymes. The purified *$EoPLA_2$*-05 insert was labeled with ^{32}P-(dCTP) and used to probe an *E. ocellatus* venom gland northern blot (RNA that had been denatured and electrophoretically fractionated and blotted onto a nylon membrane; Boehringer Mannheim). The hybridized filter was exposed to X-ray film for two weeks at –20°C. The result as shown in (Fig.3.7) revealed a broad band of 0.9kb which strongly suggests that the gene encoding the 416bp open reading frame of *$EoPLA_2$*-05 contains extensive upstream and downstream flanking sequences.

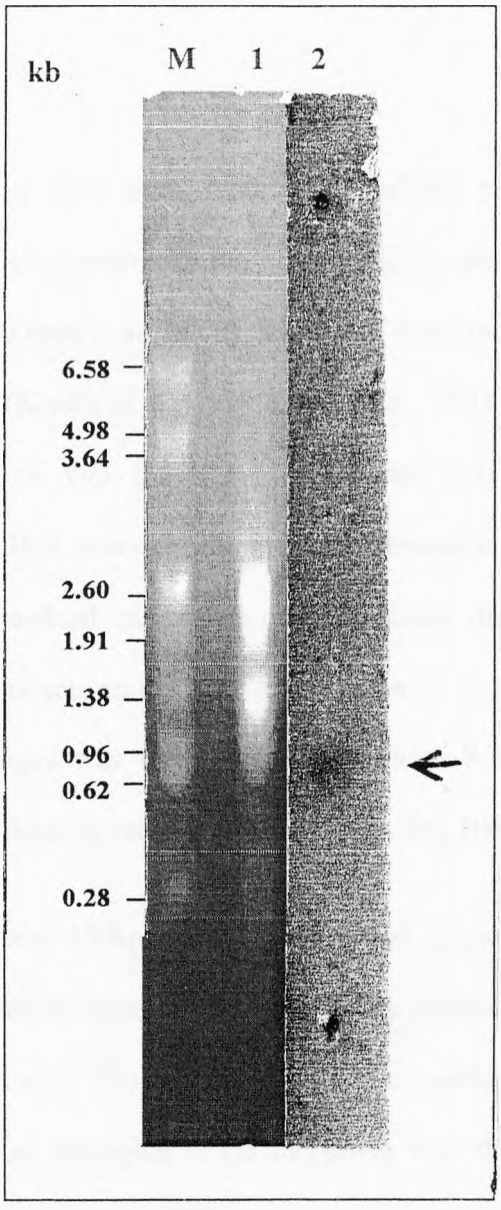


Fig.3.7. Northern blot analysis. The ^{32}P-labelled *EoPLA$_2$*-5 was used to probe 10μg of *E. ocellatus* venom gland RNA on a nylon membrane. M and 1: RNA molecular weight markers (0.28- to 6.58kb) and *E. ocellatus* venom gland RNA, respectively, were fractionated by formaldehyde-agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. Lane 2: autoradiograph of the hybridized northern blot membrane. Arrow indicates transcript *Eo*-phospholipase hybridized with the ^{32}P-cDNA labeled probe.

3.5. Discussion

Snake venom PLA_2s have been reported to exhibit such diverse toxic effects as haemolysis, myotoxicity, neurotoxicity, cardiotoxicity, anticoagulant activity and edema (Ogawa et al., 1995; Ohno et al., 2003). Several of these can be present in the venom of a single viper species (Kordis et al., 1998; Ohno et al., 2003). The overall objective of the research described in this thesis is to generate toxin-neutralizing antibodies by immunisation with DNA encoding the major haemostasis-disruptive *E. ocellatus* venom toxins. Despite the medical importance of *E. ocellatus,* there is little information in the literature and no sequences in the genetic databases on *E. ocellatus* venom PLA_2. The *E ocellatus* cDNA library was therefore screened with PCR primers complimentary to consensus 5' and 3' flanking regions of the open reading frame of viper group II PLA_2.

A PCR product of about 450bp (Fig.3.2) was ligated into a TA cloning vector (chapter 2) and this construct used to transform *E. coli.* Twenty colonies ($EoPLA_2$ 1-20) contained an insert of the correct size. The sequences from the twenty colonies (Appendix B) were identified (BLAST) as belonging to the sub-group II of the PLA_2 superfamily. The 415 nucleotides (excluding the two restriction enzymes and the two stop codons) correspond to an open reading frame that predicts a polypeptide of 138 amino acids and a molecular weight of 15.7kDa. The observed sequence discrepancies of the $EoPLA_2$ cDNAs revealed six distinct variants (Fig.3.3a-b) out of the twenty clones mentioned above. The northern blot analysis revealed a major transcript band of 0.9kb. According to the extensive literature survey of venom PLA_2 by Kini (1997), RNA of this size is within the acceptable range for the PLA_2 transcripts or genes isolated from the *Viperidae* family.

The breadth of the $EoPLA_2$-05-hybridizing band in the Northern blot probably reflects the presence of several isoforms which is consistent with the numerous $EoPLA_2$ variants obtained here by PCR.

Analysis of the deduced amino acids of the $EoPLA_2$ isoforms indicate eight distinctive segments: (1) a well-characterised, highly conserved signal peptide (composed of 16 amino acid residues) (Blobel and Dobberstein, 1975), (2) a substrate recognition domain (containing lipophilic glycine-rich residues) (Jeyaseelan et al., 2000), (3) an overlapping Ca^{2+}-binding loop, (4) sequences encoding the catalytic domain (Jeyaseelan et al., 2000), (5) a short domain of 15-amino acids encoding the anticoagulant region (Kini and Evans 1987), (6) a β-wing domain, (7) a Helix-3 domain that plays a pivotal role in the structure and pharmacological activity of PLA_2 (Kini et al., 1987 and Scott, 1997) and (8) the C-terminal cysteine rich domain, which characterises the sub-group II PLA_2 (Kini, 1997) and is important to maintain a defined conformation external to the main globular mass structure of PLA_2 molecule (Brunie et al., 1985).

The greatest discrepancy observed within the $EoPLA_2$ variants was the number of C-terminal Cys residue substitutions. These residues are important to the tertiary structure of the PLA_2 molecule (Moura da Silva et al., 1995). *$EoPLA_2$-09* to *$EoPLA_2$-12* isoforms showed a Met for Cys 133 substitution (Fig.3.2b). The other three variants, $EoPLA_2$-10- to 12, differed from $EoPLA_2$-09 by a Leu for Cys^{133} substitution. However, whether the Cys substitutions at the C-terminal region have any negative impact on the structural and/or functional properties of the protein molecules remains to be elucidated. The other very minor, amino acid substitutions of the $EoPLA_2$ variants outside the C-terminal region are

however, unlikely to affect the tertiary structure because the positions of the 14 cysteine scaffold were well conserved and likely reflect a common lineage to PLA_2 enzymes in other vipers (Moura da Silva et al., 1995).

Generally the extensive sequence similarity between the $EoPLA_2$ variants and PLA_2s from related vipers is interesting because it highlights regions of the protein sequence that are important to the structure or function of PLA_2. The multiple sequence alignment generated by MegAlign-[DNA STAR software] (Fig.3.4) showed that there are extensive regions of sequence similarity between $EoPLA_2$-05 and PLA_2 from other vipers. Moreover, since these vipers are taxonomically and geographically distinct, the conservation of these "motifs" suggests that they are related to the structure and/or function of this family of toxic enzymes. The phylogenetic tree shown in Fig.3.5 demonstrated that PLA_2 from *E. sochureki* may be the common ancestor of this group of enzymes. It also shows that the PLA_2 molecules from the related viper species may have emerged after the divergence of the $EoPLA_2$-05 molecule.

In order to assign potential functional attributes to a PLA_2 molecule, its deduced amino acid sequence is usually analysed for the existence of certain criteria including (a) the expression of Lys or Asn residues at position 64 (including the signal peptide) which is known to confer calcium-mediated catalytic activity, (b) the number of lysine residues within the anticoagulant domain and (c) pH. The latter criteria to predict PLA_2 function is controversial. Thus, although the catalytic properties of PLA_2 molecules are not correlated with their pIs (Fuly et al, 2002), Mounier et al., (2000) reported that the phospholipid-independent anticoagulant effect appears to be relevant for some basic venom $sPLA_2$.

All the $EoPLA_2$ variants examined here possessed the consensus catalytic domain, including the characteristic Asp^{64} residue (Fig.3.4). This indicates that all $EoPLA_2$s possess catalytic potential. With regards the possible linkage between PLA_2 function and pI; among the PLA_2s isoforms aligned in Fig.3.4, only the *B. asper*, *B. jararacussu* (Moura-da-Silva et al., 1995) and Ecarpholin S from *E. sochureki* (Polgar et al., 1996) showed a basic pI (Fig.3.5) despite the known functionality of PLA_2s from many of these species. Therefore, the predicted acidic pI for $EPLA_2$ may not be useful to assign a function to the encoded proteins. In the absence of isolating and determining the functional activities of *E. ocellatus* venom PLA_2, these structure/function predictions illustrate the problems of using cDNA-derived amino acid sequence data to predict functional characteristics of proteins that exist as multiple isoforms, each with the potential to effect distinct functions (Bahrati et al., 2003).

Despite the geographical distribution, taxonomic designation and pI distinctions between $EoPLA_2$-05 and PLA_2 from other viper the hydrophilic profile plot (Fig.3.6) and antigenic index (data not shown) showed that the predicted structure of $EoPLA_2$-05 was remarkably similar to PLA_2 of other vipers. This may reflect common molecular properties as well as functional attributes of these protein isoforms. In the context of the overall immunotherapeutic objective of this work, it is predicted that transfection of mammalian cells with PLA_2–encoding DNA is likely to present to the immune system molecules that faithfully represent native venom PLA_2 (Bahrati et al., 2003) and that the generated antibody has the potential to cross react and neutralize all PLA_2 variants in *E. ocellatus* and also those in venoms of related viper species.

Chapter 4

Cloning and characterization of novel Echis ocellatus cDNAs encoding C-type lectins

4.1. Introduction

Venoms of *Viperidae* contain a wide variety of proteins which affect haemostasis by activating or inhibiting the function of platelets or coagulation factors as stated in Chapter 1. These components have been classified into groups based on structure and mode of action. The snake venom C-type lectin (svCTL) group of proteins was first identified in the venom of *Bothrops atrox* (Gartner et al., 1980). The svCTL protein closely resembles the primary structure and disulphide bond arrangement of the mammalian C-type lectins (Horii et al., 2003) such as mannose-binding protein (Weis et al., 1991) and the selectins (Chou, 1996). Snake venom CTLs typically have a molecular weight of approximately 30 kDa and are commonly found as heterodimeric proteins consisting of homologous α and β subunits linked by a single inter-chain disulphide bridge (Sarray et al., 2003). Higher molecular weight forms of disulphide-linked multimers, ranging from ~50 to 150 kDa, have also been reported by several authors (Taniuchi et al., 1995; Polgar et al., 1997; Wang et al., 2001a).

A striking feature of venom CTLs is that they are structurally homologous but functionally distinct (Wei et al., 2002). Viper venom CTLs possess a range of pharmacological functions including binding to coagulation factors IX and X and inhibiting the clotting cascade or having agonistic or antagonistic effects on platelet aggregation (Wisner et al., 2002). Those exhibiting anticoagulant activities bind to γ-carboxyglutamic acid (Gla)-containing domains of the coagulation factors IX and X in the presence of calcium ions (Wang, 2001a; Atoda et al., 2002). Examples of such svCTL proteins include IX/X binding protein (bp) from the venom of the Habu snake (*Trimeresurus flavoviridis*) (Atoda and Morita, 1989), acutus IX/X-bp from the venom of *Deinagkistrodon acutus* (Cox,

1993), ECLV IX/X-bp from the venom of *Echis carinatus leucogaster* [*now known as E. leucogaster*] (Chen and Tsai, 1996) and jararaca IX/X-bp from the venom of *Bothrops jararaca* (Sekiya et al., 1993). Other, venom CTLs activate the coagulation cascade through distinct routes such as that exerted by bothrojararacin, a thrombin inhibitor from venom of *B. jararaca*, which binds the exosite of α-thrombin (Zingali et al., 1993). Snake venom CTLs also inhibit or activate platelet aggregation by modulating their binding interactions to their specific receptors, including glycoprotein (GP) Ib, α2β1, GPVI and von Willebrand factor (vWf) (Navdaev et al., 2001b; Polgar 1997; Shin and Morita 1998; Wang et al., 2001a). Most of the platelet aggregation-inhibitory CTLs bind to GPIb, including Echicetin from the venom of the saw-scaled viper, *Echis carinatus* (Peng et al., 1994), jararaca GPIb-binding protein from *B. jararaca* venom (Fujimura et al., 1996); alboaggregin-B from the venom of white-lipped tree viper *Trimeresurus albolabris* (Peng et al., 1994); Flavocetin-A from *T. flavoviridis* (Taniuchi et al., 1995); CHH-A and B from the venom of the timber rattle snake *Crotalus horridus horridus* (Andrews et al., 1996); Tokaracetin from *T. tokarensis* venom (Kawasaki et al., 1995). Other venom CTLs bind to distinct receptors including, (i) vWF (Botrocetin from *B. jararca* venom) (Andrews et al., 1989; Fujimura et al., 1991); (ii) GP1a [Aggretin from *C. rhodostoma* venom, (Huang et al., 1995)] and (iii) GPVI [Convulxin from *C.d. terificin* venom (Polgar et al., 1997)]. Some of these CTLs exhibit additional affects such as that shown by Echicetin [see Chapter-1], that blocks platelet interaction with both vWf and thrombin (Navdaev et al., 2001a).

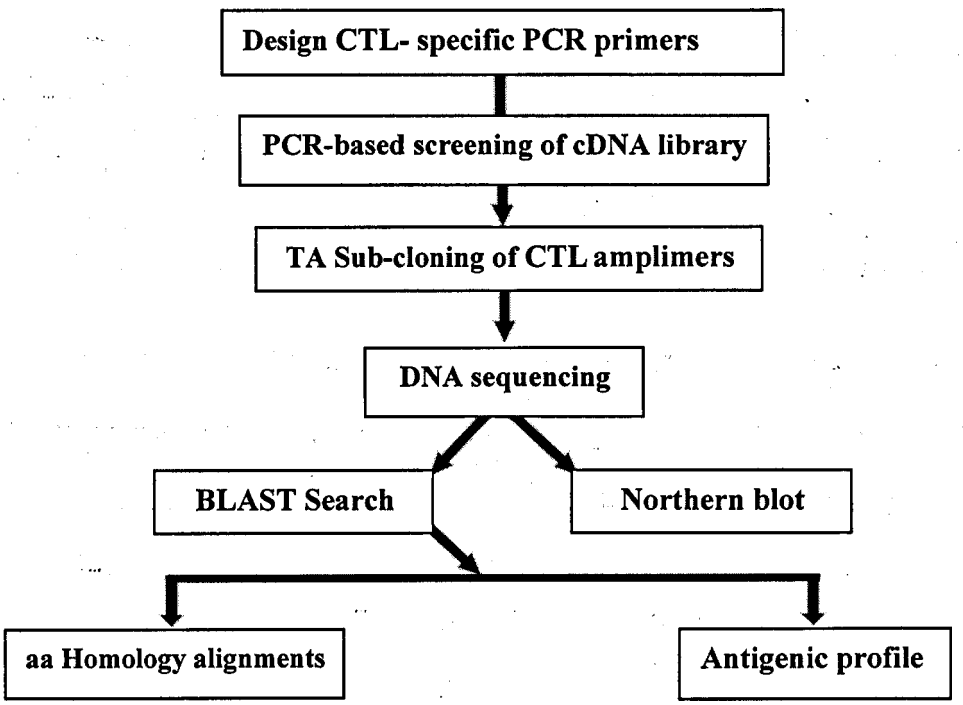
The existence of multiple forms of snake venom CTLs within a single species, as shown for example a *B. jararaca* and *E. carinatus* snake venoms, is likely to contribute to the

diverse biological effects exerted by venom of *Viperidae.* Despite the medical importance of *E. ocellatus,* comparatively nothing is known about the contribution of CTL activity to the pathology of *E. ocellatus* envenoming. Therefore, the experimental objective of this chapter was to isolate different isoforms of CTLs from the *E. ocellatus* cDNA library. The majority of the results represented here have been published (Harrison et al., 2003a)

4.2. Strategy

The experimental strategy to achieve these objectives is show in Fig.4.1.

Fig.4.1. Strategy plan for isolating EoCTL.



4.3. Designing primers

A PCR strategy was used to isolate sequences encoding CTLs from the *E. ocellatus* venom gland cDNA library. Primers complementary to the highly conserved amino-terminal signal peptide and to the less conserved carboxy-terminal domains of published CTL DNA sequences of related viper species were designed (Table 4.1)

Table 4.1. Designation of PCR primers for isolating EoCTLs

	***EoCTL* Primers Description**			
	***Bam*HI**	**Amino-terminal signal peptide [M-G-R-F-I-F]**	***Xho*I**	**Carboxy-terminal [F-V-C-K-S-P-A]**
Anti-sense 5' primer [24 bp]	**GGA-TCC**	ATG-GGG-CGA-TTC-ATC-TTC		
Sense 3' primer [30 bp]			**CTC-GAG**	CTA-TGC-CGG-GCT-CTT-GCA-GAC-GAA

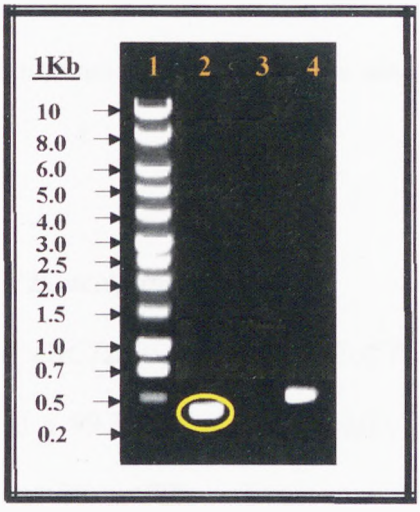
A stop codon (TAG, underlined) was inserted in the 3' primer and *Bam*H1 and *Xho*1 restriction endonuclease sites (bold) were included in the 5' and 3'primers respectively to facilitate future subcloning into mammalian expression plasmids. The two primers were synthesized commercially by Sigma-Genosys Ltd., U.K.

4.4. Results

4.4.1 Isolation of cDNAs encoding E. ocellatus CTLs

The expected PCR product of approximately 450 bp was obtained by PCR of venom gland cDNA using the two CTL-specific primers (Fig.4.2). The amplified DNA was ligated into the TA cloning vector (Invitrogen) and used to transform competent *E. coli* [TOPO 10F' One Shot (Invetrogen)]. A good yield of recombinant colonies was obtained [1:1 ratio of blue and white colonies]. *Bam* HI and *Xho* I restriction enzymes digestion was performed as a first step procedure to screen transformed white colonies, prior DNA sequencing.

Fig.4.2: PCR product of the *E. ocellatus* C-type lectin.



Analysis of PCR amplification products by 0.7% agarose gel electrophoresis. Bands were visualised using ultraviolet trans-illumination. Lane 1-molecular weight marker; Lane 2 represents the amplified PCR product (circled) of about 450bp from *E. ocellatus* venom gland cDNA; Lanes 3 and 4, represent a negative control where H_2O, instead of DNA template was used and a positive control using a (10ng) SOD [Superoxide Dismutase-from *Onchocerca ovululus*] DNA template, respectively.

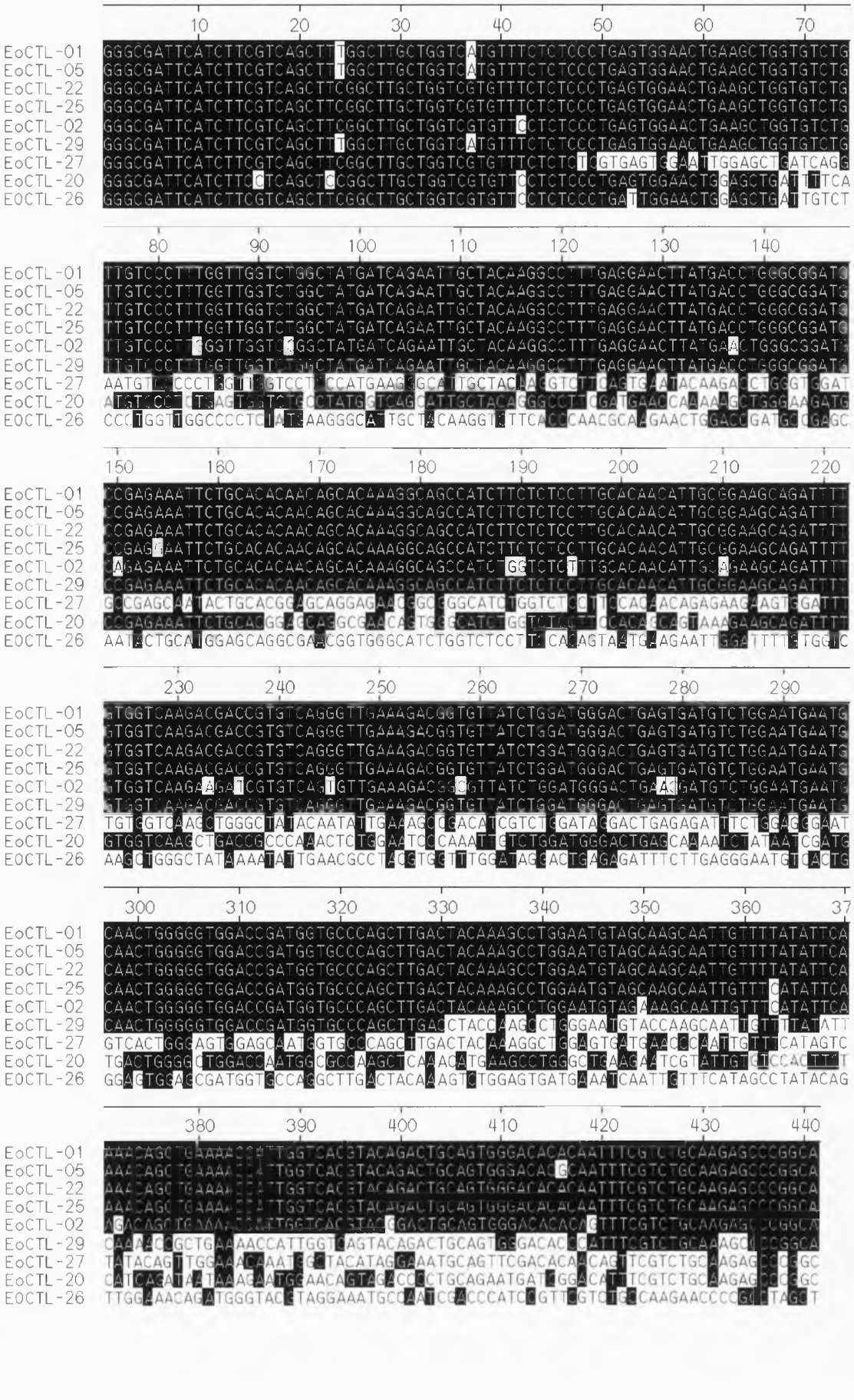
4.4.2. Analysis of the isolated cDNA-encoding E. ocellatus CTLs

Of the thirty recombinant colonies containing DNA inserts of 450 bp, twelve were found to encode full-length CTL genes (Fig.4.3a and Fig.4.3b). Nine of the thirty *EoCTL* sequences were distinct at the nucleotide sequence level. Where two or more identical sequences were obtained, a single representative cDNA was used for subsequent analysis. The predicted amino acid sequences of these cDNAs were subjected to BLAST (Altschul et al., 1997) searches of the GenBank, PDB, SwissProt, PIR and PRF databases to confirm their CTL identity (Fig.4.4). All of the *E. ocellatus* cDNA consisted of 441 nucleotides (Fig.4.3a) that were predicted to encode an open reading frame of 148 amino acids (17.2 kDa) of (Fig.4.3b). A phylogenetic tree analysis (Fig.4.3c) was used to categorise the nine *EoCTL* sequences into six distinct groups, based entirely on sequence alignment. Alignment of the predicted amino acid sequences of the nine EoCTL revealed extensive sequence variation.

4.4.3. Sequence alignment of E. ocellatus CTLs.

Group 1 contained *EoCTL-01, EoCTL-05, EoCTL-09, EoCTL-22, EoCTL-23, EoCTL-25* and *EoCTL-30*, which showed a 99.3 % sequence identity [differing by 2 residues as illustrated in Fig.4.3b]. The remaining CTL variants were represented by single EoCTL cDNAs: group 2 [*EoCTL-2*] differ from group 1 by 9 residues substitutions. In contrast to group 1 and 2, groups 3-6 show significant sequence variations which may represent distinct EoCTL isoforms. For instance, group 5 [*EoCTL-27*] did not contain the consensus methionine start codon and its amino terminal sequence differed markedly from the highly conserved signal peptide sequence (residues 1-23). Intriguingly, *EoCTL-27* had a similar

Fig.4.3a


EoCTL-01
EoCTL-05
EoCTL-22
EoCTL-25
EoCTL-02
EoCTL-29
EoCTL-27
EoCTL-20
EoCTL-26

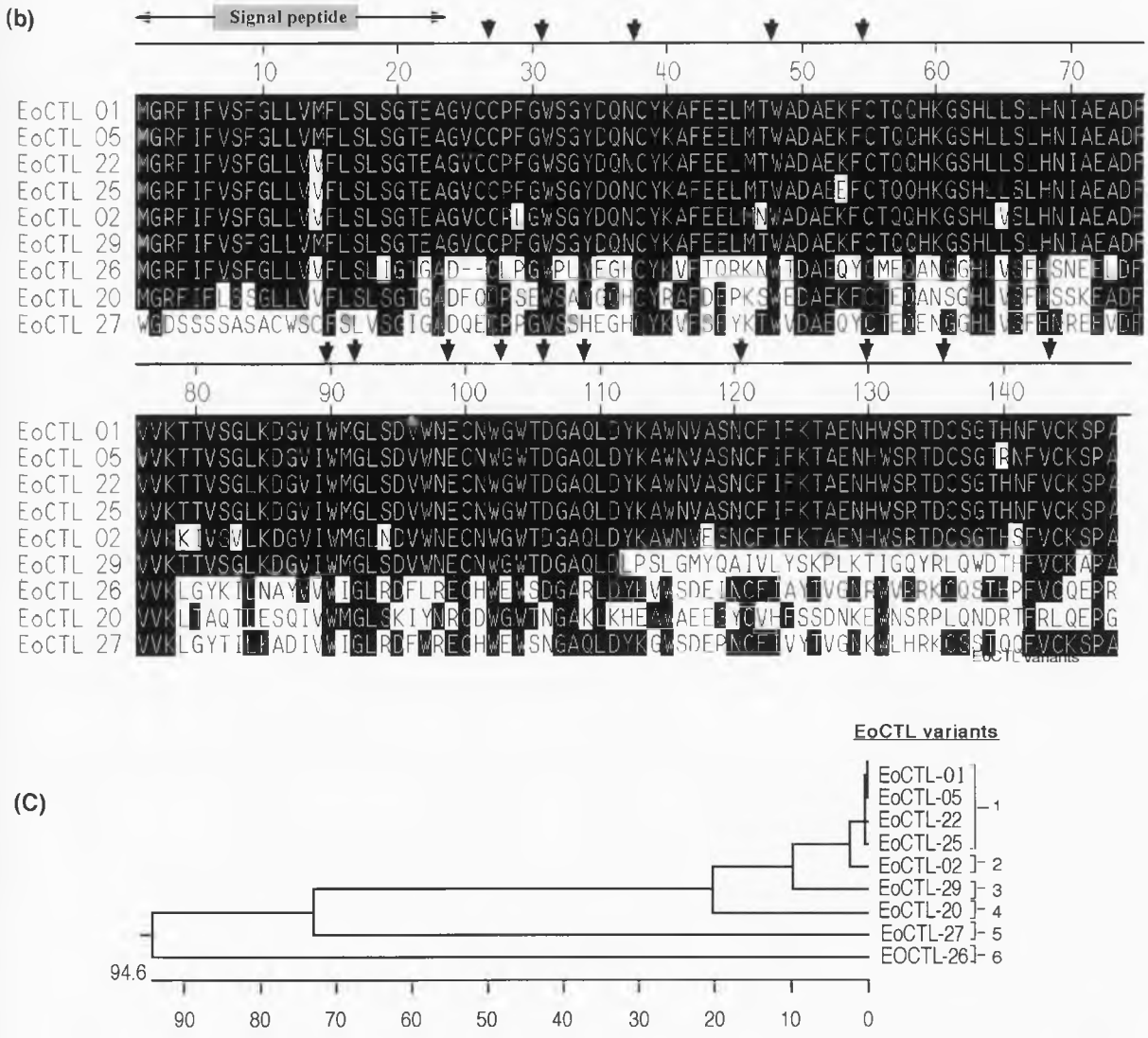


Fig.4.3. Alignment of the cDNA **(a)** and deduced amino acid sequences **(b)** of CTLs isolated from the saw scaled viper *E. ocellatus* [residues shaded in black are identical to *EoCTL-01*]. The horizontal arrows represent the signal peptide (sp) domain (residues 1-23). The vertical arrows identify amino acid residues described in the text that are associated with the carbohydrate recognition domain (CRD) of the classic CTL proteins. (C) Phylogenetic tree analysis of the *E. ocellatus* CTL deduced amino acid sequences. This analysis was used to categorise the sequences into six distinct clusters which is based on the number of amino acid substitutions.

amino terminal sequence to *EsCTL-3* from *Echis sochureki* (Harrison et al., 2003) but the down-stream sequence of *EoCTL-27* was very different (Harrison et al., 2003). The distinct 5' sequence composition of *Eo CTL-27* was therefore not considered to be the result of a cloning artefact.

The deduced amino acid sequences of all *EoCTLs* possessed the cysteine scaffold that defines the carbohydrate-recognition domain (CRD) of mammalian CTLs (Drickamer, 1988; Speiss, 1990) that bind sugars in a Ca^{2+}-dependent manner. Disulphide bridges formed between C^{27} and C^{38}, C^{55} and C^{144} and between C^{121} and C^{136} (as indicated by arrows in Fig.4.2b) are conserved in all the *EoCTL* sequences, except in those of groups 3 and 4 where the C^{121} and C^{136} were substituted with other residues as shown in Fig.4.3b. The CRD-invariant residues W^{31}, W^{48}, W^{90}-X-G^{92}, W^{105}-X-X-G^{107} and W^{131} (Wisner et al., 2002) are represented in all the *EoCTL* groups, except groups 3. Group 3 showed I substitutions for the W residue at position 105 and 131, respectively. All the *E. ocellatus* venom CTL groups showed a G^{92} and L^{110} residue substitutions for W^{92} and P^{110}, respectively that are conserved in mammalian CRD sequences. Furthermore, the *EoCTLs* cDNA sequences did not encode the E-P-N and Q-P-D triplets associated with CRD binding of the mammalian mannose and galactose CTLs, respectively.

4.4.4. Analysis of the E. ocellatus clusters with analogous CTLs in venoms of related viper species.

Viper venom CTL sequences from the *Viperidae* genetic databases that showed significant sequence similarity to EoCTLs were selected for comparison (Fig.4.4) on the basis of highest BLAST matches and their phylogenetic or geographic relatedness to *E. ocellatus CTLs*: ECLV IX/X bp β (AAB36402) from *E. leucogaster* venom (Chen and Tsai, 1996), Echicetin β (P81996) from *E. carinatus* venom (Peng et al., 1994), Convulxin β (CAA76182) from *C. durissus terrificus* venom (Leduc and Bon, 1998), Alboaggregin α subunit 4 (P81114) from *T. albolabris* venom (Kowalska et al., 1998), α (D83331) and β (D83332) IX/X bp subunits from *T. flavoviridis* venom (Matsuzaki et al., 1996), Flavocetin-A β subunit (AAN72437) also from *T. flavoviridis* venom (Shin et al., 2000), Aggretin (JC7105) from *C. rhodostoma* venom (Chung et al., 1999), α (AF176420) and β (BAB20441) subunits from *Deinagkistrodon acutus* venom (direct submissions), α (AF190827) and β (AF197915) subunits from *Gloydius halys* venom (direct submissions), *Es CTL-3* (AY254332) and *Es CTL-8* (AY254334) from *E. sochurecki* venom, *Epl CTL-4* and *Epl CTL-7 (*AY254338 & AY254340), from *E. p. leakeyi* venom, and *Ba CTL-6* (AY254328) from *B. arietans* venom (Harrison et al., 2003a).

Groups 1-3 represent novel, highly similar, CTL isomers with highest sequence similarity to CTLs isolated from *E. p. leakeyi, E. sochureki and B. arietans* (Harrison et al., 2003a). Phylogenetic tree analysis showed that groups 1-4 were most closely associated with β-subunit CTLs (Fig.4.5). EoCTL-27 and EoCTL-26 were grouped in the same clade as the β-subunit of Echicetin from *E. carinatus venom* and EoCTL-20 showed greatest sequence

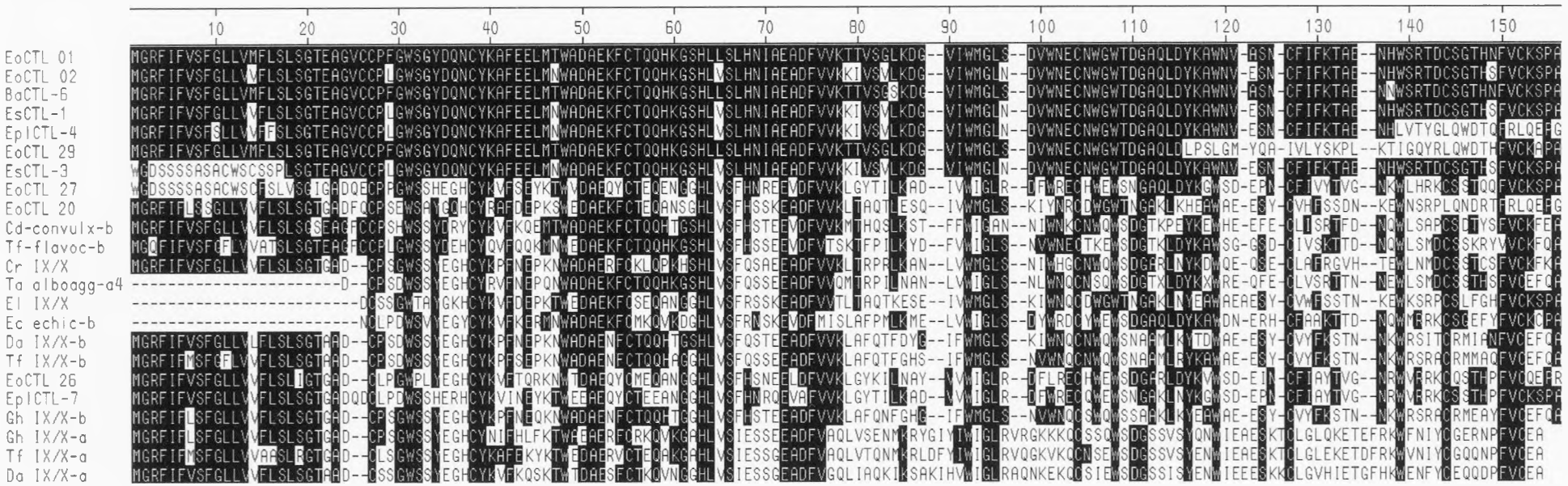


Fig. 4.4. Analysis of the amino acid sequences of the *E. ocellatus* CTL clusters by sequence similarity [residues shaded in black match *EOCTL-01*] with venom CTLs from *Echis* and other related viper species. Ec echic-b = echicetin b from *E. carinatus*; Tf-flavoc-a and-b CTL subunits from *T. flavoviridis*; Cd-convulx-b subunit of convulxin from *C. durissus terrificus*; Ta-alboagg-a subunit 4 from *T. albolabris*; Cr IX/X = aggretin, a factor IX/X binding protein from *Calloselasma rhodostoma*, Da anticoag-a and -b subunits of the anticoagulant CTL from *Deinagkistrodon acutus*; Gh Xa inhib-a and -b subunits of the factor Xa inhibiting CTL from *Gloydius halys*, El IX/X-b subunit of ECLV, the IX/X binding protein of *E. leucogaster*; Ba CTL-6 from *B. arietans; Epl CTL-4 and Epl CTL-7 from E. pyramedum leakyi; and Es CTL-3 and Es CTL-4 from E. sochureki*. Residues shaded in black are identical to *EoCTL-01* of group 1.

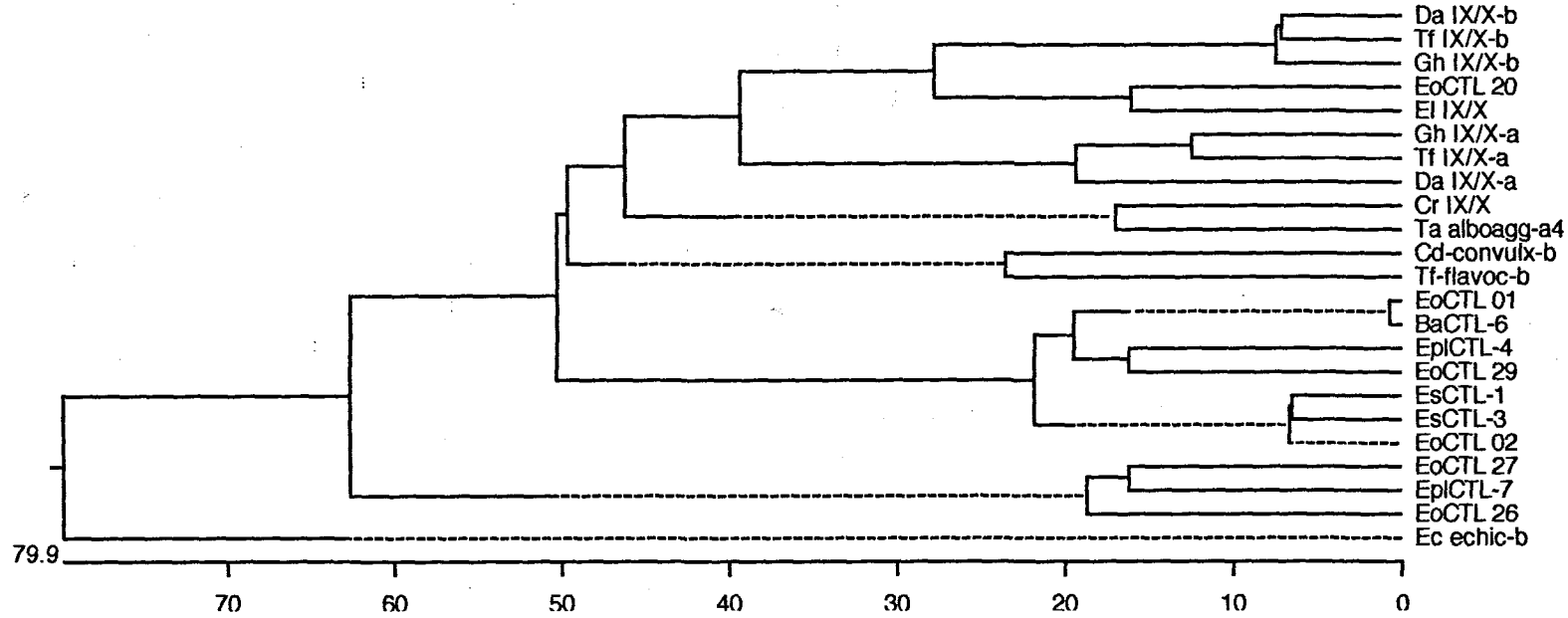


Fig. 4.5. Phylogenetic tree analysis of the *E. ocellatus* CTL groups and venom CTLs from *Echis* and other related vipers. The scale beneath the tree measures the distance between sequences (in millions of years).

similarity (64.2%) to the β-CTL subunit of the West African *E. leucogaster* venom. Of all the EoCTL groups, only groups 1 and 2 seemed to represent the highest sequence similarity of 92.5 to 98.7 % to the BaCTL-6 of unknown function of the African, viper *Bitis arietans* viper.

4.4.5. Predicted antigenic profiles of E. ocellatus CTL-01

The main objective of this research project is to develop toxin-neutralising antibodies by immunisation with DNA encoding specific venom toxins (Harrison et al., 2000b, 2002). The high degree of sequence similarity between EoCTL-1 and CTLs of *E.p. leakeyi*, *E. sochureki* and *B. arietans* suggested that immunisation with EoCTL-1 could generate antibodies reactive with CTL analogous in venoms of these phylogentically related vipers. To investigate this possibility I compared the predicted immunogenicity of these CTL sequences using an algorithm developed by Jameson-Wolf (Fig.4.6).

Line A approximately corresponds to the residues C-Y-K (38-40, Fig.4.4) and is common to all EoCTL groups, except group 4. The highly conserved domain between residues 48-55 (W-A-D-A-E-K-F-C) is not predicted to be immunogenic (Harrison et al., 2003a). Line B corresponds to the residues around H-L-L/V-S (63-66) and is common to groups 1-6 and all analogous CTL molecules. Lines C [residues around C^{145}] and (D) (V-C-K/E at positions 152-154) appear to represent immunogenic domains common to all EoCTLs and most published African viper venom CTL sequences, except group 4 EoCTL which encoded "RLQ" motif a unique carboxyl end.

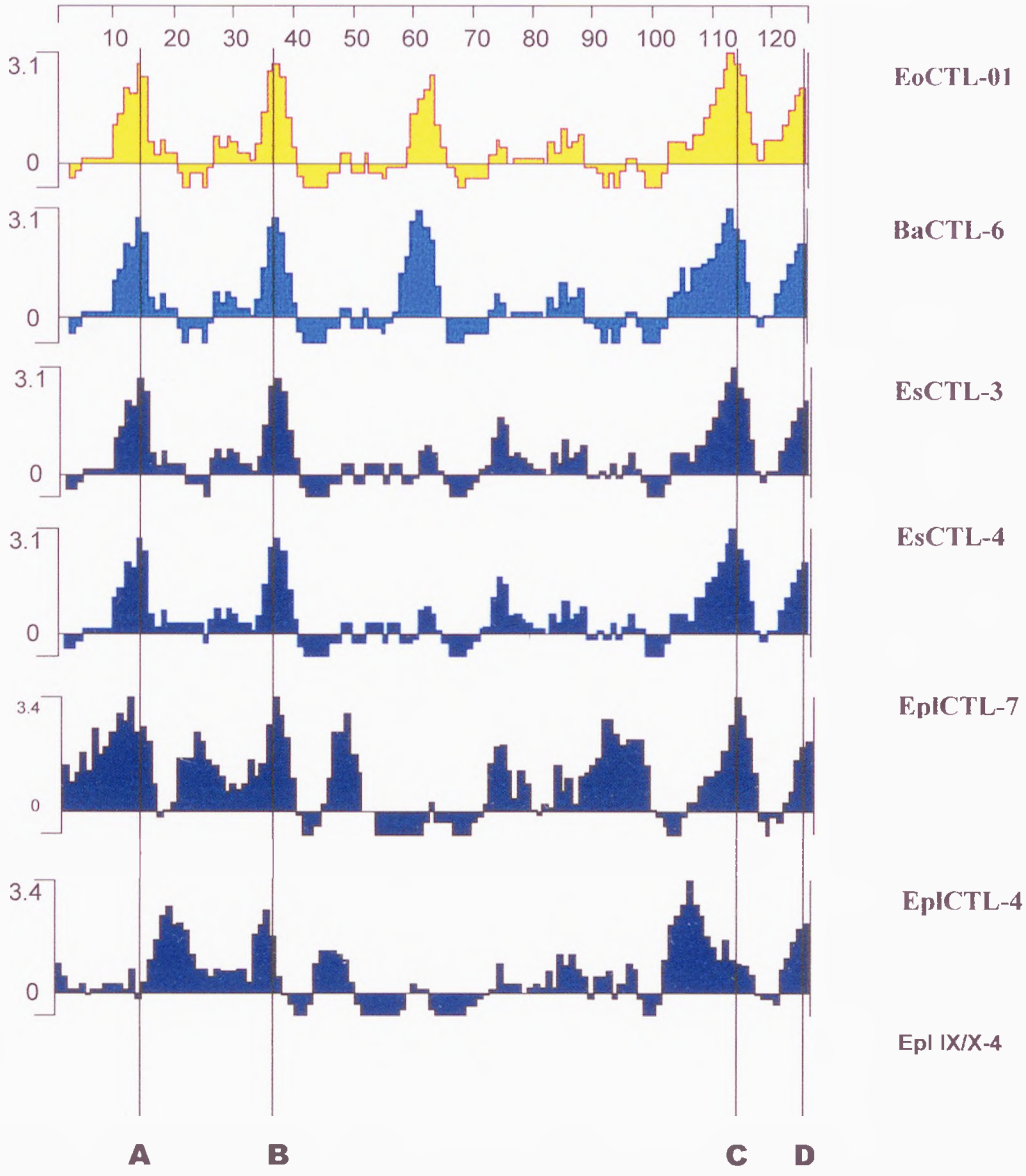


Fig.4.6. Jameson-Wolf antigenic profiles of the *E. ocellatus* CTL group1 and all known CTLs from related African vipers aligned in Fig.4.3. The top horizontal scale represents the number of amino acid residues (excluding the deduced signal peptide domain because they would be cleaved from the native proteins). The vertical scale scales represent comparative antigenic values. The four thin vertical lines (A-D) are a subjective assignation of antigenic domains that show the greatest conservation across phylogenetic and geographic boundaries.

4.5. Discussion

Viper venom CTLs possess a range of pharmacological functions that disrupt haemostasis at several distinct levels. The overall objective of my research project was to generate, by DNA immunisation, antibodies capable of neutralizing the function of the major *E. ocellatus* venom toxins. Prior to this study, there were no *E. ocellatus* CTL DNA or amino acid sequences described in the literature or genetic databases. The objective of the research in this chapter was therefore to isolate and characterise cDNA-encoding CTL from the *E. ocellatus* venom gland cDNA library.

My utilisation of a low-stringency PCR approach was successful in amplifying several sequence-distinct CTL isoforms. Most of the isolated *EoCTL* sequences described here, except those of groups 3 and 6, contained all the cysteine residues and most of the associated amino acid motifs that define the CRD domain and overall architecture of the mammalian and viper CTL sequences (Drickamer, 1988; Spiess, 1990).

The *E. ocellatus* CTL sequences of groups 1 and 2 appear to be highly similar CTL subunits that are novel to the genetic database of Serpentes as illustrated in Fig.4.4. In common with CTL β-subunits of flavocetin-A and convulxin from venoms of *T. flavoviridis* and *C. d. terrificus*, respectively (Shin et al., 2000; Leduc and Bon, 1998), CTLs of groups 1-3 contained a double cysteine motif at positions 26 and 27, which have been invoked to differentiate between β- and from α-CTL subunits (Shin et al., 2000). The existence of an additional cysteine residue at position 26 in the N-terminal of β-CTL subunits is thought to form a disulphide bridge with the C-terminal cysteine residue of α-

subunits, thereby permitting the polymerisation of these peptides into heterodimeric molecules (Wisner et al., 2002). Although they lacked the additional C26 residue, the sequences in groups 4-6 were most closely associated with β-CTL subunits. It is concluded from the absence of an initiating methionine and non-signal peptide-like N-terminal sequence of group 5 (*EoCTL-27*) that these sequences are derivatives from other genes, presumably the group IV metalloproteinases that bind CTL subunits at either the carboxyl (Bjarnason and Fox, 1994) or amino terminal ends (Kini, 1996). I have attempted to use the percent sequence similarity between the *EoCTL* sequences and those published by Harrison et al., (2003a) as well as published CTLs of known function to predict and classify the related function(s) for these new *EoCTL* isoforms. However, the sequence-function relationship of snake venom CTLs, and probably many other venom toxins, is still insufficiently understood to confidently assign a function to a molecule based solely on sequence characteristics.

The extraordinarily high degree of sequence conservation of the CTL molecules [particularly group 1CTL] between the phylogenetically and geographically distinct *E. ocellatus, E. p. leakeyi*, *E. sochureki* and *B. arietans* (Fig.4.4 and Fig.4.5) indicate the evolutionary importance of the multimeric nature of *EoCTLs.* Here, and as frequently observed in the snake venom literature (Deshimaru et al., 1996; Nakashima et al., 1993), the DNA sequences encoding these *EoCTLs* [and those of other *Echis* and *Bitis* species (Harrison et al., 2003a)] showed a higher level of sequence conservation than the deduced amino acid sequences (Harrison et al., 2003a). Accelerated evolution of snake venom toxins may reflect an evolutionary selection mechanism which provides the snake with a wider spectrum of substrates for each enzyme family and thereby increases the scope of

prey vulnerable to venom. The similarity of the inter-specific and inter-generic sequences of CTLs from the *E. ocellatus, E.p. leakeyi, E. sockureki and B. arietans* may indicate that accelerated venom toxin evolution is regulated by a sophisticated control mechanism, rather than by a system that simply accepts degenerative substitutions.

One of the objectives of this project was to evaluate the range of CTL molecules that are likely to be represented in venom of the saw-scaled viper, *E. ocellatus,* in order to guide the design of DNA-immunisation constructs required to generate toxin-specific antibodies as described in Chapter 7 of this thesis. The predicted antigenic profiles of the group 1 (*EoCTL-01*) and other viper species illustrated in (Fig.4.6) indicate that neutralisation of this complex group of toxins will require antibodies with several specificities. Thus, an antibody raised by immunisation with group 1 DNA is likely to be effective against the gene products of only group 1 and 2 CTL. Therefore, to be confident of neutralising the function of this group of venom toxins, Harrison et al., (2003a) speculated the requirement of between six to eight CTL antibody specificities. However, because of the conserved sequence characteristic of the *Echis* and *Bitis* CTL isoforms, it is possible that these isomer-specific antibodies will neutralise this important group of functionally diverse toxins in venoms of the most important African vipers across their geographic range.

Chapter 5

Serine protease variants encoded by Echis ocellatus venom gland cDNA: cloning and sequencing analysis

5.1. Introduction

Snake venoms contain a great variety of toxic proteases. Many of these components are proteases (e.g., metalloproteases, serine proteases, phospholipases A_2) and mediate their toxicity by either stimulating or inhibiting the haemostatic system of human victims or experimental animals, resulting in clinical complications of blood clotting or uncontrolled haemorrhage (Hutton and Warrell, 1993; Markland, 1998; Stocker, 1990; Matsui et al., 2000; Markland, 1998a). Several of these proteinases cleave plasma proteins of the victims in a specific manner with varying degrees of substrate specificity. Thus, while some serine proteases have both fibrinogenolytic and fibrinolytic activities, others have only fibrinogenolytic activity and are called 'thrombin-like' proteases (Markland, 1991; 1998; Pirkle et al., 1986; 1990). The latter hydrolyze fibrinogen specifically and release either fibrinopeptide A or B or both (Hutton and Warrell, 1993) resulting in the disruption of the blood coagulation system by producing abnormal fibrin clots composed of short polymers that are rapidly dispersed and no longer cross-linked by activated factor XIII. Another group of serine proteases such as Batroxobin, Crotalase and Ancrod cleave fibrinogen in manner distinct from that of thrombin. Other venom serine proteases function like mammalian kallikrein (or kininogenase) releasing bradykinin from kininogen (Bjarnason et al., 1983; Komori et al., 1998) and are called 'kallikrein-like' proteases (Bjarnason et al., 1983), an example of this is halystase (Matsui et al., 1998), a kallikrein-like serine protease isolated from *A. halys blomhoffii* venom, which cleaves the β chain at Arg^{42} and slowly degrades the α chain of fibrinogen to generate a product that is no longer converted to normal fibrin clots by thrombin; this results in both reduction of blood pressure as well as inhibiting fibrinogen clotting in the victims. Another kallikrein-like

serine protease with potent biological activity but with different physicochemical properties from those of halystase has been isolated from the same venom; a number of the other kallikrein-like serine proteases have also been isolated from the venoms of *A. caliginosus, C. atrox* and *C. viridis* (Iwanaga et al., 1976; Bjarnason et al., 1983). In addition, there have been a few reports on venom serine proteases with a unique activity, such as ACC-C, a protein C activator isolated from the *A. contortrix* venom (Parry et al., 1998), [which inhibits blood coagulation by inactivating the activated forms of factor V and VIII], a plasminogen activator such as TSV-PA isolated from the *T. stejnegeri* venom (Zhang et al., 1995: Zhang et al., 1997), PA-BJ, a platelet aggregating enzyme isolated from the *B. jararaca* venom (Serrano et al., 1995) and RVV-V, a factor V-activating enzyme isolated from the *V. russelli* venom (Tokunaga et al., 1988).

These data indicate that snake venom serine proteases comprise an enzyme superfamily with multifunctional activities that may have diverged or have undergone gene duplication resulting in alteration of their biological properties during the process of evolution thus acquiring special functions (Perona and Craik, 1997). Although a considerable amount of data is now available, no standardised grouping of these venom serine proteases has yet been documented. However, in 2001b Wang et al. compared sequences of 40 serine proteinases isolated from different snake venoms, using a constructed phylogram in which such sequences were clustered into three groups designated as coagulating enzymes, kininogenases, and plasminogen activators.

No Serine proteinases have yet been purified from venom of the West African saw scaled viper *Echis ocellatus,* in particular or for members of the *Echis* genus in general.

However, the fact that the serine protease superfamily was important in the venom of the Viperidae suggested that such enzymes should be present in the venom of *E. ocellatus* and that serine protease-specific antibodies are likely to be an important factor in *E. ocellatus* envenoming. I therefore, screened the *E. ocellatus* cDNA library in order to isolate and characterise different isoforms or variants of this enzyme superfamily.

5.2. Designing primers

To isolate sequences encoding serine proteases from the *E. ocellatus* venom gland cDNA library a PCR strategy similar to that demonstrated in Chapter 3 was employed. Primers complimentary to the highly conserved amino-terminal signal peptide and to the highly conserved carboxy-terminal domains of published serine protease DNA sequences of related viper species were designed as shown Table 5.1.

Table.5.1. Designation of PCR primers for isolating *E. ocellatus* serine proteasess

	***EoSP* Primers Description**			
		Amino-terminal		**Carboxy-terminal**
	***Bam*HI**	**[M-G-R-F-I-F]**	***Xho*I**	**[F-V-C-K-S-P-A]**
Sense primer; 5' [24 bp]	**GGA-TCC**	ATGGTGCTGATCAG AGTGCTAGCAAA		
Antisense primer; 3' [30 bp]			**CTC-GAG**	TAGTGGGGGGCAAGTC GCAGTTG

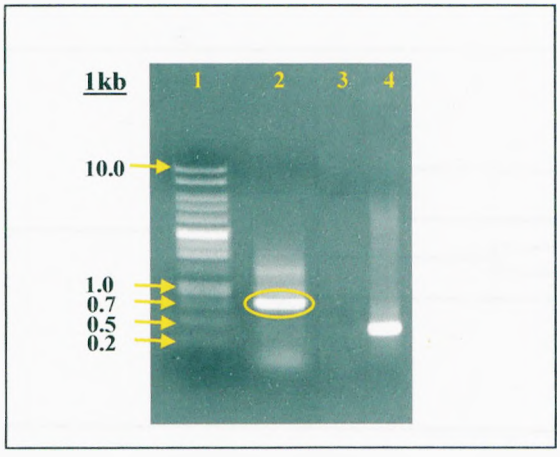
To facilitate future subcloning into mammalian expression plasmids two restriction endonuclease sites (*Bam*H1 and *Xho*1, bold) were included in the 5' and 3'primers, respectively and a TAG stop codon was inserted into the 3' primer. The two primers were synthesized commercially by Sigma-Genosys Ltd., U.K.

5.3. Results

5.3.1. Isolation of cDNAs encoding E. ocellatus serine proteases

A PCR product of the expected size, approximately 800 bp, was obtained (Fig.5.1) after amplification of the cDNA library using the two serine protease-specific primers. The amplified DNA was ligated into the TA cloning vector (Invetrogen) and used to transform chemically competent *E. coli* [TOPO 10F' One Shot (Invitrogen)]. A good yield of recombinant colonies was obtained [1:1 ratio of blue and white colonies]. *Bam* HI and *Xho* I restriction enzyme digestion was performed as a first step procedure to screen transformed white colonies prior to DNA sequencing.

Fig.5.1: PCR product of the *E. ocellatus* serine proteases.



Analysis of PCR amplification products by 0.7% agarose gel electrophoresis. Bands were visualised using the ultraviolet trans-illumination. Lane 2: represents the amplified PCR product (circled) of about 800bp from *E. ocellatus* venom glands cDNA compared with Lane 1: 1kb ladder DNA-marker bands, of known molecular weight. Lane 3 and 4, represent a H_2O negative control and a SOD positive control, respectively.

5.3.2. Nucleotide sequence analysis of E. ocellatus serine protease cDNAs

After PCR amplification and cloning, the nucleotide sequences of 15 cDNA clones were determined by DNA sequencing [Beckman Coulter CEQ™2000 XL DNA Analysis System] as illustrated in Fig.5.2a. The complete amino acid sequences were predicted from the cDNA nucleotide sequences and aligned as shown in Fig.5.2b using MegAlign-DNASTAR software. The 780-bp cDNA had an open-reading frame of 260 amino acids with a calculated molecular weight of 28.5 kDa. The sequence similarities between the 14 serine proteases from *E. ocellatus* were analyzed further by constructing a phylogram (Fig.5.3) using the MegAlign–DNA STAR program on the basis of their complete amino acid sequences. Unfortunately the result of the phylogenetic tree was not significant in grouping the 14 *EoSP* clones. Therefore, I have characterized such sequences in terms of their structural properties as an alternative means of grouping.

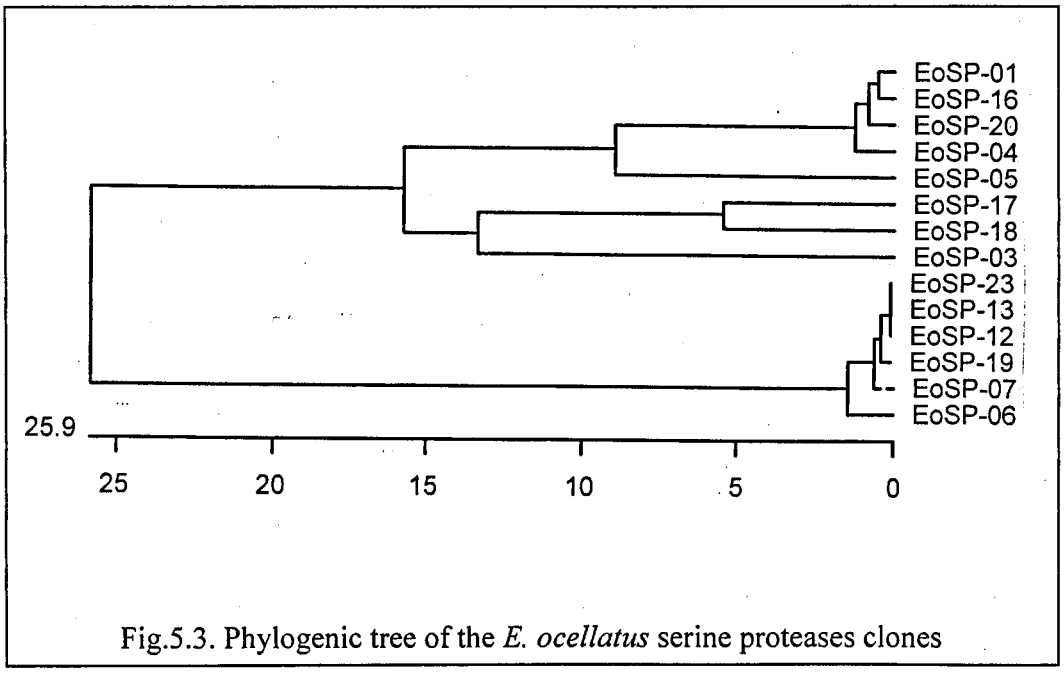


Fig.5.3. Phylogenic tree of the *E. ocellatus* serine proteases clones

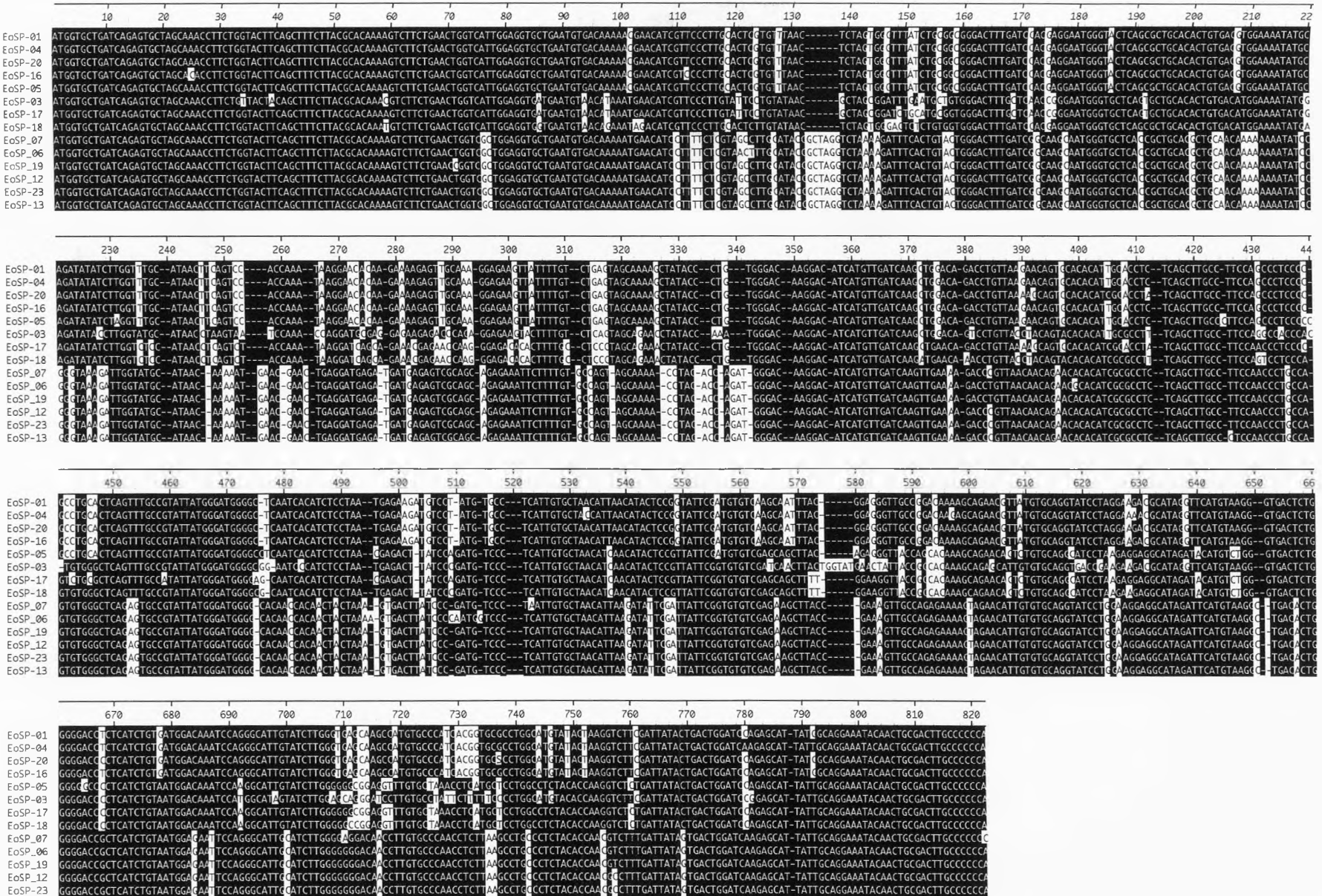


Fig.5.2a. The nucleotide sequence of the fourteen *E. ocellatus* venom gland cDNAs resulting from PCR amplification.

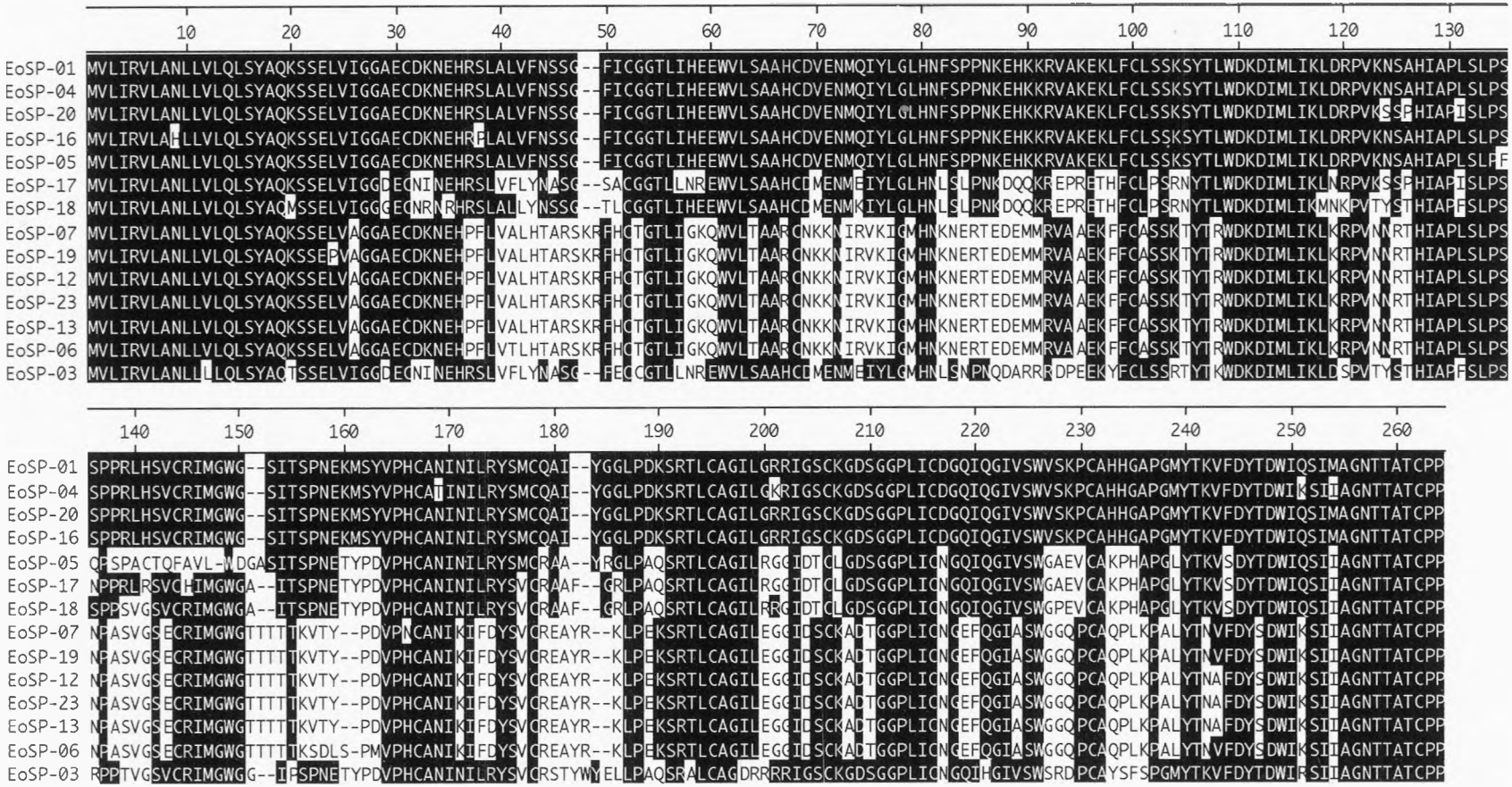


Fig.5.2b. Amino acid sequences of *E. ocellatus* venom gland serine proteases deduced from its cDNAs

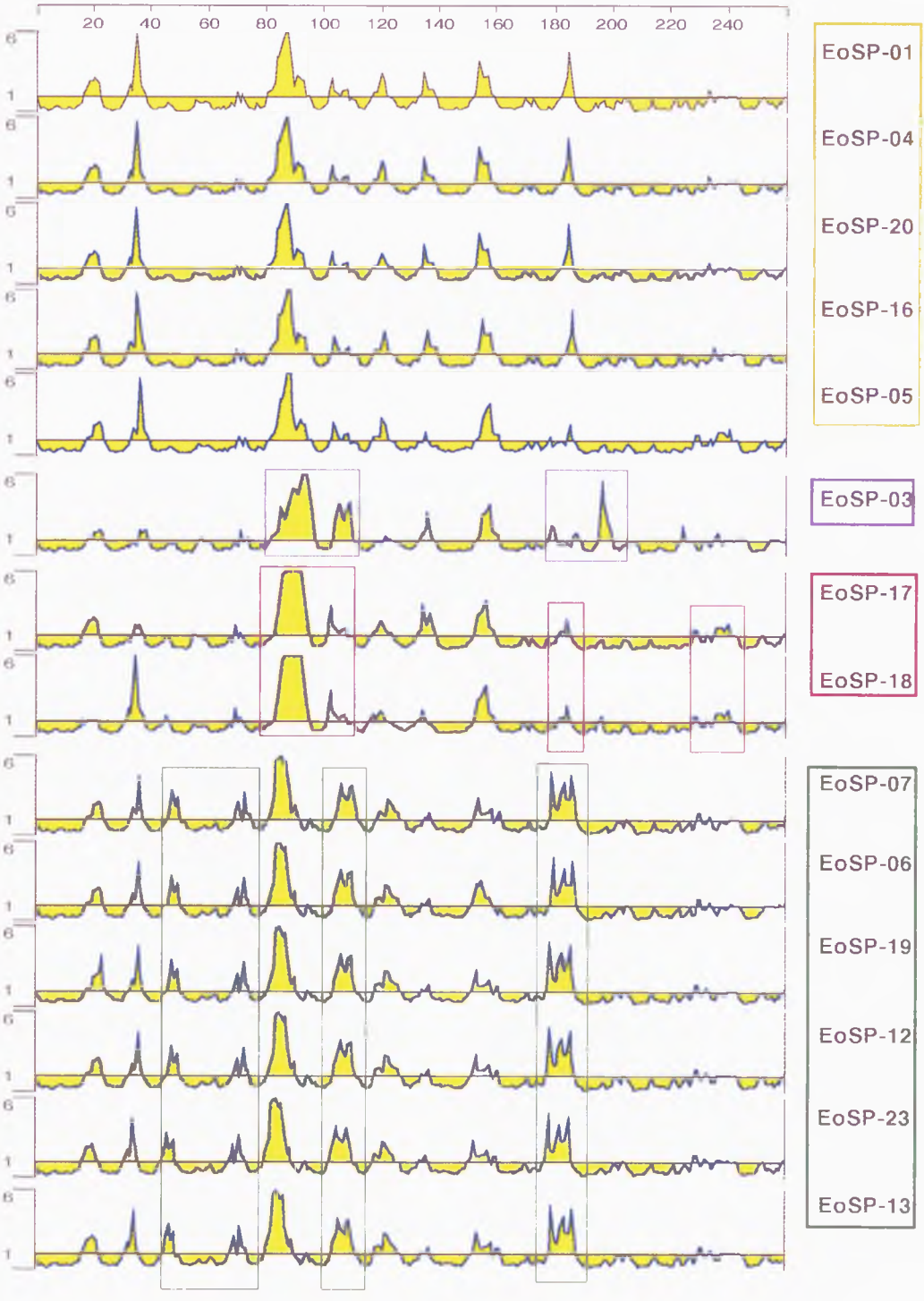


Fig.5.4. Differentiation of the fourteen cDNA-encoding *E. ocellatus* venom serine proteases. The predicted surface (Emin algorithm-DNASTAR, USA) of the 14 *E. ocellatus* serine protease cDNAs were aligned. The boxed areas indicate group specific structural motiffs

5.3.3. Structural properties of EoSP variants.

An analysis of surface probability using the amino acid sequences of all *EoSP* clones were performed using Protean software (DNASTAR, USA). This was successful in grouping the 14 *EoSP* clones into four distinct groups (Fig.5.4). A single clone (*EoSP-01, EoSP-03, EoSP-07* and *EoSP-17*) from each group was selected as a representative clone and used for the subsequent analyses.

5.3.4. BLAST search of the predicted amino acid sequence

The predicted amino acid sequences of the *EoSP-01, EoSP-03, EoSP-07* and *EoSP-17* were submitted to BLAST searches of the genetic data bases and their similarity to published viper serine protease (table 5.2, Fig.5.5) confirmed that the EoSP cDNAs encoded serine proteases.

5.3.5. Comparison of E. ocellatus cDNAs with analogous serine proteases from other viper species

The complete amino acid sequences of the *EoSP* variants were aligned with those of other venom serine proteases (Fig.5.5). All the *EoSP*-variants contained the serine protease-consensus 24 amino acid signal peptide sequence (Fig.5.5 arrows), including the six-amino acids activated motif. The signal peptide residues were followed by a protease domain of 236 residues. The deduced primary structures of all *EoSP* cDNA clones include the requisite, highly conserved, 12 cysteine residues that form the 6-disulphide bonds responsible for the characteristic tertiary structure of venom serine proteases.

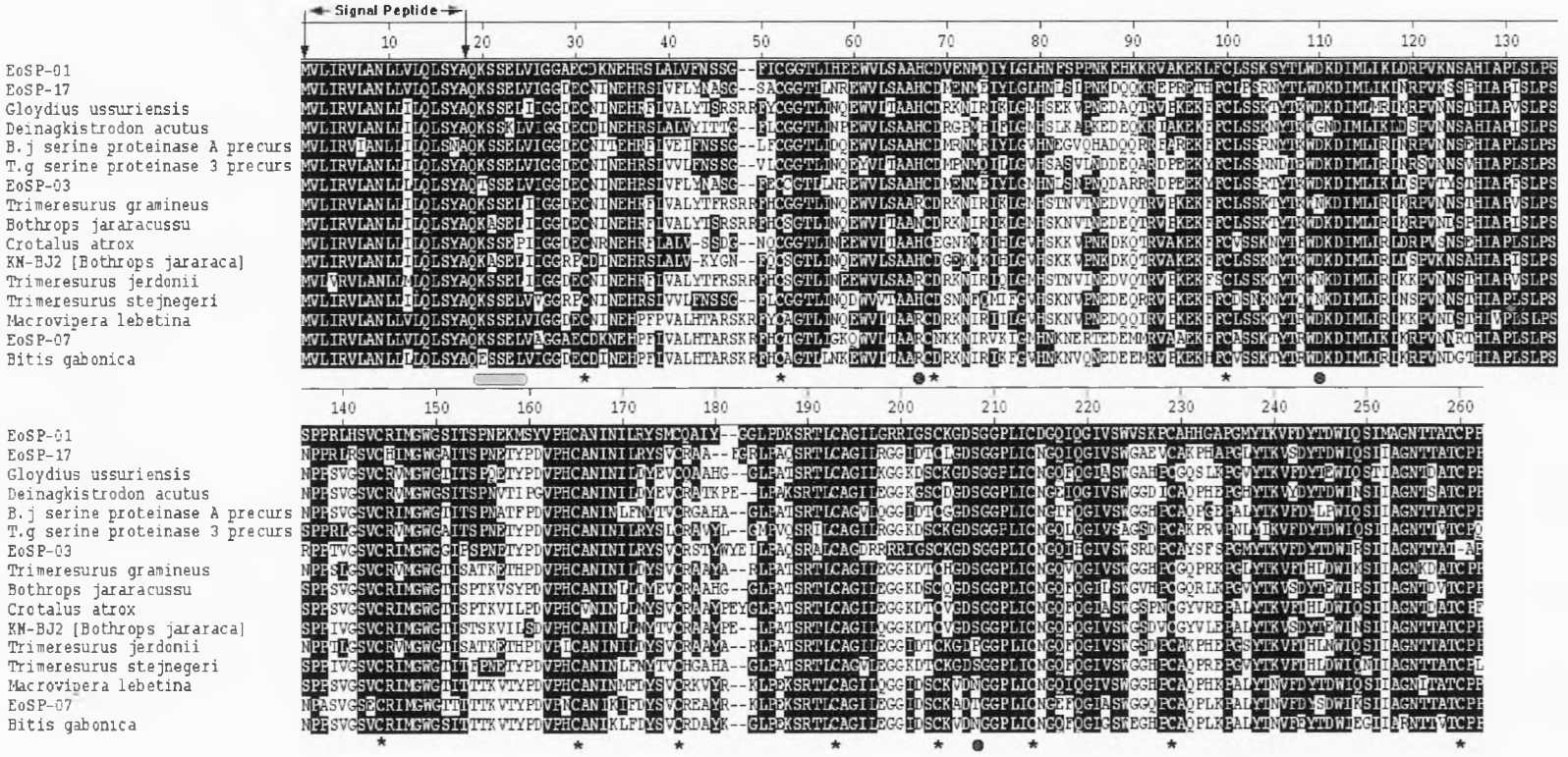


Fig.5.5. Amino acid sequence similarity between *EoSP* variants and serine proteases from related vipers. The residues shaded in black correspond to residues that are identical to *EoSP-01*. The asteriks [*] represented the tweleve conserved cysteine residues. The catalytic traid **His/Arg** (67), **Asp** (110) and **Ser** (208) are represented in red circles ●. Activated peptide where the mature protein cleaved is represented by ▭

Table. 5.2. Percent sequence similarity between *E. ocellatus* serine proteases and analogous molecules from related viper species.

Percent Identity

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
	75.2	67.4	71.3	68.2	71.3	71.7	65.1	63.2	65.5	68.5	65.5	66.7	65.5	62.0	62.4	1	EoSP-01
		70.9	72.5	74.4	76.0	77.1	70.5	68.2	65.5	69.3	70.2	69.4	69.0	62.0	65.1	2	EoSP-17
			73.6	74.0	72.1	68.1	86.2	88.1	72.2	72.8	81.5	74.8	78.8	71.9	75.0	3	Gloydius ussuriensis
				70.9	69.8	69.8	72.5	70.9	68.6	73.9	70.2	70.2	68.6	64.3	64.7	4	Deinagkistrodon acutus
					72.9	68.2	73.6	70.9	70.9	71.2	71.3	78.3	72.1	66.3	68.6	5	B.j serine proteinase A precurs
						71.3	71.3	69.0	67.8	67.3	69.8	74.4	70.2	61.2	65.9	6	T.g serine proteinase 3 precurs
							65.8	64.6	62.5	65.0	66.5	66.3	65.8	59.6	63.5	7	EoSP-03
								85.4	73.7	72.4	90.4	75.2	79.6	73.5	75.8	8	Trimeresurus gramineus
									73.4	76.3	80.4	72.9	79.2	72.3	78.1	9	Bothrops jararacussu
										82.9	70.7	71.7	68.7	62.9	65.3	10	Crotalus atrox
											71.6	72.0	70.0	62.3	65.0	11	KN-BJ2 [Bothrops jararaca]
												72.9	78.1	71.5	73.5	12	Trimeresurus jerdonii
													72.9	65.9	69.4	13	Trimeresurus stejnegeri
														80.4	85.4	14	Macrovipera lebetina
															82.3	15	EoSP-07
																16	Bitis gabonica

Table 5.3. Comparison of amino acid motifs which are responsible for the potent effects and characterisation of some published venom serine proteases with the four EoSP cDNAs

Amino acid	TSV-PA	Batroxobin	Ancrod	EoSP-1	EoSP-17	EoSP-3	EoSP-7	References
H/R	H^{57}	H^{57}	H^{57}	H^{67}	H^{67}	H^{67}	R^{67}	Braud et al., (2000)
D	D^{102}	D^{102}	D^{102}	D^{112}	D^{112}	D^{112}	D^{112}	
S	S^{195}	S^{195}	S^{195}	S^{208}	S^{208}	S^{208}	T^{208}	
H	H^{192}	G^{192}	N^{192}	K^{205}	L^{205}	K^{205}	K^{205}	
F	F^{193}	G^{193}	S^{193}	G^{206}	G^{206}	G^{206}	A^{206}	
D	D^{189}	D^{189}	D^{189}	G^{202}	D^{202}	G^{202}	D^{202}	Guinto et al., (1999)
P	P^{225}	P^{225}	P^{225}	P^{235}	P^{235}	P^{235}	P^{235}	
P	P^{219}	P^{219}	P^{219}	P^{228}	V^{228}	P^{228}	P^{228}	Braud et al., (2000)
D	D^{96}	N^{96}	R^{96}	Y^{106}	Y^{106}	Y^{106}	Y^{106}	Zhang et al., (2002)
D	D^{97}	V^{97}	T^{97}	T^{107}	T^{107}	T^{107}	T^{107}	
E	E^{98}	I^{98}	S^{98}	L^{108}	L^{108}	K^{108}	R^{108}	

HDS = Catalytic Traid; H/F = substrate specificity; **D & P** = Architecture of water channel; P =Evolutionary region to kalikrien; **DDE** = substrate specificity to plasminogen.

5.3.6. Antigenic profile analysis of E. ocellatus serine proteases with analogous molecules.

Since the future aim of this research project is to develop toxin-neutralising antibodies by immunisation with DNA encoding specific toxins in venom of the *E. ocellatus*, I next compared the algorithm-predicted immunogenicity of the *E. ocellatus* serine protease cDNA sequences with those of (BLAST) analogous toxin molecules from other vipers. The predicted antigenic profiles (Jameson and Wolf, 1988) of the published and new *E. ocellatus* serine proteases were analysed as shown in Fig.5.6 using Protean software (DNASTAR, USA). The deduced signal peptide domains of the *EoSP* variants are separated by a vertical dotted line, as these would normally be cleaved from the native proteins during posttranslational. The thin vertical boxes depict the residues comprising the catalytic traid, H/R/N, D/G/N and S/P/N/T (67, 110 and 208), that show the greatest phylogenetic conservation as demonstrated in Fig.5.5.

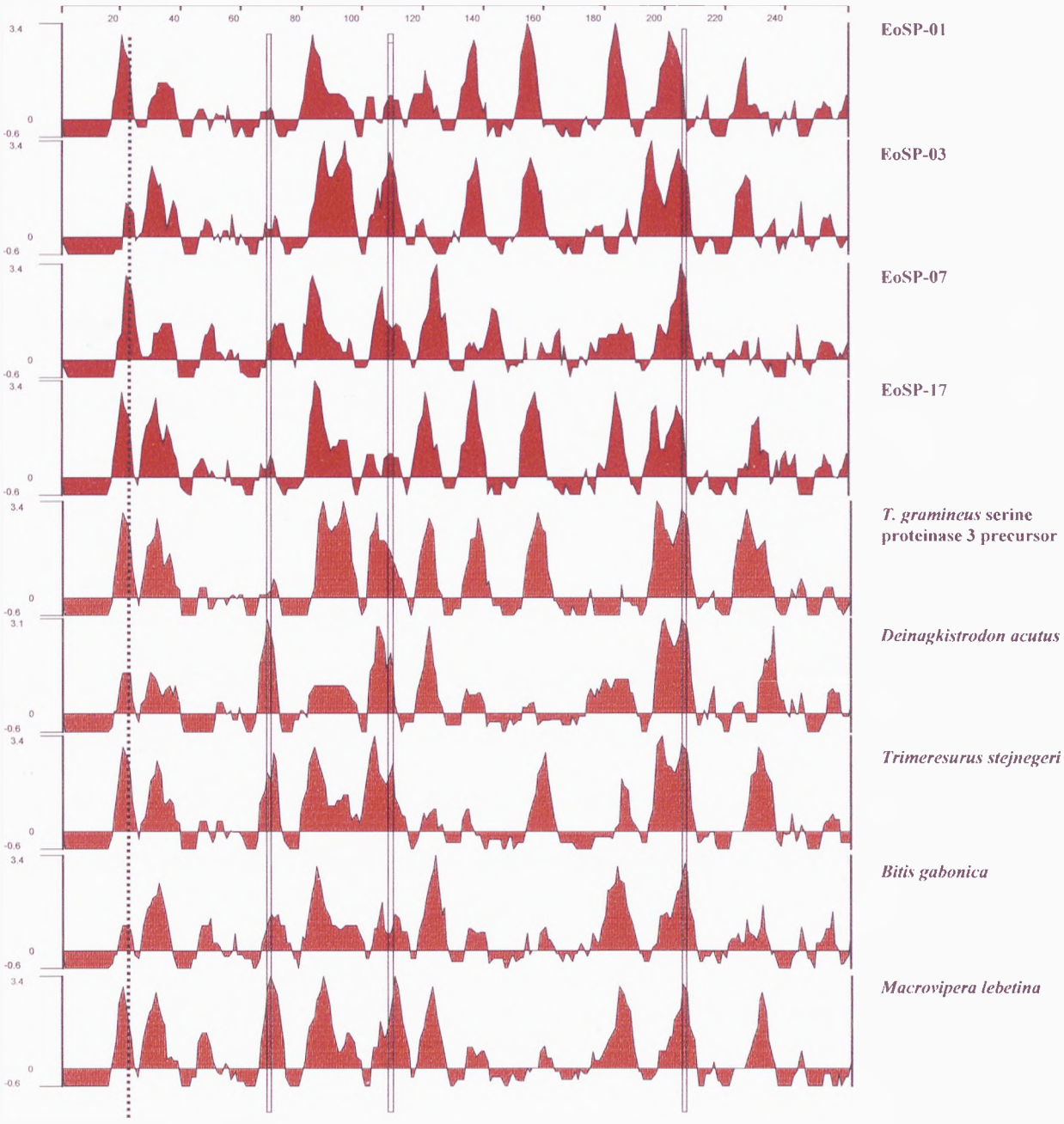


Fig.5.6*. Comparison of antigenic profile of the EoSP variants with analogous serine proteases used in fig.5.5.* The top horizontal scale represents the number of amino acid residues. The conserved signal peptide is separated from the mature protein by a vertical dotted line. The three vertical boxes were drawn to indicate the conserved catalytic traid regions described in the text.

5.5. Discussion

The existence of multiple forms of serine proteases in the venom of a single viper species is likely to contribute to the diverse biological effects exerted by the whole venom. Therefore screening the *E. ocellatus* cDNA library to isolate different isoforms or variants of serine proteases was the aim of this research chapter.

The results obtained in this chapter provide the first molecular sequence data for *E. ocellatus* serine proteases they also reveal that the serine protease composition of *E. ocellatus* is as complex as that of the better characterised *Viperidae* species.

The utilization of PCR amplification of *E. ocellatus* venom gland cDNA with the new viper serine protease-specific primers was successful and produced fourteen cDNAs sequences that were identified (BLAST) as belonging to the serine protease enzyme family. All *EoSP* cDNAs were of similar total length (approximately 0.80 kb) and encoded 260 amino acids (Fig.5.2b) with a predicted molecular weight of 28.5 kDa.

To differentiate between the isolated EoSP clones a surface probability algorithm was used to assign the 14 *E. ocellatus* serine protease cDNAs into four main groups (Fig.5.3). A single representative clone from each group was chosen for further analyses as described in section 5.3.3.

The sequence similarity between the *EoSP* variants proteins was less than 60% for the mature protein-coding region but over 90% for regions coding both the signal peptide and the carboxyl-terminal end. Thus the latter two regions are highly conserved, which

explains why the PCR experiment to amplify the cDNAs-encoding EoSP clones was successful.

The EoSP cDNA sequences were confirmed by BLAST searches as encoding serine proteases (Fig.5.5). The greatest sequence similarity was between EoSP-7 and *B. gabonica* and *V. labetina* (80% and 85%) with the remaining EoSP cDNAs showing 60-76% sequence similarity with other snake venom serine proteinases as illustrated in Table 5.2. From the proteins with known biological activity, sequence similarities of the EoSP variants (i.e., *EoSP-01*, *EoSP-03*, *EoSP-07* and *EoSP-17*) were 62-69% with the kinin-releasing and fibrinogen-clotting serine protease (KN-BJ) from venom of *B. jararaca* (Serrano et al., 1998) (Table 5.2).

The putative 18 amino acid signal-peptide of the *EoSP* variants was as conserved (over 90% sequence similarity) as that in the serine proteases of other viper species (Fig.5.5) (arrows). Following the signal peptide all the *EoSP* variants contained the predicted six-amino acid cleavage (activation) site Q-K/T/M/E-S-S-E-L/P (Fig.5.5 in green) as proposed for batroxobin (Itoh et al., 1987); thus cleavage generates a hydrophilic zymogen peptide, based on the processing site of pre-peptides of mammalian serine proteinases (Swift et al., 1982; MacDonald et al., 1982; MacDonald et al., 1982).

Comparison of the EoSP variants with analogous members of the serine protease family revealed that all EoSP variants encoded the presumed catalytic triad, which is common to venom serine proteases H^{67}, D^{110} and S^{208} as shown in Fig.5.5. Such residues were highly conserved in groups 1-3, except proteins of group 4 (Fig.5.2b, Fig.5.5) which contain R instead of H at the same position (Fig.5.5). Furthermore, comparison of the EoSP amino

acid sequence alignment with analogous venom serine proteases (Fig.5.5) revealed a conserved consensus active site of L-T/S-A-A-H/R/N-C corresponding to position 63–68, as previously determined by Brenner (1988).

Using the primary structure of EoSP variants (Fig.5.5) the putative N-linked glycosylation sites, Asn-X-Thr/Ser (Bell et al., 1984; Wang et al., 1985), were found and are located at two different positions. *EoSP-01, EoSP-03 and EoSP-17* [N^{44}-X^{45}-S^{46} and N^{257}-X^{258}-T^{259}] and EoSP-07 [N^{124}-**R**^{125}-T^{126} and N^{257}-T^{258}-T^{258}]. Although such motifs are thought to be needed for protein stabilization rather than for the catalytic function of the venom enzymes (Komori and Nikai, 1998), confirmation of the roles of such motifs in venom proteases remain to be investigated.

All serine proteases have a common pattern of 6-disulphide bridges (Nikai et al., 1995). This was found in all EoSP clones (Fig. 5.2b) that encoded the common 12 cysteine residues in which are strongly conserved forming putative disulphide bridges which are located at Cys^{31} Cys^{52}, C^{68}, C^{100}, C^{145}, C^{165}, C^{176}, C^{204}, C^{214}, C^{229} and C^{260} (Fig.5.5). This suggests that the EoSP proteins possess a similar tertiary structure to that of other serine proteases which are well characterized.

Despite such sequence and structural conservation, viper venom serine proteases show very divergent effects on haemostasis as previously stated in section 5.1. In some cases certain amino acid sequences have been shown to be responsible for such effects as demonstrated in Table 5.3. Although such table gives a preliminary prediction of the functional characterization of the EoSP cDNAs in comparison with well known characterized venom serine proteases, it can not be considered as a functional

confirmation or even a categorization strategy to differentiate between the four EoSP cDNAs. However, from Fig.5.5 and Table 5.3 it can be generally concluded that such comparison demonstrates that the enzymes encoded by the four EoSP cDNAs confer multiple haemostasis-disruptive activities to *E. ocellatus* venom. Furthermore, the sequence and predicted structural similarities of these four EoSP groups suggest that an antibody generated to one group may be capable of neutralizing the other group of EoSPs. To examine this permeability the sequences of EoSP groups were subjected to a more specific algorithm that predicted amino acid motifs of high immunogenicity.

A protein structure-predicting algorithm [Jameson and Wolf, 1988] has been used (i) to identify domains of strong antigenic potential in the toxin gene product and (ii) to determine whether these domains are conserved in analogous venom toxin gene products of related vipers. The signal peptide was separated from the mature protein by dotted line as would be cleaved post-translationally.

The peaks shown by the EoSPs profile indicate the numerous domains predicted to have a surface location and potential for antibody induction. Although the antigenic peaks of the catalytic traid of the EoSPs showed less similarity with that of the analogous venom SPs particularly those at residues 67 and 110, many antigenic residue similarities of EoPs are shared with other SVSPs of related vipers. Therefore, it is likely that antibodies raised by EoPS DNA immunisation are likely to possess considerable cross-reactivity and might competitively inhibit the function of these domains in the similar venom toxins of related vipers. However, binding of antibodies specific to conserved antigenic domains without a known function are equally as likely to disrupt protein function by virtue of steric

hindrance. The veracity of these speculations need to be confirmed experimentally and thus is a focus of our current research.

In conclusion the predicted Jameson-Wolf antigenic profiles (DNASTAR, USA) of the *EoSP* variants aligned with very low identity to their (BLAST) analogous serine proteases. This observation strongly suggests that an antibody raised by immunisation with group one *EoSP* DNA is likely to be less effective against the gene products of groups 2, 3 or 4. Therefore additional antibodies generated against antigenic index that showed less conservation will be required.

Chapter 6

Cloning and characterization of a novel cDNA encoding an Echis ocellatus prothrombin activator-like metalloproteinase

6.1. Introduction

Venoms from viperid and crotalid snakes exert broadly similar local and systemic effects. The literature indicates that the snake venom zinc-metalloproteinases (SVMPs) are arguably the most clinically significant group of viper toxins (Bjarnason and Fox, 1994; 1995; Gutierrez and Rucavado, 2000).

SVMPs have been shown to be a major cause of local pathology such as haemorrhage, oedema and ischaemia (Kamiguti et al., 1996; Laing et al., 2003). Haemorrhage represents an especially difficult problem because it develops quickly after venom injection causing extensive microvascular damage and blood loss (Borkow et al., 1993). In addition to haemorrhage, some viperine SVMPs have been shown to induce skeletal muscle damage and myonecrosis, which seems to be secondary to the ischaemia that ensues in muscle tissue as a consequence of bleeding and reduced perfusion (Gutierrez et al., 2000). Furthermore, SVMPs are also capable of initiating a cascade of pro-inflammatory responses indirectly via the cleavage of pro-tumour necrosis factor [TNF] to biologically active TNF (Van Dyk et al., 1997; Moura-da-Silva et al., 1996). SVMPs are therefore considered one of the major causative agents of permanent tissue loss following snakebite (Gutiérrez et al., 1995; Gutiérrez and Rucavado, 2000).

SVMPs, as reviewed in Chapter 1, are members of the reprolysin Zinc-metalloproteinases, a family of zinc-containing endopeptidases (Bjarnason and Fox, 1995) ranging from 20 to 100 kDa and form, together with the ADAM ('**A** **D**isintegrin **A**nd **M**etalloprotease') group, the subfamily of metzincins. They share a common overall structural organisation (Gutierrez and Rucavado, 2000; Omari-Satoh et al., 1995) but differ in substrate specificity, cellular sources

and inducibility (Moura-da-Silva et al., 1996). SVMPs are categorised into four groups dependent upon molecular mass and the presence of catalytic, disintegrin and cysteine-rich domains (Bjaranson and Fox, 1994) that exert distinct effects.

While SVMP DNA and peptide sequences have been reported for other *Echis* species, it was only during the course of this project that our group published the first report of the purification and functional characterization of *E. ocellatus* SVMPs (Howes et al., 2003); the main objective of this chapter was to screen the *E. ocellatus* venom gland cDNAs library to isolate cDNA encoding SVMP from this viper species. the intention was to utilize the E. ocellatus SVMP cDNA sequences to generate toxin-specific antibodies by DNA immunization. To achieve this objective I exploited the highly conserved nature of the nucleotide and amino acid sequences of viper SVMP propeptide and carboxyl domains (Paine et al., 1992a; 1994; Selistre de Araujo and Ownby, 1995) to PCR-amplify analogous sequences from a cDNA library constructed from the venom glands of adult *E. ocellatus*. Since (i) the propeptide domain is cleaved from the nascent zymogen (Kini and Evans 1992) and (ii) my objective is generate antibodies to neutralize protein function, the PCR experiments were designed to amplify cDNA sequences encoding the mature peptide and not the full length open reading frame.

6.2. Experimental design

The experimental strategy was the same as that described in Chapter 3.

6.3. Design of PCR primers

Screening of the cDNA libraries was performed using PCR (Israel, 1993). I exploited the extensive sequence similarity of the group III viper SVMPs (Bjarnason and Fox, 1994; Paine et al., 1994; Selistre de Araujo and Ownby, 1995) to design polymerase chain reaction (PCR) primers complimentary to highly conserved regions within the N-terminal propeptide (VEDHCY) and C-terminal (CVDVNTAY) domains. *E. ocellatus* (origin: Kaltango, Nigeria) venom gland cDNA was subjected to PCR [using program #4, chapter 2] with the 5' [**GGATCC-ATG**-GTT-GAG-GAT-CAC-TGC-TAT; incorporating a restriction endonuclease (*Bam*HI) site and a methionine start (ATG) codon, in bold, respectively] and 3' [**CTCGAG-TCA**-GTA-ATC-ATC-CAG-GTC-ATC-ACC-CCT; incorporating a restriction endonuclease (*Xho*I) site and a stop codons (TGA), in bold, respectively to facilitate future subcloning] primers which were synthesized commercially by Sigma-Genosys Ltd., U.K.

6.4. Results

6.4.1. Isolation of cDNA encoding E. ocellatus SVMP (EoMP)

A PCR product of the expected size about 1.5 Kb, was obtained using the SVMP- specific primers (Fig.6.1). The PCR amplicon was excised from the gel, purified from other components and sub-cloned into the TA cloning vector, pCR 2.1-TOPO, (Invitrogen) and the ligation mixture used to transform chemically competent *E. coli* (TOP10F), under ampicillin selection, following the manufacturer's instructions [as described in Chapter 2]. The ligation and transformation of the PCR product resulted in a good yield of recombinant colonies. On average, the overall ratio between the blue and the white colonies was 1:1. Plasmid DNA was extracted using a commercially available kit (Qiagen, Hilden, Germany) and restriction enzymes [*Bam* HI and *Xho* I] digestion was performed as a first step procedure to screen transformed white colonies prior to DNA sequencing. However, only eight of the sixteen colonies screened were confirmed to be positive and sent for DNA sequencing.

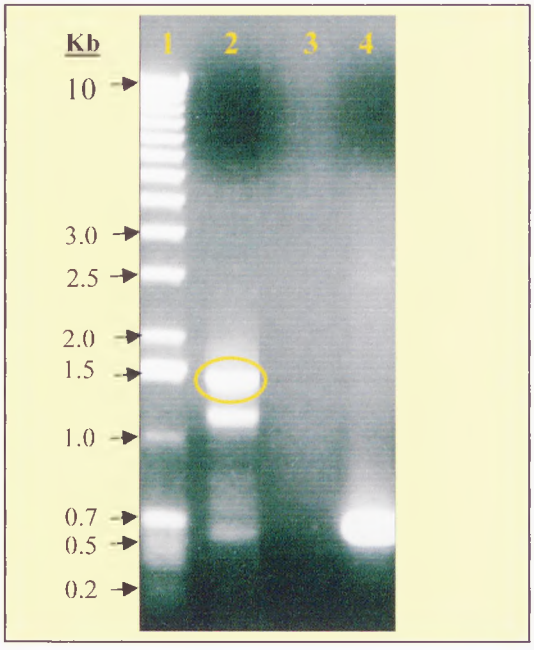
6.4.2. DNA sequencing

DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method [Sanger et.al., 1977] in a Beckman Coulter CEQTM 2000 XL DNA Analysis System.

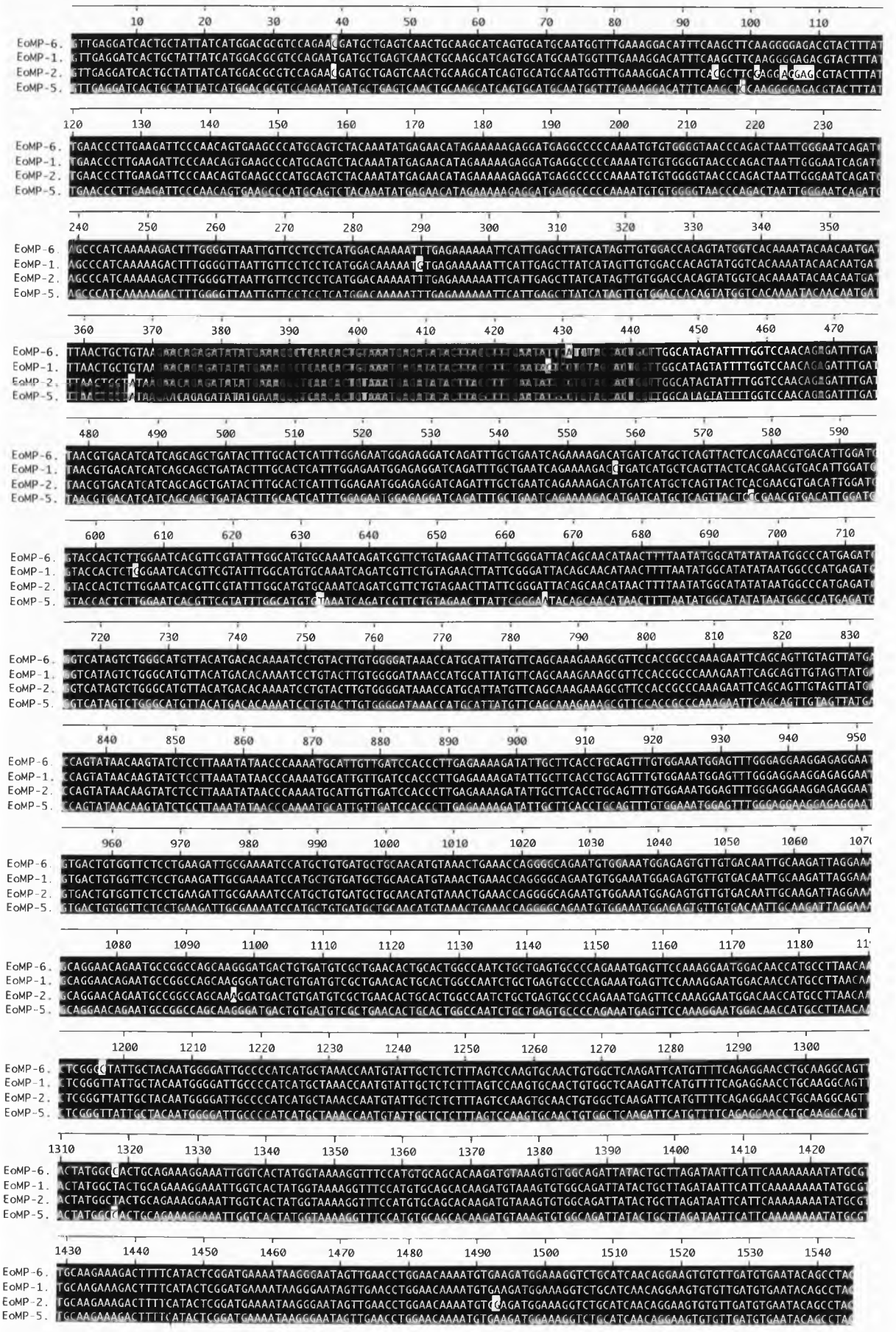
DNA sequencing resulted in four sequences that had an open reading frame of 1543 base pairs (Fig.6.2a) predicting proteins of 514 amino acid residues (Fig.6.2b). The molecular weight of each protein was calculated from their amino acid sequences and found to be of 57 kDa. The DNA sequences of all *EoMP* (termed *EoMP-1, EoMP-2, EoMP-5, EoMP-6*) clones

were virtually identical (98% sequence similarity) (Fig.6.2a and Fig.6.2b). Therefore, the sequence data of EoMP-06 was chosen for further analysis.

Fig.6.1. PCR product of the *E. ocellatus* MP.



Analysis of PCR amplification products by 0.7% agarose gel electrophoresis. Bands were visualised using the ultraviolet trans-illumination. The amplified PCR product (1550bp, the upper band) from *E. ocellatus* venom glands cDNA shown in Lane 2 was compared with marker bands of known molecular weight (Lane 1). Lane 3 represents a negative control where H_2O, instead of DNA template; Lane 4, demonstrate the use of the positive control, SOD DNA, template (10ng) (Appendix A).


EoMP-6.
EoMP-1.
EoMP-2.
EoMP-5.

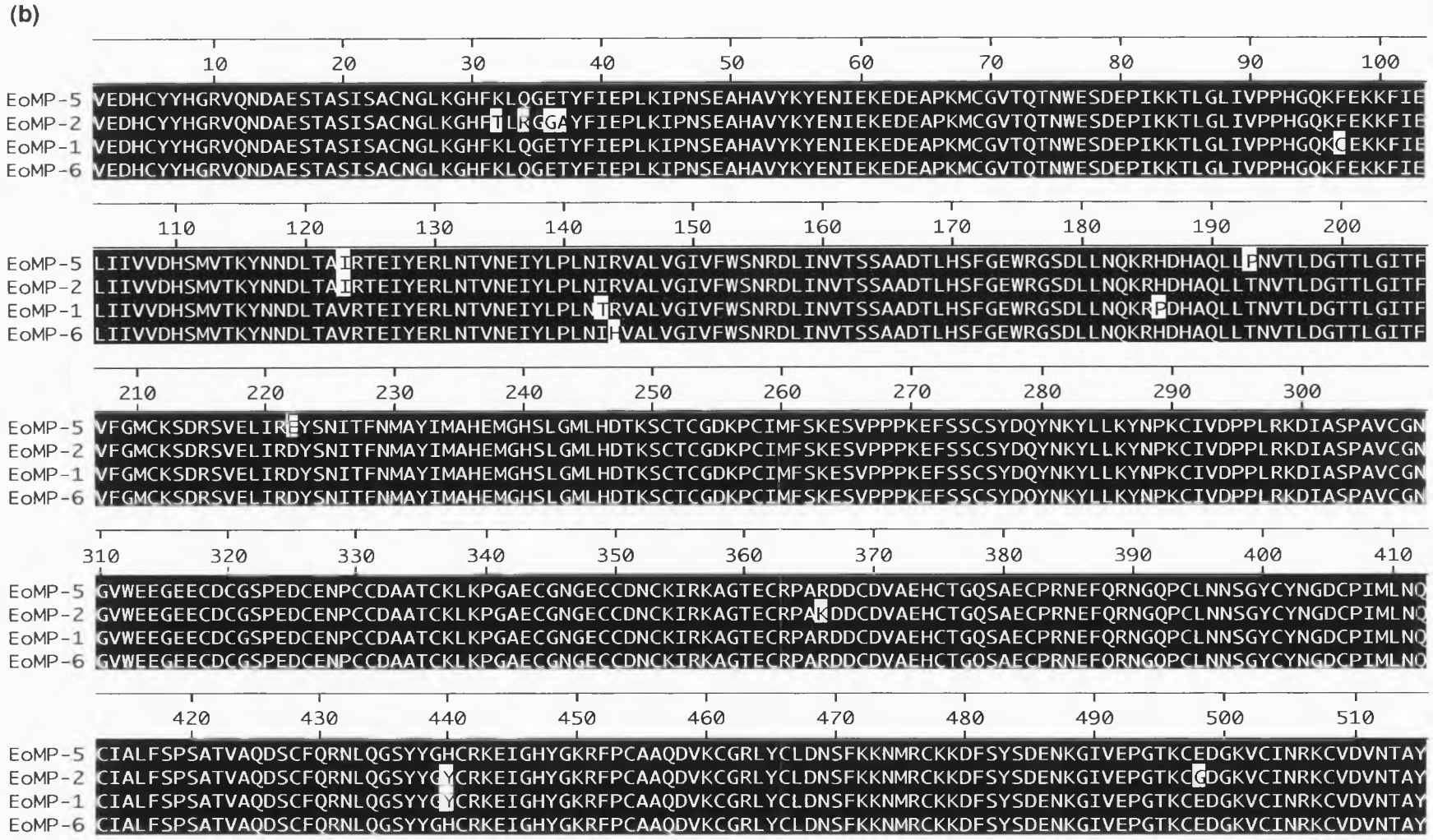


Fig.6.2. The nucleotide (a) and deduced amino acid sequence (b) of cDNAs encoding *E. ocelltus* SVMPs. *EoMP-6*. Residues shaded in black are identical to the consensus sequences.

6.4.3. Blast Search

The deduced amino acid sequence of *EoMP-6* [GenBank accession number: AY261531] was subjected to BLAST (Altschul et al., 1997) searches of the genetic databases. These searches revealed the deduced amino acid sequence of *EoMP-6* to be 91% and 59% similar to the prothrombin-activating SVMPs of *E. pyramidum leakeyi* [Ecarin, A55796 (Nishida et al., 1995)] and *Bothrops erythromelas* [Berythractivase, AAL47169 (Silva et al., 2003)] respectively. *EoMP-6* also showed high levels of sequence similarity to haemorrhagic SVMPs from *Bothrops jararaca* [jararhagin, 59%, P30431 (Paine et al., 1992)], *Agkistrodon contortrix laticinctus* [Aclh, 58%, AAL47169 (Selistre de Araujo and Ownby, 1995)], *Crotalus atrox* [Catrocollastatin, 59%, S55270 (Zhou et al., 1995)], *Protobothrops flavoviridis* [Hr1a, 58%, BAB92013 (Kishimoto and Takahashi, 2002)] and *E. pyramidum leakeyi* [Ech-I, 54%, S48160 and Ech-II, 56%, S48169, (Paine et al., 1994)]. The inferred amino acid sequence of *EoMP-6* was aligned to the above sequences using the CLUSTAL W program (Thompson et al., 1994) with PAM 250 residue weight matrix (Fig.6.3) and the perecent sequence similarity described in Table 5.1.

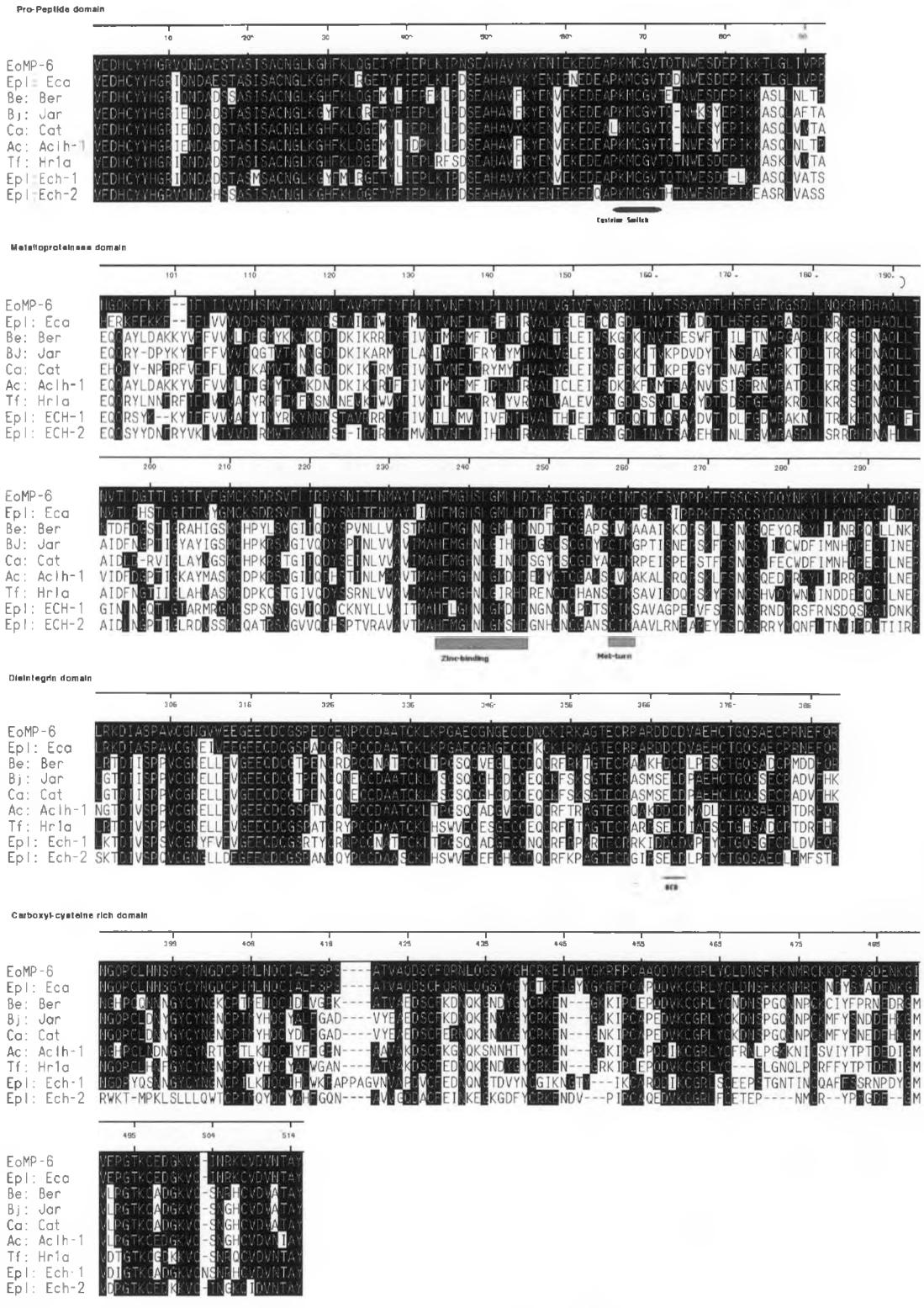


Fig.6.3. Alignment of the predicted amino acid sequence of *EoMP-6* with SVMPs of related vipers. The alignment is divided into the four structure/function domains of SVMPs. Epl:Eca - Ecarin from *E.p. leakeyi* venom; Be:Ber - Berythractivase from *B. erythromelas* venom; Bj:jar - Jararhagin from *B. jararaca* venom; Ca:cat - Catrocollastatin from *C. atrox* venom; Ac:Aclh - SVMP from *A. contortrix laticinctus* venom; Tf:Hrla - SVMP from venom of *P. flavoviridis* (previously termed *Trimesurus flavoviridis*); Epl:Ech-1 and Epl:Ech-2 - SVMPs from *E.p. leakeyi* venom. The numbeers indicate the amino acid sequence of EoMP-6. Gaps have been inserted to optimise the sequence alignment. Residues identical to *EoMP-6* are shaded in black. Motifs of structural and catalytic significance are indicated.

6.4.4. Sequence alignment of the EoMP-6 with Group III SVMP from related viper species

As illustrated in (Fig.6.3), *EoMP-6* has the same modular organization as the class III SVMPs (Kini and Evans 1992, Silva et al., 2003) with the expression of pro-peptide, metalloproteinase, disintegrin-like and cysteine-rich domains.

The *EoMP-6* contains a typically highly conserved pro-peptide domain with a cysteine switch motif (PKMCGVT) located at position 66-72 in the carboxyl-terminal portions of the pro-peptide domain (Fig.6.3).

The *EoMP-6* zinc chelating motif (236-248) was identical to that of the *E.p.leakeyi* prothrombin-activator, Ecarin. Both sequences expressed substitution of S for N^{241} and L for H^{245}. The functional significant of these substitution is not known.

In common with the other viper SVMPs shown here (Fig.6.3), the *EoMP-6* sequence also encoded a DCD motif located at 368-371 instead of the RGD sequence typical of the integrin-binding domain of the true snake venom disintegrins. Some of the viper SVMP encoded a ECD in this motif. the functional implication of these E or D substitutions is not known.

As shown in Fig.6.3, a significant number of conservative cysteine residues were observed. *Eo-MP-6* encoded the 36 cysteine residues that form 18 disulphide bridges.

Table 6.1. *% of amino acid sequence similarity of EoMP-6 structural domains with analogous SVMP molecules from related vipers*

	EoMP-6			
Viper species	**Pro-peptide**	**Metalloprotienase domain**	**Disintegrin domain**	**Carboxyl-cysteine rich**
Epl: Eca	95	86	94	94
Be: Ber	82	47	64	62
Bj: Jar	80	48	63	63
Ca: Cat	82	48	63	62
Ac: Aclh-1	81	49	67	55
pf: Hrla	83	48	63	57
Epl:ECH-1	80	46	63	45
Epl: ECH-2	86	51	60	42

Key: **Epl:Eca**=*E. pyramidum leakeyi*: Ecarin. **Be:Ber**=*Bothrops erythromelas*: Berythractivase. **Bj:Jar**=*Bothrops jararaca:* jararhagin. **Ca:Cat**: *Agkistrodon contortrix laticinctus:* Aclh. **Ac:Aclh-1**=*Crotalus atrox:* Catrocollastatin. **pf:Hrla=** *Protobothrops flavoviridis:* Hr1a, **Epl:ECH-1 & EplL ECH-2**=*E. pyramidum leakeyi:* Ech-I, Ech-II.

6.4.5. Antigenic profile of EoMP06 with analogues MP molecule in related viper species

The overall research objective is to develop a rational immunotherapy for snake bite by generating toxin-specific antibodies with potential to react and neutralize the activity of analogous molecules in the venom of other *Echis* species, using DNA immunization technology (Harrison et al., 2000; 2002),.

The antigenic index as illustrated in Fig.6.4, (Jameson and Wolf, 1988) was constructed to guide the design of the DNA immunization constructs and to evaluate the potential inter-specific and inter-generic immunoreactivity of each toxin-specific antibody (thereby estimating the geographic limits of their potential clinical usefulness).

The peaks in the *EoMP-6* profile (Fig.6.4) indicate the numerous domains predicted to have a surface location and potential for antibody induction. The thin vertical lines (A- M) illustrate that many of the antigenic residues of *EoMP-6* are shared by SVMPs of related vipers and that antibodies raised by *EoMP-6* DNA immunisation are likely to possess considerable cross-reactivity. As expected, the antigenic profile of *EoMP-6* was very similar to that of Ecarin and, to a lesser extent, to Ech-I and Ech-II from *E. p. leakeyi* venom. The latter viper is a phylogenetically close relative of *E. ocellatus* (Lenk et al., 2001) with an East African distribution. Line D corresponded to the catalytic site (position 234 –246, Fig.6.4) and appeared to be highly immunogenic in all the SVMPs. Line H corresponded to the DCD motif in the disintegrin-like domain (position 368-370, Fig.6.3).

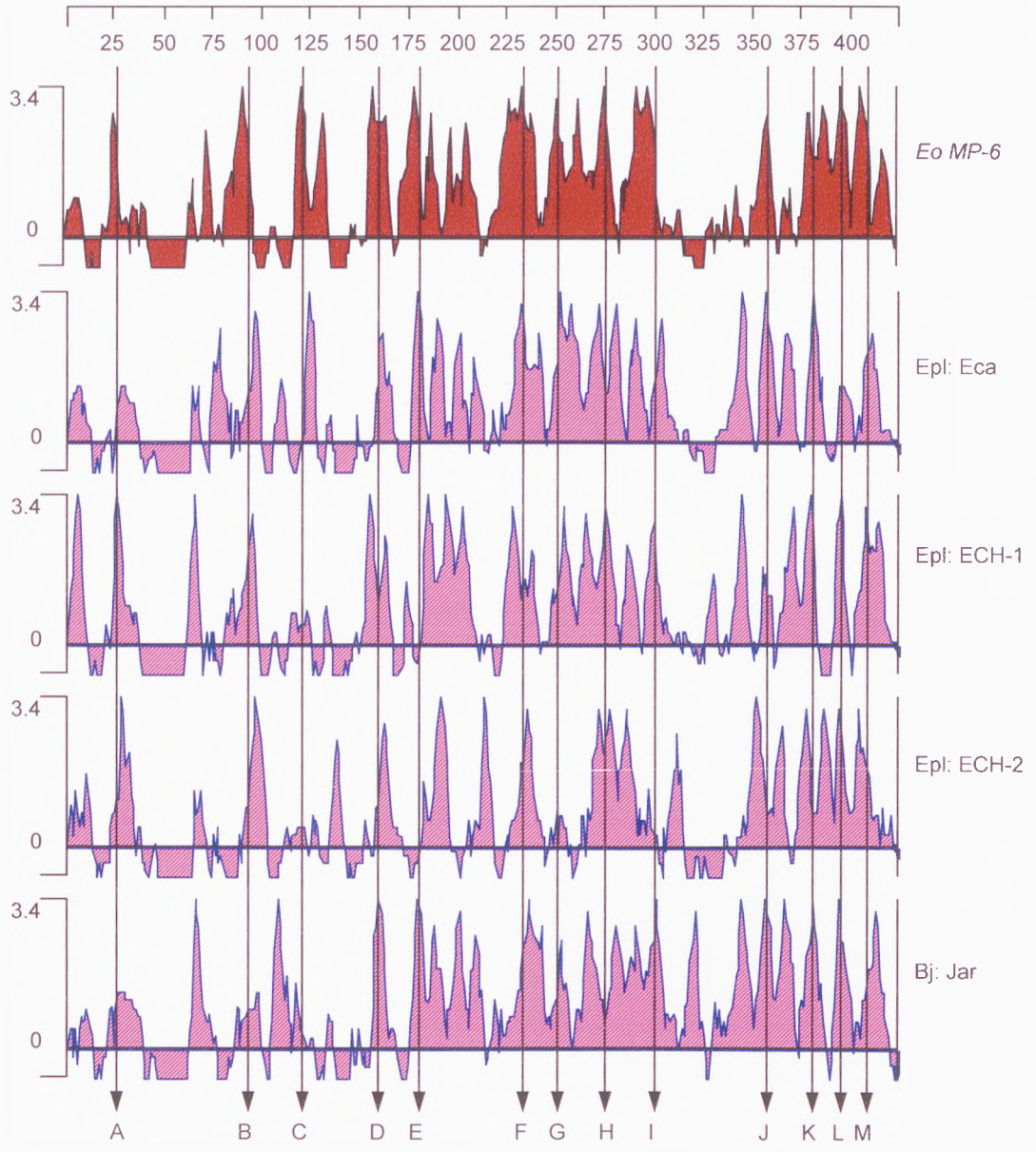


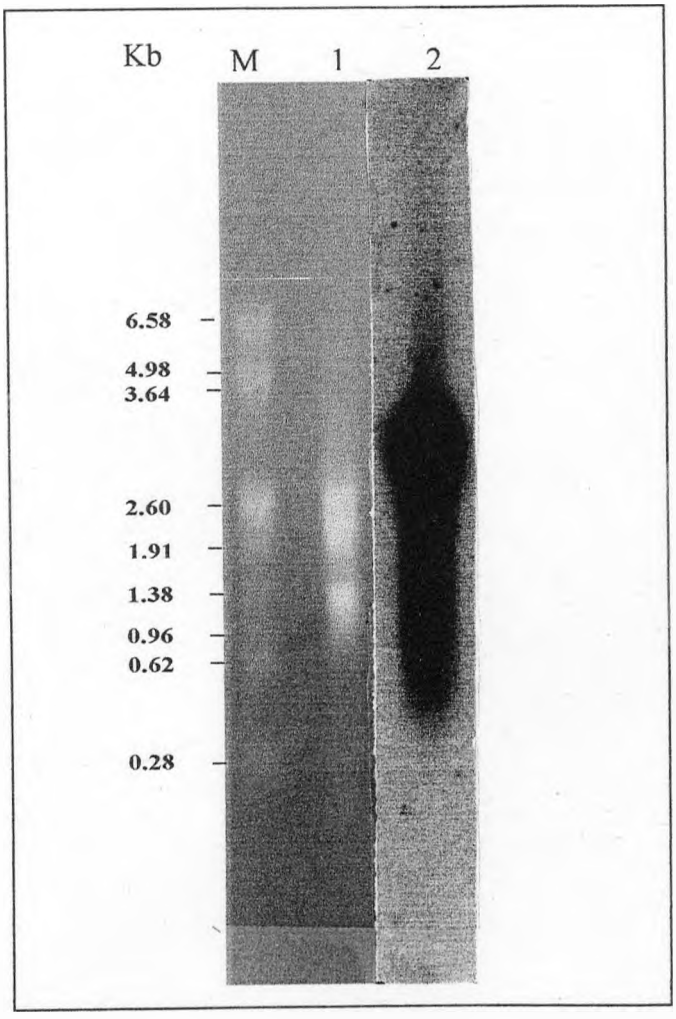
Fig.6.4. Jameson and Wolf antigenic profiles of *EoMP-6* and SVMPs from related vipers. The top horizontal scale represents the number of amino acid residues of the *EoMP-6* sequence. The vertical scales represent comparative antigenic values. The thin vertical lines (A-M) are a subjective assignation of antigenic domains that exhibit the greatest phylogenetic conservation. Lines D and H correspond to the catalytic zinc binding and DCD motifs, respectively.

6.4.6. *Northern blot analysis*

Northern blot analysis was performed to determine the size and number of EoMP transcripts using total RNA extracted from the *E. ocellatus* venom gland. The *EoMP*-6 DNA was chosen as a representative clone to probe the venom gland total RNA on northern blots. The result as shown in (Fig.6.5) revealed a broad band from 0.6 kb to 3.5 kb.

Fig.6.5. ***Northern blot analysis of E. ocellatus MP-06.***

The *EoMP-6* cDNA was labelled with ^{32}P isotope to probe *E. ocellatus* venom gland total RNA (10μg) northern blot; M; RNA size marker (0.28- to 6.58kb); Lane 1, formaldehyde-agarose gel electrophoresis of *E.ocellatus* venom gland fractionated total RNA; lane 2, autoradiograph of the hybridized northern blot membrane.



6.5. Discussion

The utilisation of PCR amplification of *E. ocellatus* venom gland cDNA with SVMP-specific primers was successful in amplifying four *E. ocellatus* cDNA sequences encoding SVMP molecules of about 1.5 Kb that showed over 98% nucleotide sequence similarity (Fig.6.2a).

All the isolated *EoMP* sequences described here have the same modular organization as the class P-III SVMPs from related vipers with a typically highly conserved pro-peptide domain with a cysteine switch motif (PKMCGVT) located at position 66-72 in the carboxyl-terminal portions of the pro-peptide domain (Fig.6.3). The cysteine switch motif in this domain is thought to prevent proteolytic activity of the zymogen (Silva et al., 2003; Siigur et al., 1996). In the latent form of the zymogen, a free thiol group in this sequence is thought to coordinate the catalytic zinc ion at the active site to prevent proteolytic cleavage of the zymogen (Silva et al., 2003; Siigur et al., 1996).

The propeptide domain of *EoMP-6* is shorter (94 residues) than that of the other SVMPs (data not shown) as a result of the design of the PCR primers. Consequently, the amino acid sequence number [e.g., of functionality important domains] discussed in this chapter are different from those used in the literature on full-length SVMPs.

The zinc-chelating consensus sequence **X-His-Glu-X-X-His-X-X-Glu-X-X-His** in the metalloprotease domain (Bode et al., 1992; Gomis-Rüth et al., 1993) of SVMPs and the Astacins (Nishida et al., 1995) is the active site responsible for the haemorrhagic activity of these proteases (Bjarnason and Fox, 1994) toward different target substrates (Kini and Evans, 1992; Moura-da-Silva et al., 1996; Sugita, 1999). The zinc chelating motif (236-248) of *EoMP-6* was very similar to that of the other SVMPs (Fig.6.3) and identical to that of the *E.*

p. leakeyi prothrombin activator, Ecarin. Both sequences showed a typical substitution of S for N^{241} and L for H^{245}.

EoMP-6 encoded the consereved (**C-I/V-M**) sequences at position 258-260 as illustrated in Fig.6.3. This motif is thought to promote hydrophobic interactions within the protein structure thereby ensuring the structural integrity of the zinc-binding site (Selistre de Araujo and Ownby, 1995).

In common with the other viper prothrombin activator, Ecarin and Berythractivase, and in contrast to most of the haemorrhagic SVMPs, the *EoMP-6* sequence included three N-linked glycosylation sites **N-X-S/T** at position N^{160}, N^{194} and N^{225}, respectively (Fig.6.3). To my knowledge this is the first time that this observation has been made and suggests that while the functional significance of the N-glycosylation sites (NXS/T) remains unclear, it suggests a sequence motif to distinguish between prothrombin activator as SVMPs from the other, highly sequence-analogous SVMPs of distinct function.

The *EoMP-6* sequence also encoded the carboxy-terminal disintegrin-like domain. In common with other SVMPs (Fig.6.3), *EoMP-6* encoded a DCD motif at 368-371 instead of the RGD sequence typical of the integrin-binding domain of the true snake venom disintegrins. Furthermore, *EoMP-6* shows a cysteine-rich carboxyl extension of the disintegrin domain. Such an inter-link extension may reflect that both domains function as a single contiguous unit with an adhesive role. This domain is characterised by the presence of a high percentage of cysteine residues displayed in a distinctive pattern (Jia et al., 1996). This group (Jia et al., 2000) expressed the cysteine-rich domain of Atrolysin A (a haemorrhagic SVMP from the venom of *C. atrox*) and demonstrated its ability to inhibit collagen-induced

platelet aggregation. It also inhibited the adhesion of MG-63 cells to collagen type I. These findings suggest a synergistic effect of platelet targeting and haemorrhagic effect by SVMPs (Jia et al., 2000). As shown in Fig.6.3, a significant number of conservative cysteine residues were observed. *Eo-MP-6* encoded the 36 cysteine residues that form 18 disulphide bonds which may be responsible for their protein structural and biological characterisation as well as the cleavage of the putative domains (Calvete et al., 1992; Kini and Evans 1981).

Despite the conserved regions within the pro-peptide and the functional domains, there are two interesting points to outline. The first is the high variability found in the amino acid regions that encode the mature protein of *EoMP*-6. The same pattern of variability also seems to be operating in related venom molecules from other snake species as illustrated in Table 6.1. Such a high variability within this corresponding domain may reflect one of the features of selection during the evolution of these snakes. Thus, the amino acid variability within the catalytic domain may provide the snake with a device of new enzymatic activities with which the spectrum of the venom's action will be increased by its variable interaction with different but related substrates. The second point is that despite this sequence variability, all viper SVMPs retain the consereved cystein scaffold that ensures the protein retain a similar tertiary structure.

In the disintegrin domain, it is unclear whether the D for E substitution in *EoMP-6* has functional significance. The disintegrin-binding RGD motif is frequently substituted with E/DCD in many snake venom metalloproteinases such as jararhagin (Paine et al., 1992), ecarin (Nishida et al., 1995), HR1a (Kishimoto, and Takahashi, 2002), Ca: Cat (Zhou et al., 1995), ECH-I and ECH-II (Paine et al., 1995) as illustrated in (Fig.6.3). However, it may play

a role in the adhesion of venom metalloproteases to target molecules, possibly to integrins that are located on the cell surface membrane of the vascular endothelium (Paine et al., 1994). Evidence for this was strengthened by the discovery of non-RGD motif in the disintegrin domain of mammalian sperm proteins including PH30-α and PH30-β, which bind to the integrins located on the egg surface during fertilization (Kratzschmar et al., 1996, Blobel et al., 1992). Furthermore, on closer observation within the disintegrin domain a high degree of amino acid sequence similarity was observed, as illustrated in (Table 6.1), in comparison with the high sequence variability found within the catalytic domain. However, the functional significance of this sequence similarity is not clear, as the removal of segments from the amino terminal portion of this domain does not significantly affect integrin inhibitory activity (Kini and Evans, 1992). cDNA sequences indicate that some venom metalloproteinases such as that of classes P-II and P-III are synthesised containing metalloproteinase and disintegrin-like domains and they are post-translationally processed to remove the latter domain (as in the case of Atrolysin E from Western diamondback rattlesnake [*C. atrox*] venom and the haemorrhagic factor HR2a from Habu *Trimeresurus flavoviridis* venom (Fox and Bjarnason, 1998). Such processing may be autocatalytic or may be performed by other proteinases (Gutiérrez et al., 2000).

It can be seen clearly that obtaining sequence similarities of each structural domain [as illustrated in Table 6.1] promotes the prediction of structure/function relationships between the *EoMP-6* and its analogous molecules. It also provided a satisfactory differentiation between two related proteins based on functionally important domains. This is explained further in the following paragraph.

There are several types of prothrombin activators in snake venom, which have been divided

into four groups based on their structural properties and their mechanism of prothrombin activation (Kini, 2001). Although the amino acid sequence similarity and the structural organization between *EoMP-6* and its analogous proteins were significant, without functional assays it is difficult to predict the functional characteristic of *EoMP-6.* For instance, the berythractivase showed a 70% sequence similarity to jararhagin, however, their biological actions were considerably different. Silva et al., (2003), demonstrated that although berythractivase was capable of modulating the endothelial cell, it has a very low fibrinolytic-activity in degrading the α-chain of fibrinogen, and it does not contribute to the synthesis of vWF (vonWillebarnd factor).

To guide the design of the DNA immunization constructs and to evaluate the potential inter-specific and inter-generic immunoreactivity of each toxin-specific antibody (thereby estimating the geographic limits of their potential clinical usefulness), I have used a protein structure-predicting algorithm (Jameson and Wolf, 1988) to (i) identify domains of strong antigenic potential in the toxin gene product and (ii) determine whether these domains are conserved in analogous venom toxin gene products of related vipers. Amino acid sequences differ only slightly does not however have a negative impact on the achievement of the main objective-the development of toxin-neutralizing antibodies by DNA immunisation. The deduced primary structures of EoMPs include the requisite, highly conserved, 36 cysteine residues that form the 18 disulphide bonds responsible for the characteristic tertiary structure of class III SVMP. It is therefore predicted that transfection of mammalian cells with encoding-EoMP DNA is likely to present to the immune system molecules that faithfully represent native SVMP and strongly suggests that an antibody raised to any of these domains may have the potential for neutralizing SVMPs from other viper species, which are

responsible for the acute medical problems in many areas of rural Africa.

It is tempting to predict that antibodies generated against clinically significant domains in *EoMP-6* might competitively inhibit the function of these domains in venom toxins of related vipers. However, binding of antibodies specific to conserved antigenic domains without a known function (Fig.6.4; Lines A-M, except D and H) are equally as likely to disrupt protein function by virtue of steric hindrance. The veracity of these speculations need to be confirmed experimentally; a focus of my future activity. Furthermore, the truncated peptide sequence (KTLDSFGEWR) derived from a purified haemorrhagic fraction of *E. ocellatus* venom (Howes et al., 2003) differed from that *EoMP-6* (**TLHSFGEWR**, Fig.6.3). Together, these studies suggest a multimeric composition of *E. ocellatus* SVMPs and indicate the need for a more global analysis of the *E. ocellatus* venom gland transcriptome.

The northern blot analysis (Fig. 6.5) revealed a broad transcript band from 0.6 kb to 3.5 kb. This strongly suggests the existence of numerous EoMP transcripts varying in size from 0.6 to 3.5 Kb. Therefore the *EoMP-6* could be transcribed from such numerous transcript containing flanking regions, which may be cleaved by a post-translational processing. Moreover, this also agrees with the literature of group I-IV SVMPs and my own PCR result (Fig.6.1).

Chapter 7

Immunisation with cDNA encoding haemostasis-disruption E. ocellatus venom toxins

7.1. Introduction

Echis envenoming causes massive haemorrhagic and necrotic morbidity in the short term and often in the long term, also permanent disfigurement and renal dysfunction. The only effective treatment is the administration of antivenom. Antivenom production for sub-Saharan Africa has declined to dangerous low levels, thus exacerbating the already severe medical problem that envenoming poses to rural, financially-disadvantaged communities of the tropics that have limited access to frequently inadequate health resources (Lalloo and Theakston, 2003).

Although life-saving, conventional antivenoms suffer from deficiencies imposed by the mode of preparation. Currently, antivenoms are prepared by purifying the sera of large animals, usually horses, immunised with either an individual venom or a range of venoms. Since venoms contain numerous molecules, only some of which are toxic, antivenoms raised against these consist of numerous antibodies with no therapeutic function. Furthermore, because the toxicity of a venom molecule is unrelated to its immunogenic potential, the most potent antibodies in antivenoms are not necessarily targeted to the most pathogenic molecules. The objective of the work described in this chapter is to generate a bank of murine polyclonal antibodies specific to the most haemostasis-disruptive molecules in the venom of *E. ocellatus* using the new cDNA sequences [as described in earlier chapters] as immunizing material.

Harrison et al., (2000b) demonstrated for the first time that DNA immunisation with JD9, the carboxyl-disintegrin and cysteine-rich domain of single venom metalloprotease (jararhagin) gene, can be utilized to generate high titre murine IgG responses capable of

inhibiting venom-induced haemorrhagic activity caused by the Brazilian pit viper, *B. jararaca*. Harrison et al., (2003) have since shown that antibody from the JD9 DNA-immunised mice reacted immunologically with molecules of the same molecular weight in (i) the homologous venom and (ii) the venoms of snakes of diverse species and genera. The propose of this research chapter was to use the above strategy to generate antibodies to neutralize envenoming by *E. ocellatus*.

The cDNAs encoding *E. ocellatus* toxins used for DNA immunisation

The major part of the work described in this thesis was spent isolating and characterizing novel cDNAs encoding *E. ocellatus* PLA_2, CTL, SVMP and serine protease molecules. The rest of the time, was spent manipulating cDNAs encoding the latter three constructs to immunize mice. While mice immunised with SVMP DNA raised satisfactory antibodies, unfortunately the mice immunised with the CTL and serine protease DNA constructs failed to seroconvert. Consequently, this chapter describes the methodology and results of SVMP DNA immunisation. The techniques used for the latter were identical for those used in preparing the CTL and serine protease DNA immunisation constructs.

I utilized DNA encoding the carboxyl-disintegrin and cysteine-rich domain (EoDC-2) of *EoMP-6*, a prothombin activator-like metalloproteinase in the venom of *E. ocellatus* for DNA immunisation.

EoDC-2 of the *EoMP-6* possesses, like the JD9 DNA immunisation construct, a 'DCD' collagen receptor binding motif in the disintegrin domain and the highly conserved cysteine scaffold present in the cysteine-rich domain. The rationale of using the DC

domains as opposed to the whole molecule was that expression of EoDC-2 in mammalian cells was considered to be less injurious to the host than expression of the whole molecule containing the catalytic metalloprotease domain. Secondly it was thought that, antibody bound to the DC domain as in the case of JD9 (Harrison et al., 2000), may prevent substrate binding (Kamiguti et al., 2004) and/or catalytic function (Jeon et al., 1999) of the whole molecules, thus achieving the objective.

7.2. Experimental strategy

The experimental design strategies of this research chapter was as follows:

(i) To prepare the EoDC-2 DNA immunisation construct (Fig. 7.1).

(ii) To determine the using ELISA seroconversion efficiency of EoDC-2 delivered intradermally (ID) into mice or by Gene Gun (GG) delivery or intramuscularly (IM).

(iii) To assess the cross reactivity of antibody raised by EoDC-2 immunisation to analogous molecules in venoms of other *Echis* species by immunoblotting.

(iv) To analyse the venom neutralizing efficacy of antibody raised by EoDc-2 immunisation.

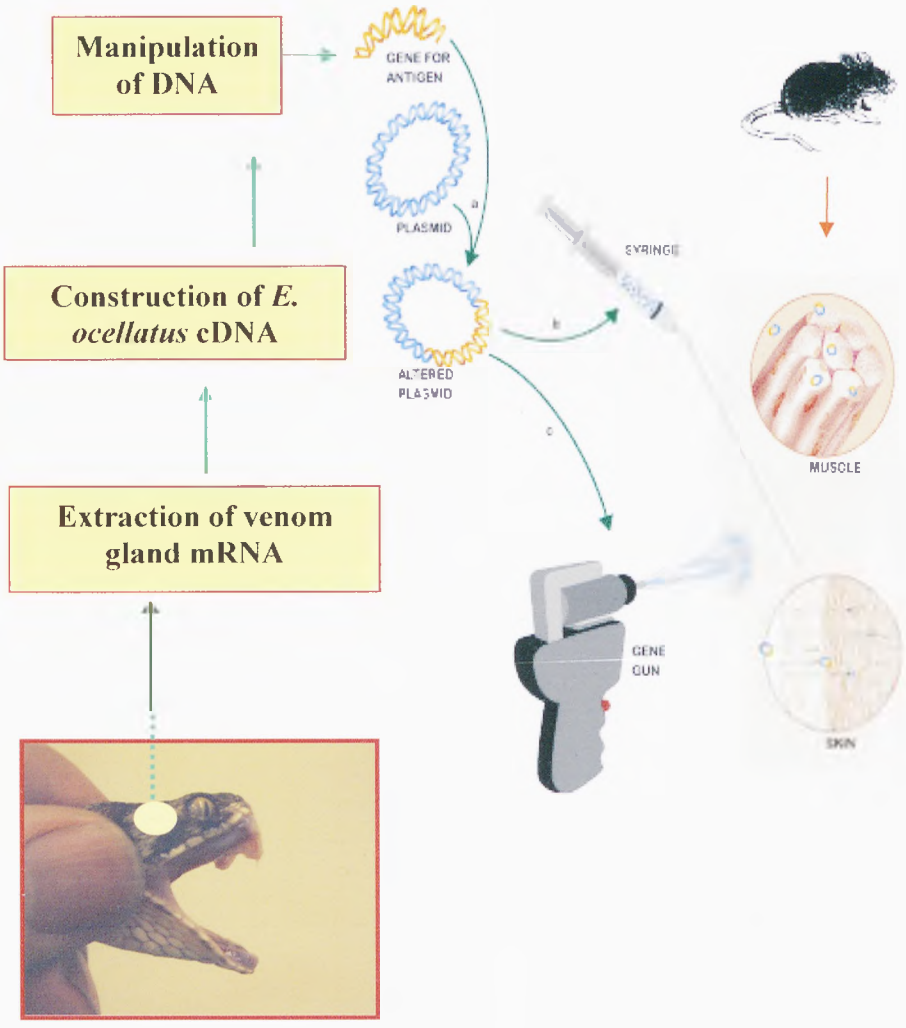
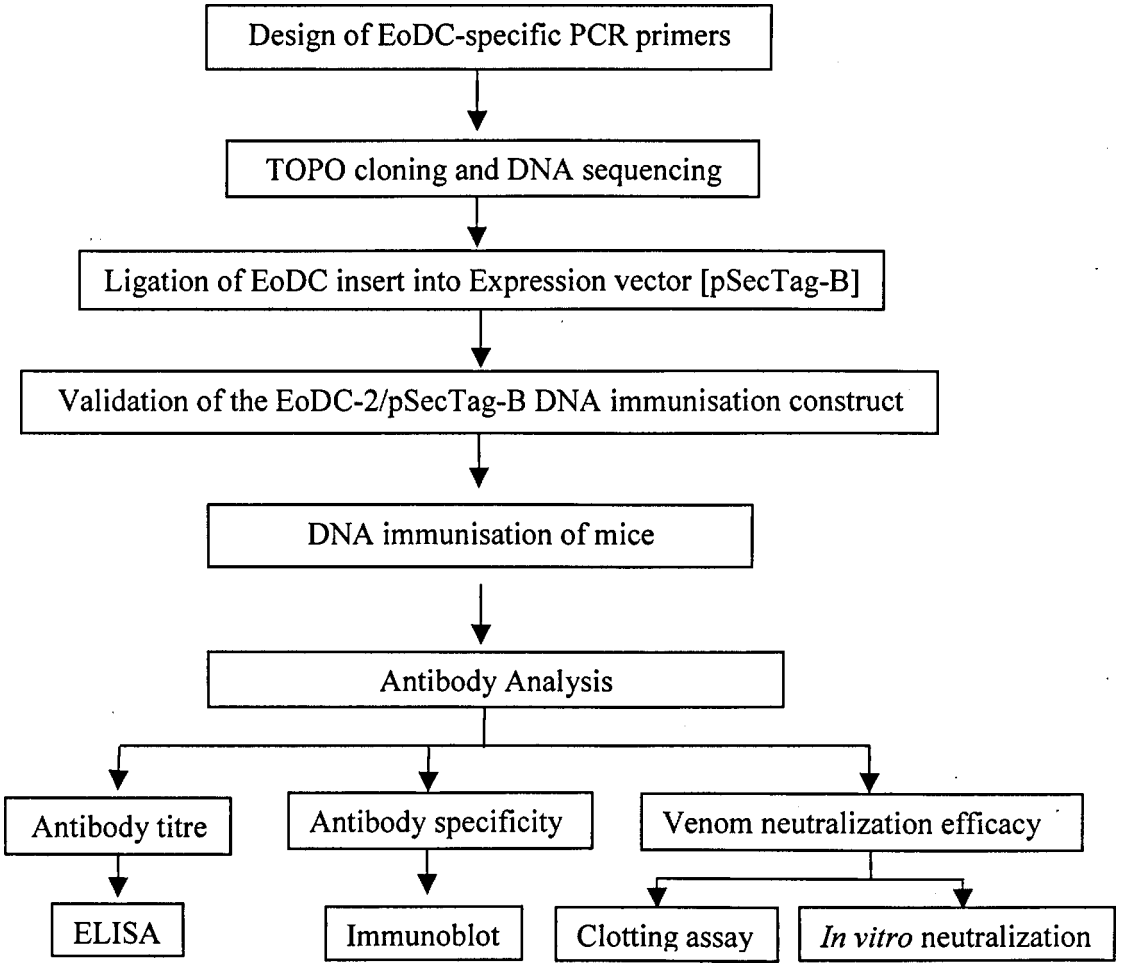


Fig.7.1. Schematic diagram of DNA immunisation showing different routes of DNA delivery used in this research chapter. *Diagram adapted from R. Harrison (personal communication).*

The following experimental procedures were used to achieve the objectives.



7.3. Materials and methods

7.3.1. Isolation and analysis of disintegrin and cysteine-rich (DC) domains from EoMP-6 clone.

The DC domain of *EoMP-6* encoding a novel *E. ocellatus* prothrombin activator (Hasson et al., 2003) was PCR amplified using primers complementary to nucleotides 890 – 912 (5' primer) and nucleotides 1523 - 1545 (3'primer) [Chapter 6] as demonstrated in (Fig.7.2). The amplicon was sub-cloned into the TA cloning vector (pCR2.1-TOPO; Invitrogen, Groningen, The Netherlands) and used to transform chemically competent *E.coli* cells (TOP10F', Invitrogen) under ampicillin selection. Plasmid DNA was extracted (Mini-spin prep kit, Qiagen, Hilden, Germany) and digested with *Bam*H1 and *Xho*1 at 37°C to select plasmids containing inserts of the predicted size for DNA sequencing.

DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method in a Beckman Coulter CEQ™2000 XL DNA Analysis System. Only one clone showing an open reading frame (ORF) identical to the DC domain of *EoMP-6* was selected. The EoDC2/TOPO clone was digested with *Bam*HI and *Xhol* and the EoDC-2 insert was electrophoretically isolated from TOPO [as described in Chapter 2] and ligated into the mammalian expression vector pSecTag-B [Invitrogen, Netherlands] as described in the following section.

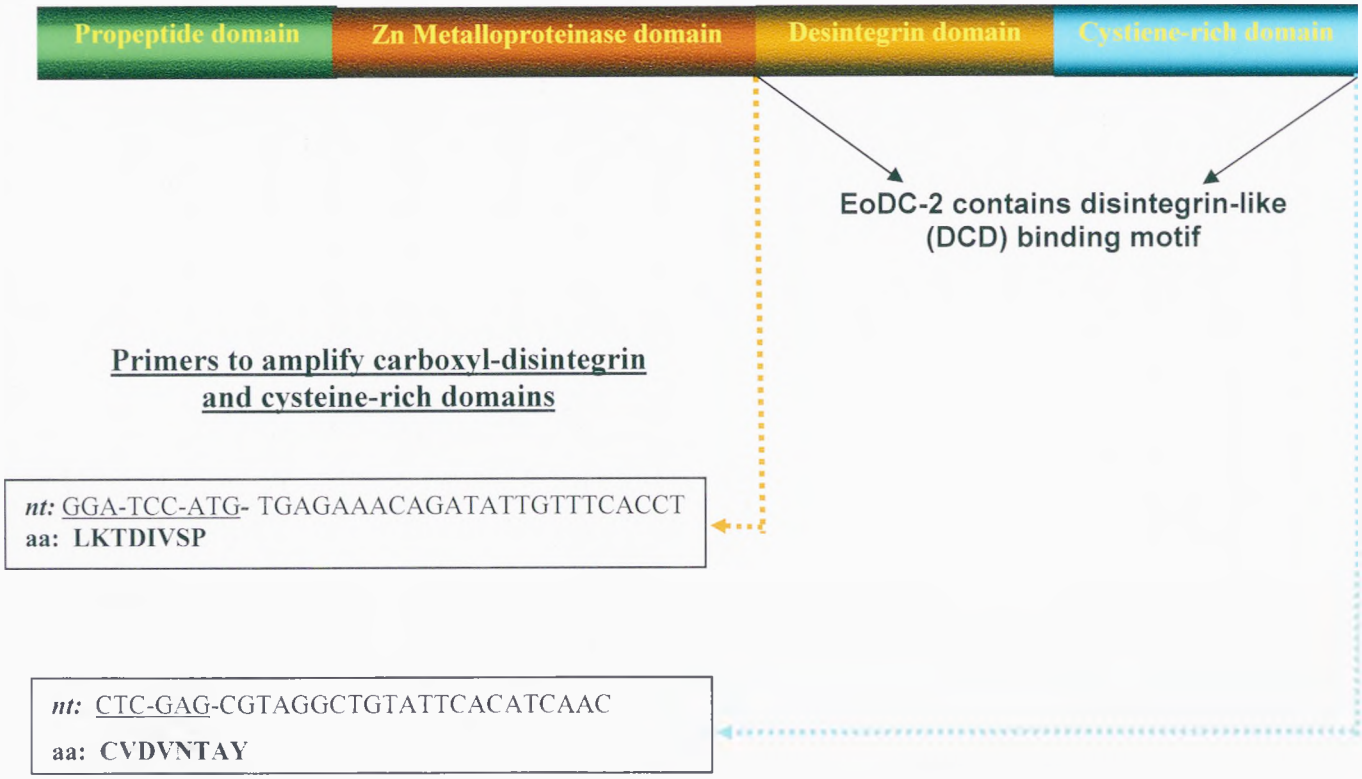


Fig.7.2. Schematic diagram demonstrating PCR primers design. Underlined nucleotides represent restriction digest sites of *BamHI* and *Xho*l respectively.

7.3.2. Plasmid construction and clone isolation

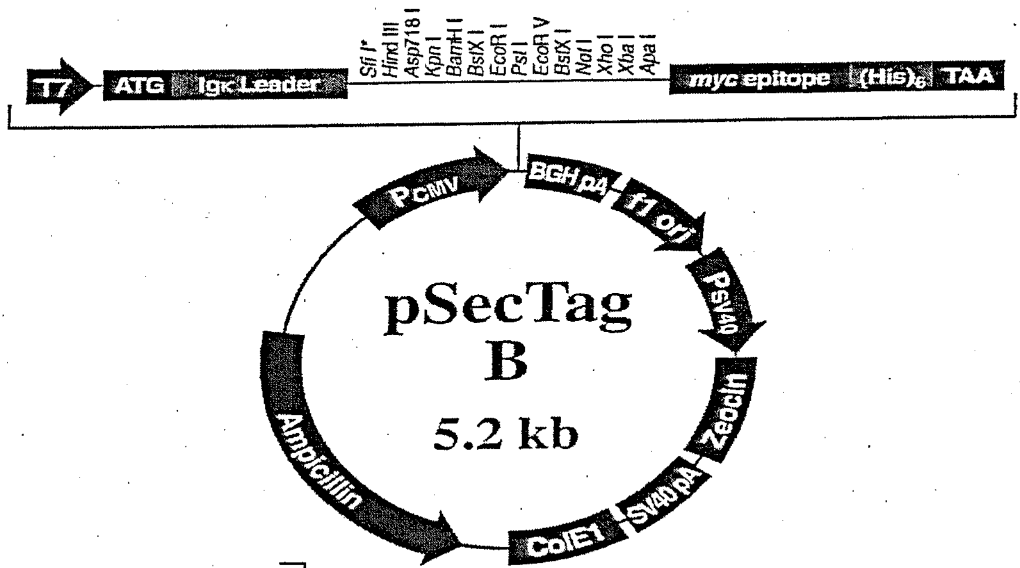
7.3.2.1. Description of the pSecTag-B DNA immunisation plasmid

The mammalian expression vector pSecTag-B (Invitrogen, Netherlands) used in this study has all the required components for successful protein expression from DNA [see Chapter 1]. The map for pSecTag-B is demonstrated in Fig.7.3. The expressed protein is under the control of the human cytomegalovirus (CMV) immediate-early promoter/enhancer that provides efficient, high-level expression of recombinant protein (Boshart et al., 1985); this has been utilised in numerous DNA vaccination studies (Donnelly et al., 1997). The up-stream murine immunoglobulin k-chain leader sequence favours secretion of the fusion protein from the transfected cell (Invitogen, Netherlands). The down-stream bovine growth hormone polyadenylation signal promotes efficient transcription termination and polyadenylation of fusion protein mRNA, thereby increasing the stability of the mRNA and reducing the chance of premature mRNA degradation (Invitrogen, Netherlands).

7.3.2.2. Ligation reaction of EoDC-2 into pSecTag-B

The amplified (EoDC-2) product from section 7.3.1 was ligated as illustrated in Table 7.1. The required quantity of the purified PCR product required for the ligation reaction was determined by the following equation which illustrates the conversion of molar ratio to mass ratios for both pSecTag-B=5.2kb plasmid and D-C= 700kb insert DNA fragment.

(ng) of vector x kb size of insert / kb size of vector = (ng) of insert for ligation




```
                 enhancer region (3' end)
699   AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG TAACAACTCC

                 CAAT                                     TATA        3' end of hCMV
759   GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT AAGCAGAGCT

                 putative transcriptional start           T7 promoter primer binding site
819   CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT

                                                  Ig κ-chain leader sequence
879   AGGGAGACCC AAGCTGGCTA GCCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG
                                   Met Glu Thr Asp Thr Leu Leu Leu Trp

                                                                 Sfi I
932   GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC GCG GCC CAGCCG
      Val Leu Leu Leu Trp Val Pro Gly Ser Thr Gly Asp

                                Hind III  Asp718 I  Kpn I   BamH I      BstX I  EcoR I
980   GCCAGGCGC [GCGCGCCG] TACGAAG CTTGGTACCG AGCTCGGATC CACTAGTCCA GTGTGGTGGA

           Pst I  EcoR V      BstX I  Not I    Xho I   Xba I      Apa I
1040  ATTCTGCAGA TATCCAGCAC AGTGGCGGCC GCTCGAGTCT AGAGGG CCC GAA CAA AAA
                                                             Glu Gln Lys

                  myc epitope                                Polyhistidine tag
1102  CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT
      Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His His His His His

                                 pcDNA3.1/BGH reverse priming site
1153  CAT TGA GTTTAAACCC GCTGATCAGC CTCGACTGTG CCTTCTAGTT GCCAGCCATC
      His ***

1209  TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT GACCCTGGAA GGTGCCACTC CCACTGTCCT

      BGH poly (A) addition site
1269  TTCCTAATAA AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGT
```

Fig.7.3. Schematic diagram illustrating the main features of the expression vector pSecTag-B, downloaded from www.invitrogen.com.

Table 7.1. Ligation reaction

Reaction components	Reaction tube
Ligase 10 Buffer	1μ
T4-ligase	1μ
D-C insert DNA fragment	40ng
pSecTag-B vector	100ng
Nuclease-free water to final volume	10μ

The reaction tube was incubated at 4°C overnight. Different volumes of the reaction mixture were then used to transform One TOPO Shot chemically competent *E. coli* cells. Transformation and plating of the bacterial cells was performed as previously described [chapter 2], except that no X-gal or IPTG were used because pSecTag-B does not support blue/white selection. Therefore, clones were selected randomly. Several clones were amplified in LB culture medium over night and the purified plasmid digested with *Bam*HI and *Xho*I. Clones that showed appropriate insert sizes were submitted for DNA sequencing which was performed as previously described in section 7.3.2.1 using the T7 and Bgh primers incorporated into pSecTag-B (Fig.7.3).

Plasmid DNAs for immunisations were purified from large-scale *E. coli* cultures using Qiagen Megaprep kits, according to manufacturer's instructions [previously described in Chapter 2]. The purified DNA was stored at -20°C until ready for use.

7.3.3. *In vitro* EoDC-2 protein expression

Before proceeding to DNA immunisation, the ability of the pSecTag-B/EoDC-2 construct to express the EoDC-2 DNA fragment was confirmed *in vitro*. Two different assays were used as described below.

7.3.3.1. *In vitro* translation of mammalian COS-7 with pSecTag-B/EoDC-2

In vitro transfection is the process of bringing foreign DNA into cells to permit analysis of protein expression. The main reagent used in this transfection /expression procedure is FUGENE 6 (Roche). FUGENE 6 is a multi-component lipid that complexes with and transports DNA into mammalian cells.

The transfection procedure was performed according to FUGENE 6 protocol (Roche).

7.3.3.1.1. Materials for COS-7 culture.

A. Immortalised cell line culture.

COS-7 cells are immortalised fibroblast endothelial cells isolated from monkeys they were kindly donated by Dr A. Craig, Liverpool School of Tropical Medicine. The stock of COS-7 cells is kept at –80°C.

B. Preparation of cell culture Medium

+ 500ml DMEM [Dulbecco's modified minimal essential medium]
+ 50ml FCS [foetal calf serum]
+ 5ml P/S [penicillin/streptomycin]
+ 5ml Glutamine

All components were prewarmed at 37°C and mixed before use. The remaining medium was stored at 4°C.

C. Setting up a COS-7 culture

A bottle of culture medium (DMEM + reagents see B above) was pre-warmed to 37°C. One aliquot of COS-7 cells was thawed and added to a 15 ml Falcon tube containing 10ml culture medium. Cells were pelleted by centrifugation at 2000rpm for 5 minutes. The supernatant was discarded and the cells re-suspended in 12 ml culture medium. The cells were then transferred into sterile 25ml flasks (4ml per flask). Cells were checked for their viability using light microscopy and incubated at 37°C (CO_2) overnight. The following day, the culture medium was changed and incubated for another 24 hours at 37°C (CO_2).

D. Passaging COS-7 culture

Once the culture achieved confluency, the cells were split into new culture flasks. The culture medium was discarded, and the cells washed with 5 ml sterile PBS. Two millilitres Trypsin (prewarmed) was added and swirled horizontally and gently. To ensure that the monolayer was evenly coated with trypsin, cells were incubated at 37°C for 2 to 5 minutes and the dissociation of the monolayer checked by microscopically. Cells were then transferred into a sterile 15ml Falcon tube, followed by addition of 5ml fresh culture DMEM to dilute the trypsin. Cells were pelleted by centrifugation at 1500 rpm for 5 minutes the supernatant discarded and cells were then resuspended in 10ml fresh, prewarmed culture medium. Resuspended cells were distributed in 3ml aliquots into

sterile culture flasks. An additional 7ml fresh (prewarmed) DMEM was added to each flask. Cells were then incubated in the CO_2 incubator overnight at 37°C. After cells of the third passage achieved 80% confluency, they were ready for transfection.

E. Transfection of COS-7

The first step is to spread cells at a low density (40% per well) into each well of a 35mm six-well plate (Nunc). Cells were then incubated overnight in the CO_2 incubator overnight at 37°C. Culture medium was aspirated from each well and replaced by serum-free DMEM. The following reaction mixture was assembled for each well: 100µl serum-free medium (RT) was pipetted into a 15ml Falcon tube, 4µl FUGEN6 was added to the centre of the serum-free media, [avoiding contact the sides of tube], gently mixed and incubated at RT for 5 minutes. 1µg DNA was added as droplets the tube, with gentle but continuous mixing. The reaction was incubated at RT for 30 minutes. Twenty microlitres of the reaction mixture was spread as droplets into two wells. Wells were gently swirled and incubated in the CO_2 incubator overnight at 37°C.

F. Harvesting of recombinant protein

The supernatant of each well was harvested 72 hours after transfection and transferred into a sterile 5ml tube and stored immediately at -20°C. Two millilitres of 0.5mM EDTA was added to each well followed by incubation at RT for 10 minutes. This is to loss cells into suspension phase. Two millilitres 10% TCA (Appendix A) was added, followed by further incubation on ice for 30 minutes. Cells were centrifuged for 15 minutes at 15,500 g at 4°C, the supernatant discarded and 10 ml pre-chilled acetone added to wash the cells

which were then centrifuged as above. The supernatant was discarded and the cell pellet was air dried, resuspended in 2x Protein loading buffer (appendix A) and stored at -20°C

7.3.3.2. Analysis of EoDC-2 recombinant protein

Analysis of protein expression of EoDC-2 DNA fragment was performed using SDS-PAGE and Western blot assays. Both assays were performed as described in Chapter 2.

7.3.4. Gene Gun immunisation

7.3.4.1. Preparation of DNA-coated gold beads for GeneGun immunisation

Coating of 1.6μm gold beads (Bio-Rad) with EoDC-2/pSecTag-B DNA construct and the control pSecTag-B plasmid was performed according to the manufacturer's instruction. (BioRad, Hercules, CA). The quantity of gold powder and DNA was adjusted to provide immunisation 'shots' of 1μg DNA/0.5 mg gold.

All the preparation steps were performed at RT. The gold beads were resuspended in a carrier protein solution of 0.05M spermidine and sonicated for 6 seconds to separate the beads. The DNA was added and the suspension mixed vigorously. The DNA was precipitated onto the gold beads by addition of 1.0M $CaCl_2$ for 2 minutes followed by vigorous mixing and the beads left settle for 15 minutes at RT. The sample was centrifuged for 1 minute and supernatant was removed without disturbing the pellet of gold beads. Three 1 minute spin washes with 700 μl of 100% ethanol were performed to remove the excess spermidine, $CaCl_2$ and H_2O. The final 1ml of gold beads [in 100%

ethanol] was added to the required amount of 100% ethanol to provide a suspension of 0.5mg gold per cm Tefzel tubing according to manufacturer's instructions.

7.3.4.2. Preparation of cartridges ('shots')

Tefzel tubing was cut to required length. A 10ml sterile syringe was joined to one end. The DNA-coated gold bead ethanol suspension was vortexed and sonicated for 6 seconds and mixed again vigorously by hand for 20 seconds. Beads were then aspirated into tubing using the syringe leaving an air gap at both ends [making sure no air bubbles were trapped in the tubing]. This step was performed in a single smooth motion. The tubing was inserted into the tube roller (Fig.7.4a) and the beads allowed to settle for 3 minutes. Very slowly (1sec/cm) the ethanol was aspirated from the tubing by the syringe and the latter removed. The tube was rotated to evenly distribute the beads in the inner surface of the tubing for about 20-30 seconds. The remaining ethanol was evaporated by a stream of nitrogen gas for 3 minutes. The tubing was rotated throughout the latter step. The coated tubing was examined to verify even distribution of the gold beads and then cut into 1cm sections using the Bio-RAD Tubing Cutter. The 'shots' were stored in a glass container containing desiccant capsules, wrapped with parafilm and stored at 4°C until used.

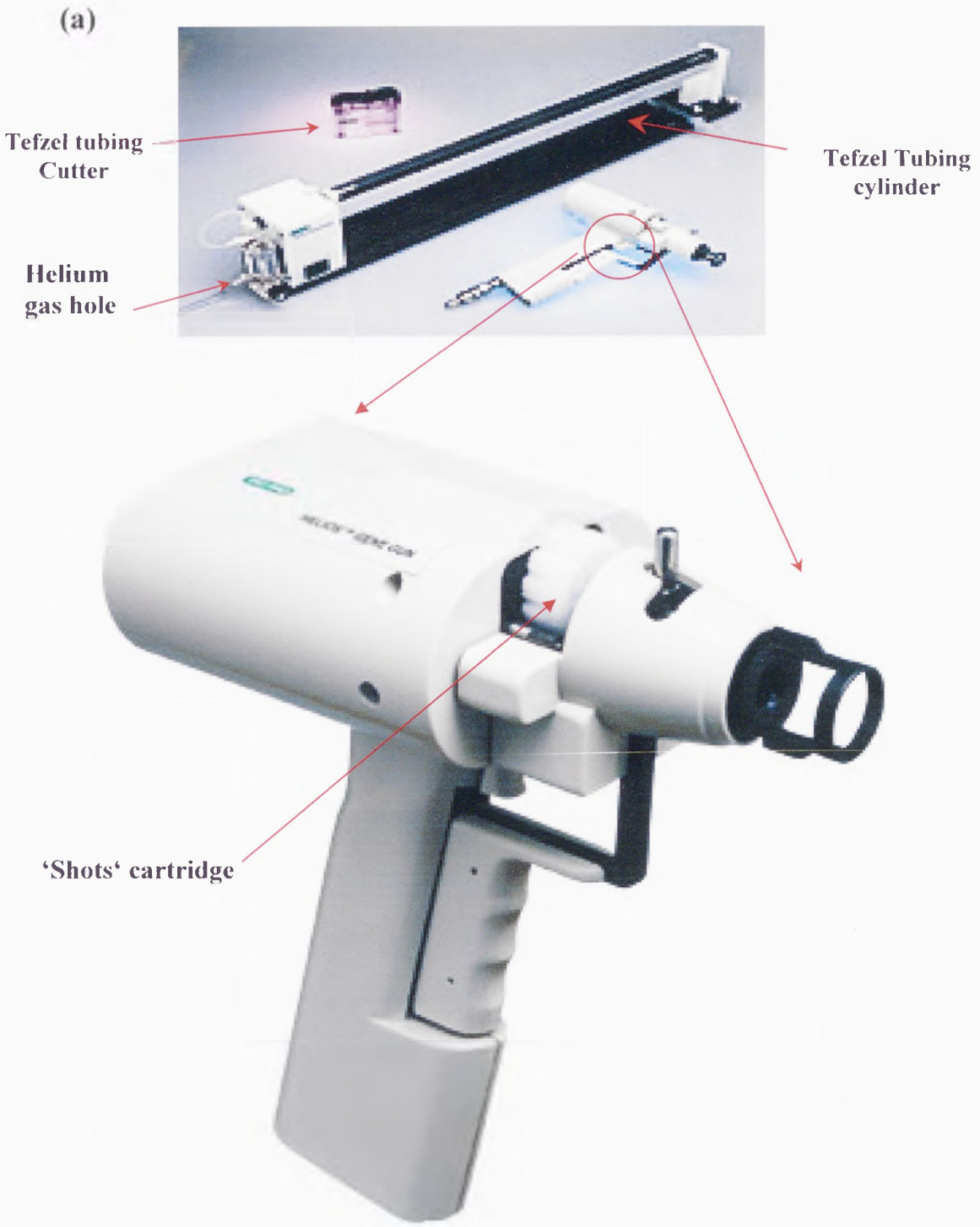


Fig.7.4. Illustrates (a) tube roller (b) The Helios GeneGun. (Bio-RAD).

7.3.4.3. Confirmation of DNA coated of the gold bead 'shots'

To verify DNA loading of the gold beads, single 1cm 'shots' were selected at random from each preparation and placed in 1.5 Ependorf tube containing 100% ethanol and vortexed vigorously for 2 minutes and then incubated overnight at RT. Shots were vortexed vigorously and centrifuged for two minutes at 13000 rpm to pellet the gold beads. The tubing and ethanol were removed and the sample centrifuged again for 1 minute to remove residual ethanol. The pellet was air dried for 15 minutes and then resuspended in 10µl ddH_2O and incubated at 55°C for five minutes to resuspended the DNA. Two microlitre of 6 SLOB (Appendix A) was added and samples loaded on to a 0.7% agarose gel.

7.3.5. BALB/c mice

8-10 week-old male BALB/c mice were purchased from Harlan Olac (U.K) or Charles River Laboratories (U.K) or were produced from breeding colonies in the Biomedical Services Unit, University of Liverpool. All mice were housed in designated areas of the Biomedical Services Unit of the University of Liverpool.

7.3.6. DNA immunisation of mice by Gene Gun

The Helios GeneGun (Fig.7.4b) was attached by metal tubing to a pressurised helium cylinder and the pressure set at 350 pounds per square inch (psi) according to Harrison et al. (1999). Previous studies had shown that this level of pressure delivered the gold beads into the epidermal layer [required for delivery of antigen to antigen-presenting cell (APC)] and that DNA-transfected cells were shown to be transcriptionally active by RT-PCR for

three days (Harrison et al., 1999). The 1cm 'shots' were loaded into the cartridge supplied the manufacture (Fig7.4b) and inserted into the GG. The abdomens of the mice were shaved using electric hair clippers and they were lightly anaesthetised using a mixture of halothane and oxygen (5%). The barrel of the GG was then placed on the shaved abdomen and discharged three times, delivering a total of 3μg of the pSecTag-B/ EoDC-2 DNA construct or the vector alone per mouse. Immunisations were given on three occasions, two weeks apart and the sera examined 8 weeks later as described by Harrison et al. (1999; 2000). Two further immunisations were performed at 4 week intervals.

7.3.7. Intradermal and intramuscular injections of DNA

pSecTag-B/EoDC-2 DNA construct and the control pSecTag-B were adjusted to 100μg DNA/50 μl distilled water and 25μl was injected intrademally into two sites on the back of anaesthetised mice [ID] or into the rectus femoris muscle of each hind leg [IM] of mice with a 25G needle. Immunisations were given three times with two week intervals between immunisations with a further two immunisations given at four week intervals.

7.3.8. Collecting of sera from immunised mice

Blood was collected from each mouse after each immunisation by tail snip or, at the end of the experiment, by cardiac exsanguinations under terminal anaesthesia (Harlow and Lane, 1988). The blood was incubated overnight at 4°C to allow formation and contraction of the blood clot which was then removed. Serum samples were then

centrifuged at 13,000 rpm for 5 minutes. Sera was transferred into sterile 1.5ml Eppendorf tube and stored at -20°C.

7.3.9. Enzyme-linked immunosorbant Assay (ELISA)

ELISA was used to determine the titre of antibodies to *E. ocellatus* whole venom from the immunised mice. Methods were based on those of Theakston et al. (1977) and Harrison et al. (2000). The ELISA plates (Maxisorp, NUNC, Denmark) were coated with 1mg/ml whole *E. ocellatus* venom in coating buffer (0.015M Na_2CO_3, 0.035M $NaHCO_3$, 3mM NaN_3, pH 9.6) and left overnight at 4°C. The plates were then washed three times with washing buffer (0.077M NaCl, 1mM Tween 20 in distilled water). One hundred microlitre of appropriately diluted test and control sera were added to the wells and incubated for 2 hours at 37°C in an incubator (Sanyo Gallenkamp, Leicestershire, UK). The wells were then washed as above and 100µl of a 1:1000 dilution of goat anti-mouse or goat anti-rabbit alkaline phosphatase conjugate (SIGMA, Poole, UK) was added. The plates were then incubated for two hours at RT before rewashing. Chromogenic substrate, 2,2'-azino-bis (2-ethylbenzthiazoline-6-sulphonic acid tablets, Sigma, Poole, UK), was then added and the plates developed at RT for 30 minutes. The absorbances of the wells were read at 405 nm using a model 450 microplate reader (BioRad, Hemel Hempstead, UK).

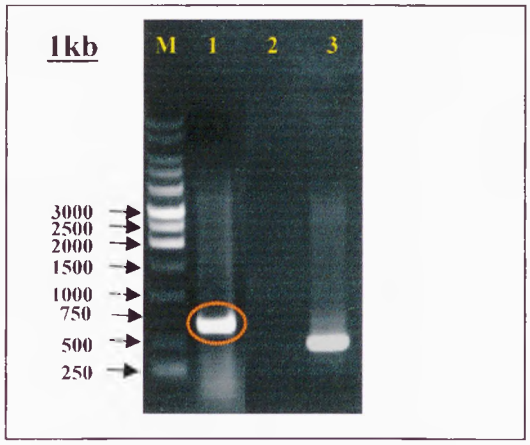
7.4. Results

7.4.1. PCR amplification of DC domain of the prothrombin activator EoMP-6

The isolated DNA *EoMP-6,* (Hasson et al., 2003) was used as the template for PCR amplification using the EoDC-specific forward and reverse primers (Fig.7.2). A PCR product of approximately 700bp was obtained (Fig.7.5a) and cloned into the TOPO-TA vector (Invitrogen, Netherlands). The TOPO-EoDC plasmids were purified from bacterial cell and subjected to BamHI and Xho1 digestion. The clones containing inserts (EoDC) of the correct size (Fig.7.5b) were submitted for DNA sequencing. The clone that shows the right insert with a good ORF was selected for ligation reaction into the pSecTag-B expression vector. Clones of pSecTag-B were examined first by endonuclease restriction digest (Fig.7.5c). The four clones (Fig.7.5c) were submitted for DNA sequencing for ORF confirmation. Clone EoDC-2 was selected for DNA immunisation as it gave a better ORF as shown by sequence alignment (Fig.7.6a and b).

Fig.7.5. 0.7% agarose gels showing the preparation of pSecTag-B/EoDC-2 DNA immunisation construct. (a) Amplification of the DC domain from the *EoMP-6* cDNA for insertion into TOPO pCR2.1 vector (circled). (b) Digestion of EoDC/TOPO colonies for ligation into pSecTag-B. Six EoDC/TOPO colonies (Lanes 1-6) were digested with BamH1 and Xho1. Four of these had inserts of the correct size (circled). Insert of 1 was used for ligation into pSecTag-B. (c) Digestion of PSecTag-B/EoDC-2 colonies. All the selected colonies (Lanes 1-4) were analyzed with BamH1/Xho1 and contained inserts of the correct size. DNA from lane 2 (pSecTag-B/EoDC-2) was used for immunisation.

(a)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2500
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750
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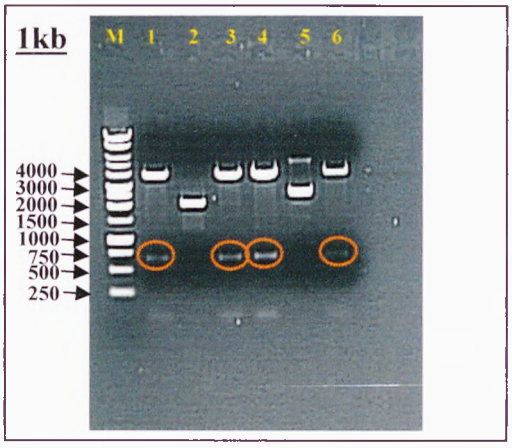
(b)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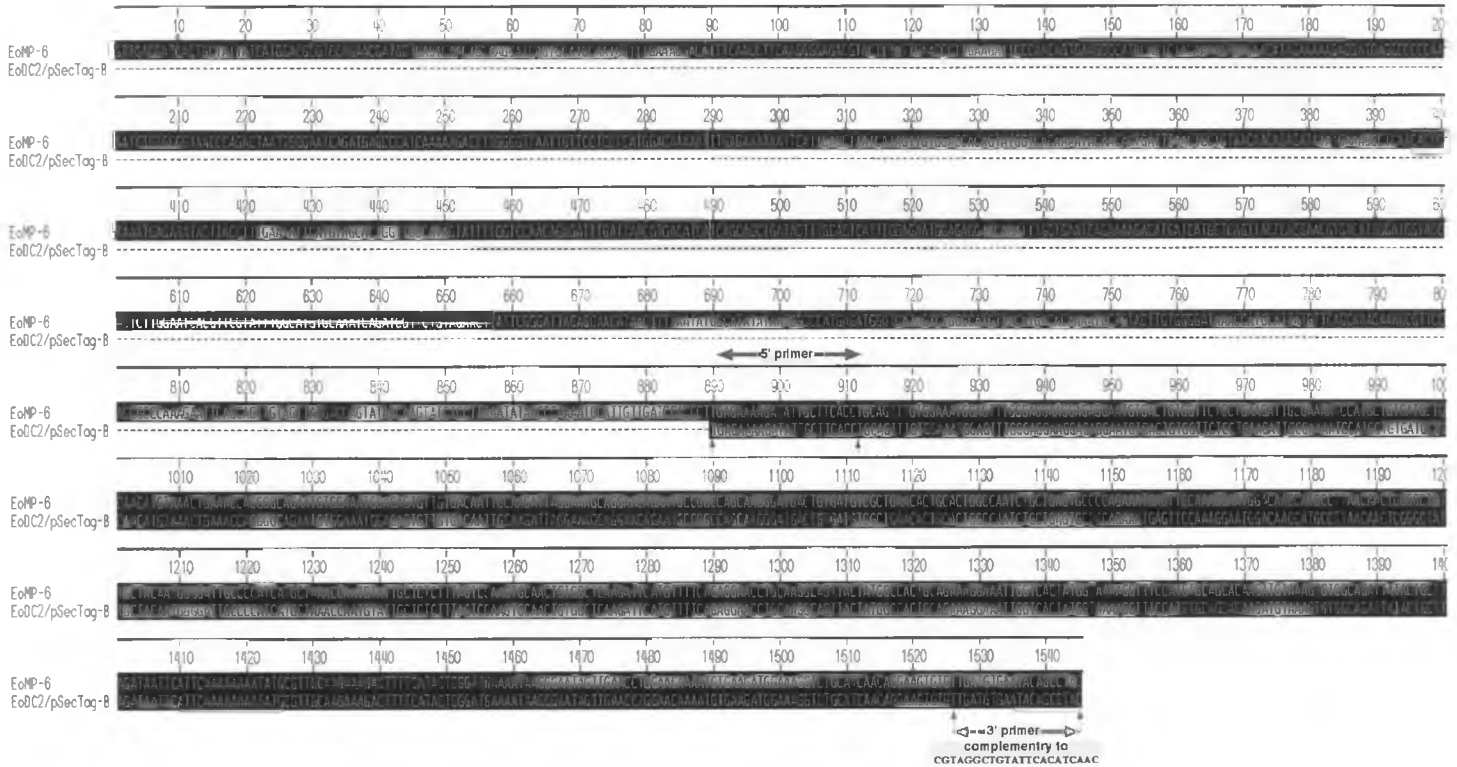


Fig.7.6a. Alignment of the DNA sequence of the EoDC insert [craboxyl-disintegrin/cystein-rich] with domain with the EoMP-6. The Site of the EoDC-specific 5' and 3' primers are highlighted.

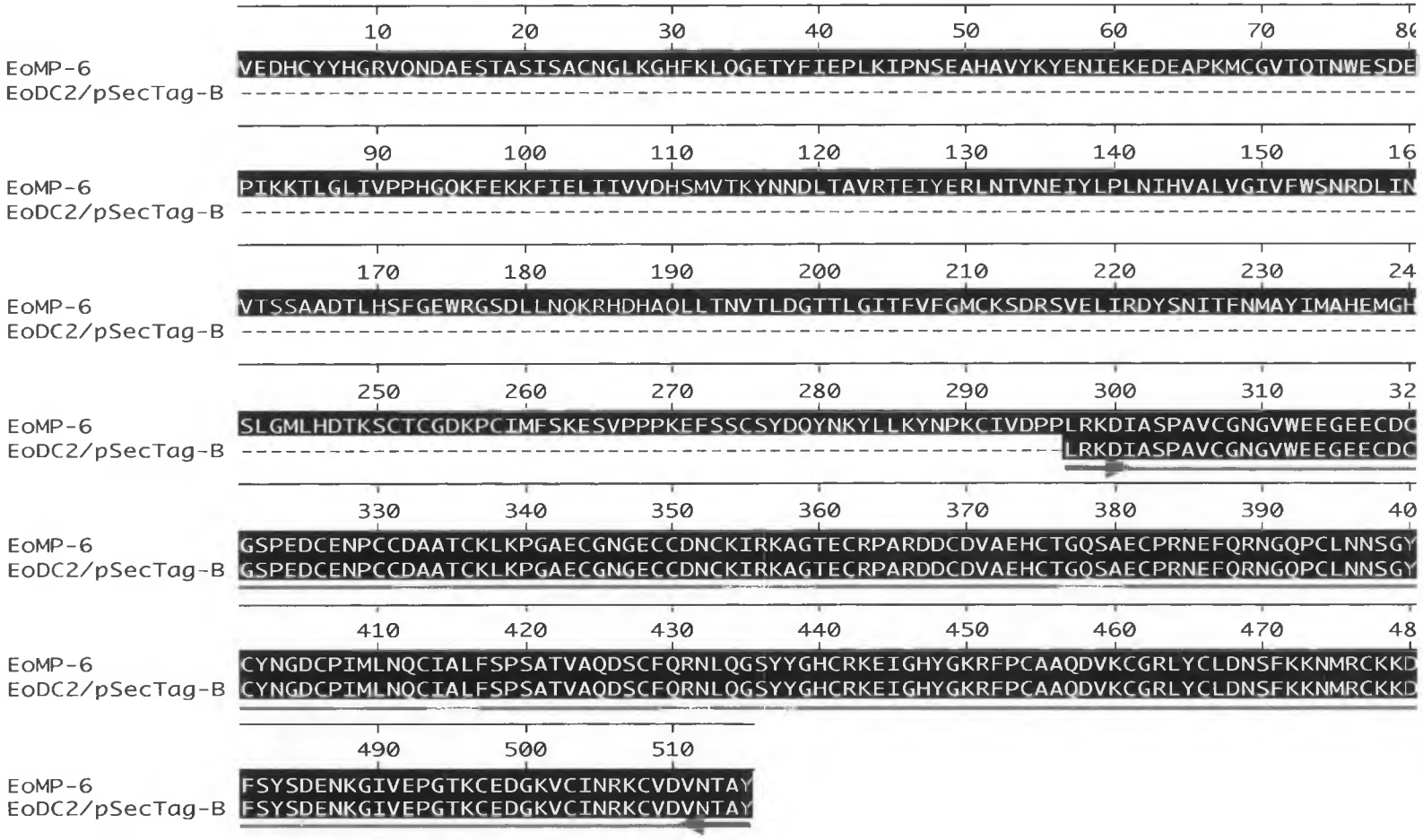


Fig.7.6b. Deduced amino acid sequence of the EoDC2 [carboxyl-disintegrin/cysteine-rich] insert domain with the *EoMP-6*. Red line indicates the isolated domain.

7.4.2. Confirmation of the transcriptional and translational validity of EoDC-2/pSecTag-B construct plasmid.

Transient transfections of COS-7 [a mammalian fibroblast cell line donated by Dr. A. Craig] were carried out to demonstrate both the transcription and translational validity of the EoDc-2/pSecTag-B immunisation construct.

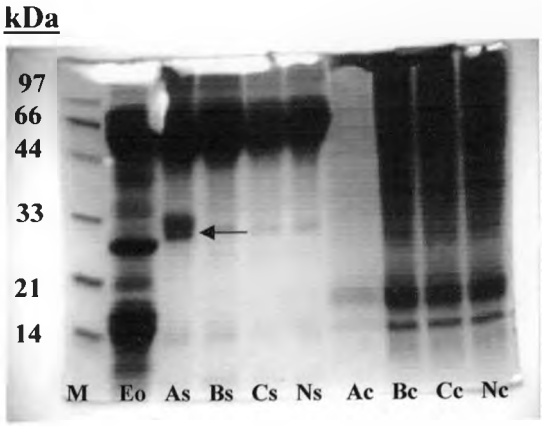
In conventional assays of this sort, the transfected COS-7 cells are maintained in FCS. However, for the period of transfection, serum-free medium was used instead of FCS. The proteins in the supernatant and the pelleted cells were precipitated by 10% TCA and examined by SDS-PAGE (Fig.7.7a). To facilitate detection of the EoDC-2 protein, duplicate SDS-PAGE gels were transfected to nitrocellulose membrane that were probed with rabbit anti-*E. ocellatus* venom (Fig.7.7b) and NMS (Fig.7.7c). The latter immunoblot was performed to discount background activity. The faint bands illustrated by the arrows in the SDS-PAGE (Fig.7.7a) and rabbit antivenom immunoblot (Fig.7.7b) confirmed that EoDC-2/pSecTag-B construct was capable of EoDC-2 expression.

Fig.7.7. Examination of the cellular protein and culture supernatant of COS-7 cells transfected with EoDC-2/pSecTag-B by (a) 12% SDS-PAGE and immunoblotting with rabbit anti-*E. ocellatus* venom (b) and normal mice serum [NMS] (c).

Figure (a). Lane M-protein molecular weight marker; Eo- *E. ocellatus* venom; As, Bs, Cs-proteins in the supernatant of COS cells transfected with EoDC-2/pSecTag-B (As), pSecTag-B (Bs), FUGENE 6 alone (Cs) or normal cell (Ns); Ac, Bc, Cc and Nc- cellular proteins recovered from COS cells transfected with EoDC-2/pSecTag-B (Ac), pSecTag-B (Bc), FUGENE 6 alone (Cc) or non-transfected COS-7 cells (Nc).

Figure (b) and (c). Lane A- protein molecular weight marker, Lane B- *E. ocellatus* venom. Lanes C-F – proteins in the supernatant of COS-7 cells transfected with EoDC2/pSecTag-B (C), pSecTag-B (D) FUGENE 6 alone (E). Lane F, supernatant of non-transfected COS-7cells. Lanes G-I- cellular proteins of COS-7 cells transfected with EoDc2/pSecTage-B (G), pSecTag-B (H), FUGENE 6 alone (I). Lane J- cellular proteins of non-transfected COS-7 cells

(a)


kDa
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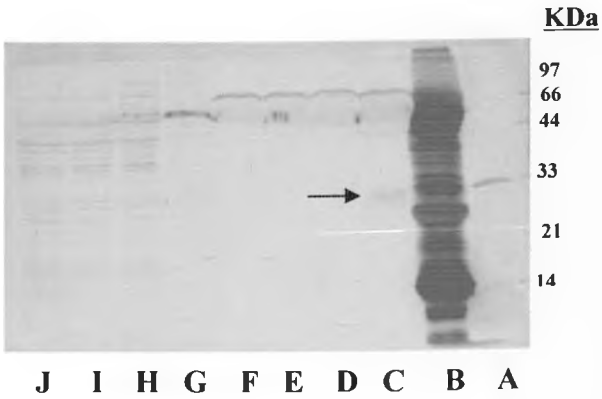
66
44
33
21
14
M Eo As Bs Cs Ns Ac Bc Cc Nc

(b)


KDa
97
66
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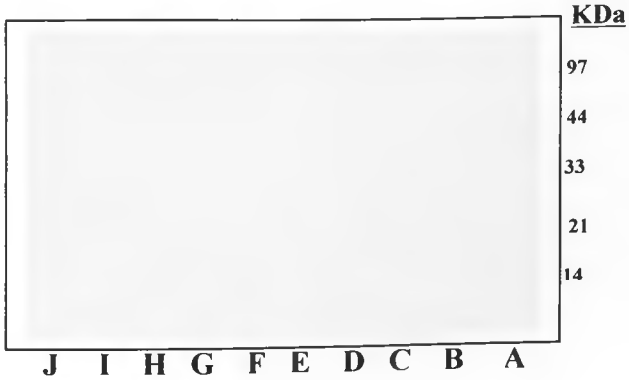
J I H G F E D C B A

(c)


KDa
97
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J I H G F E D C B A

7.4.3. Validation that gold beads used to immunized mice contained EoDC-2/pSecTag-B DNA

In order to verify that the process of 'shot' construction had proceeded correctly, the DNA content of gold beads in randomly selected 'shots' were resuspended in H_2O and resolved on 0.7% agarose gel and visualized by ethidium bromide staining (Fig.7.8).

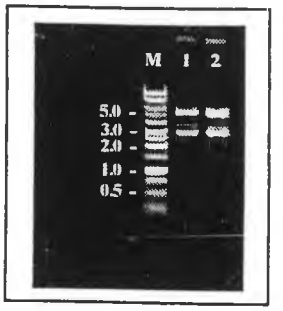


Fig.7.8. Validation that gold beads used to immunize mice contained EoDC-2/pSecTag-B DNA. M; 1kb DNA ladder (Bio-Rad): Lane 1; PSecTag-B: Lane 2; EoDC-2/pSecTag-B.

7.4.4. Responses of BALB/c mice immunised with EoDC-2/pSecTag-B DNA

Mice received five immunisations of 3µg pSecTag-/EODC-2 DNA by GG and 100µg by either ID or IM routes, respectively. The first three immunisations were performed at two week intervals, with serum samples taken two weeks post immunisation. The fourth and fifth immunisations were performed at four week intervals. Sera collected at various intervals throughout the experiment from the DNA-immunised and non-immunised control animals were examined by ELISA to determine the titre of antibodies to *E. ocellatus* venom.

In the first instance the sera from each group was serially diluted with PBS and the titre determined (Fig.7.9).

Serial dilutions of sera taken from mice 12 weeks after the final DNA immunisation were tested by ELISA to determine levels of EoDC-2 specific IgG (Fig.7.9). The OD values displayed were calculated by subtracting the OD value (blank) from the OD value of each well. Each line represents the mean OD of each group. This figure demonstrates that GG is the most efficient DNA delivery method for generating antibody to EoDC-2. The ID method was less efficient than GG and IM route was inefficient.

The next assay was a time course ELISA to determine the relative contribution of each of the five immunisations (Fig.7.10). Time course assay was performed at 1:20 dilution as it showed highest antibody titre in Fig.7.9. Sera were taken from mice two weeks after each immunisation. The OD values displayed were calculated by subtracting the OD value (blank) from the OD value of each well. Each line represents the response of each group of five mice for GG and four mice for IM and ID, respectively. Figure 7.10 (i) confirms that GG was the most efficient DNA-delivery route and (ii) also showed that satisfactory antivenom titres were only achieved after the final immunisation.

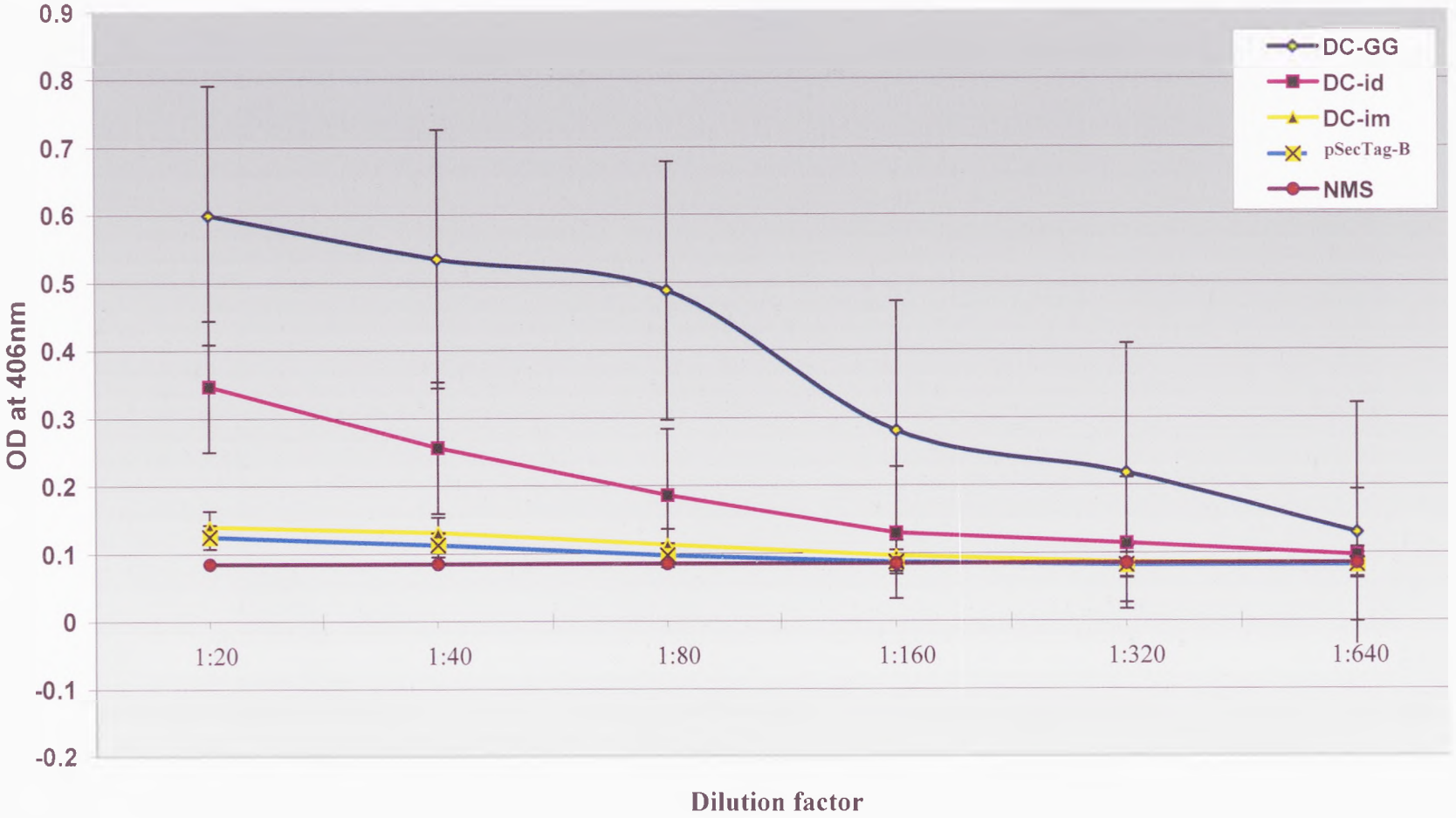


Fig.7.9. Antibody responses to *E. ocellatus* venom of the DNA immunised mice as determined by ELISA

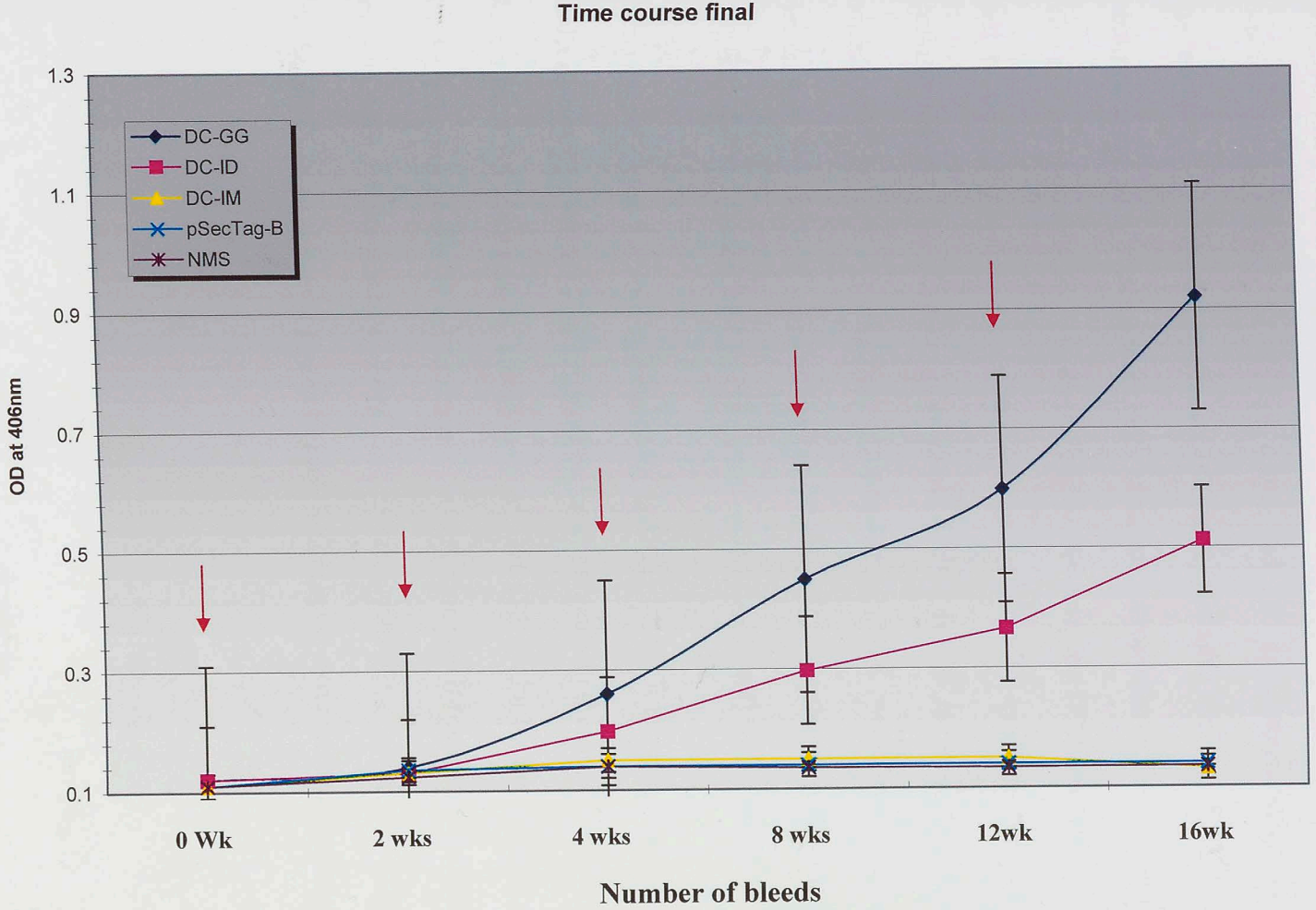


Fig.7.10. Antibody response to *E. ocellatus* venom of 1:20 diluted sera from the DNA immunised mice collected at intervals throughout the experiment. Arrows indicate the time and number of immunisation

7.4.5. Cross-reactivity of the EoDC-2 antibody with venoms from Echis snakes of different geographical areas.

Immunoblotting with antibody from the EoDC-2 DNA-immunised mice was performed (i) to assess the specificity of the antibodies and (ii) to experimentally confirm the hypothesis that the resultant antibodies, although generated from an *E. ocellatus* DNA construct, were able to cross-react with analogous molecules in the venom of related *Echis* species. Accordingly, the sera (diluted 1:100) from the DNA-immunised mice were used to screen immunoblots containing venoms from *E. ocellatus*-[Nigeria] (1), *E. ocellatus*-[Ghana] (2), *E. pyramidum*-[Saudi Arabia] (3), *E. pyramidum*-[Oman] (4), *E. pyramidum*-[Iran] (5), *E. pyramidum leakeyi*–[Kenya] (6), *E. pyramidum*-[Tunisia] (7), *E. pyramidum*-[Mali] (8), *E. multisquamatus*-[Russia] (9), *E. carinatus sochureki*-[Pakistan] (10), *E. coloratus*-[Saudi Arabia] (11) and *E. colaratus*-[Israel] (12) (Fig.7.11).

It can be seen that antibody generated by GG (Fig.7.11a) was cross reactive with high molecular weight bands [52 kDa] in all venoms, particularly those in *E. ocellatus* venom. As expected sera from mice immunised with EoDC-2 by the ID route (Fig.7.11b) were fainter than those from GG-immunised mice. Sera from the IM-immunised mice (data not shown) were as non-reactive as the control mice. However, bands of venom proteins below 33kDa have been recognised by all blots except in that where normal mouse serum was used.

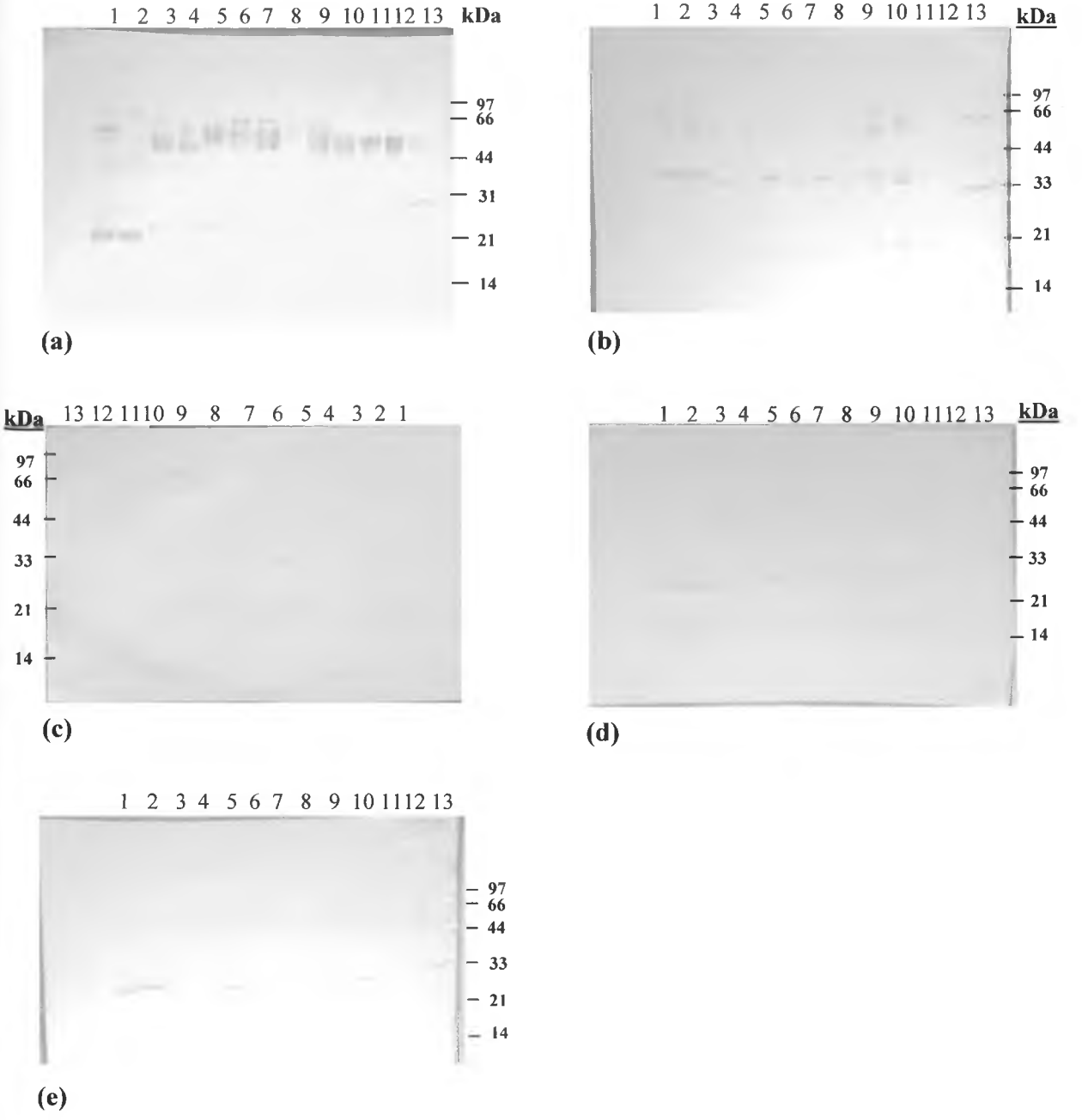


Fig.7.11. Reactivity of sera from EoDC-2 DNA immunised mice to components in venoms of various *Echis* species. Identical immunoblots of venoms from [1-12, stated in text], were probed with sera from mice immunised with EoDC-2 by GG (a), ID (b) or IM (c). Immunoblots (c), (d) and (e) were probed with sera from mice immunised with pSecTag-B by GG and ID, respectively. Whereas immunoblot (e) was probed with sera from normal mice. Lane 13 is the low molecular weight marker (BiorRad, UK).

7.5. DNA immunisation using cDNAs encoding other toxins in venom of *E. ocellatus.*

I also attempted to generate antibodies to *E. ocellatus* C-type lectin and serine protease by immunisation with cDNAs encoding EoCTL-1 (Chapter 3) and EoSer-20 (Chapter 4). The strategy involved was only slightly different from that described for EoDC-2 because the whole ORF of the CTL and serine protease were used for immunisation. Because the pSecTag-B vector contains a mammalian signal peptide, the venom sequences prepared for DNA immunisation were PCR amplified without the native signal peptide (Fig.7.12 and Fig.7.13).

Subsequent TOPO subcloning and pSecTag-B ligation experiments were performed as for EoDc-2/pSecTag-B. The EoCTL-1i/pSecTag-B and EoSer20i/pSecTag-B constructs were used to immunize mice by the GG, ID and IM routes. Unfortunately, the results (data not shown) showed that no seroconversion was obtained by any of the three DNA delivery routes with either the EoCTL-1i or the EoSer-20i. The results were unexpected after the successful seroconversion obtained by DNA immunisation with EoDC-2/pSecTag-B.

The sequence data indicated that the EoDC-1i and EoSer-20i cDNAs were correctly [in frame] inserted into the pSecTag-B plasmid. The DNA gold beads were shown to contain as much DNA as that recovered from the EoDC-2 coated gold beads (data not shown).

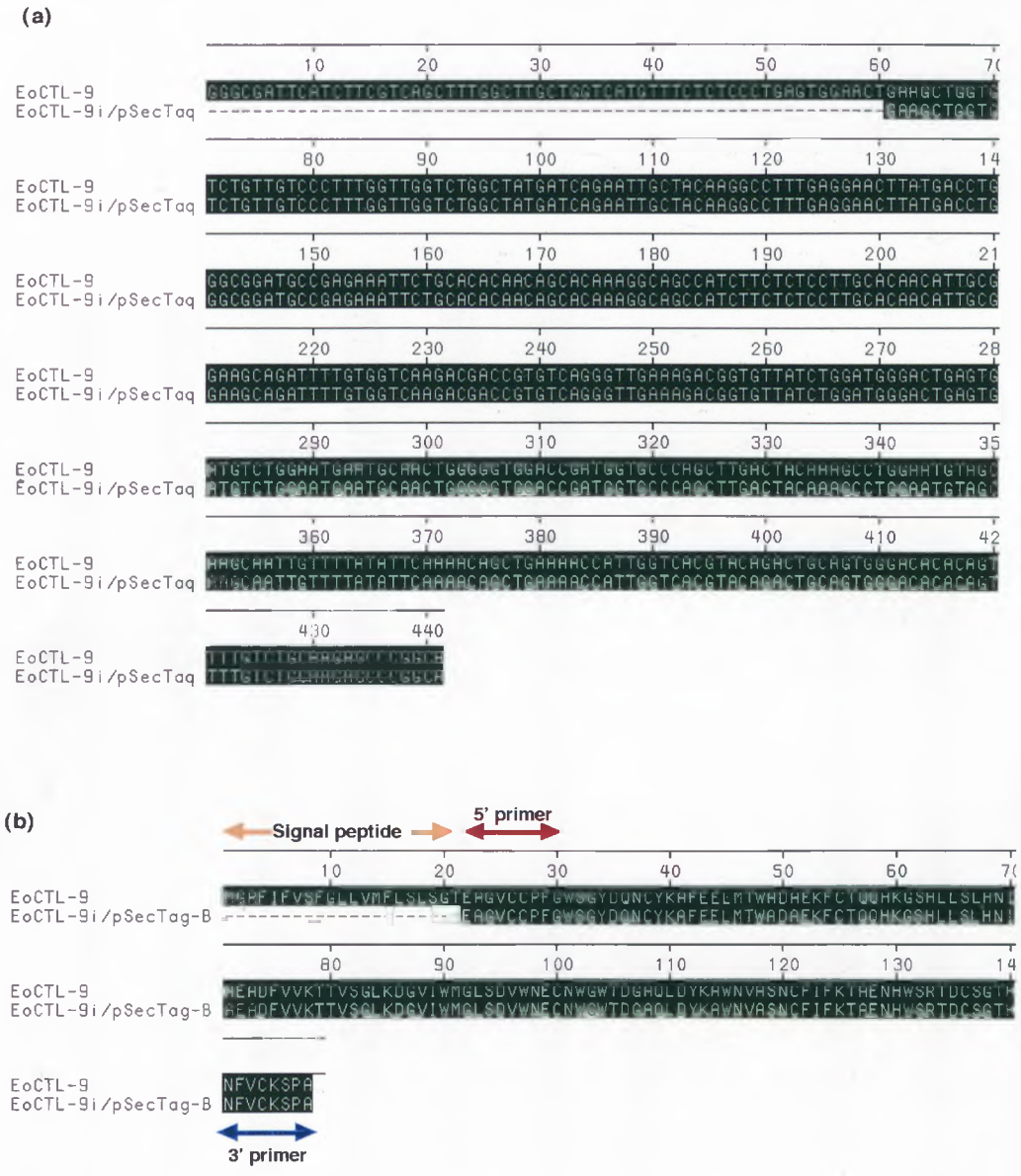


Fig.7.12. Alignement of neucleotides (a) and deduced amino acid (b) of EoCTL-1i /pSecTag-B and EoCTL-1. Sites of PCR primers were used to amplify the CTL ORF are indicated.

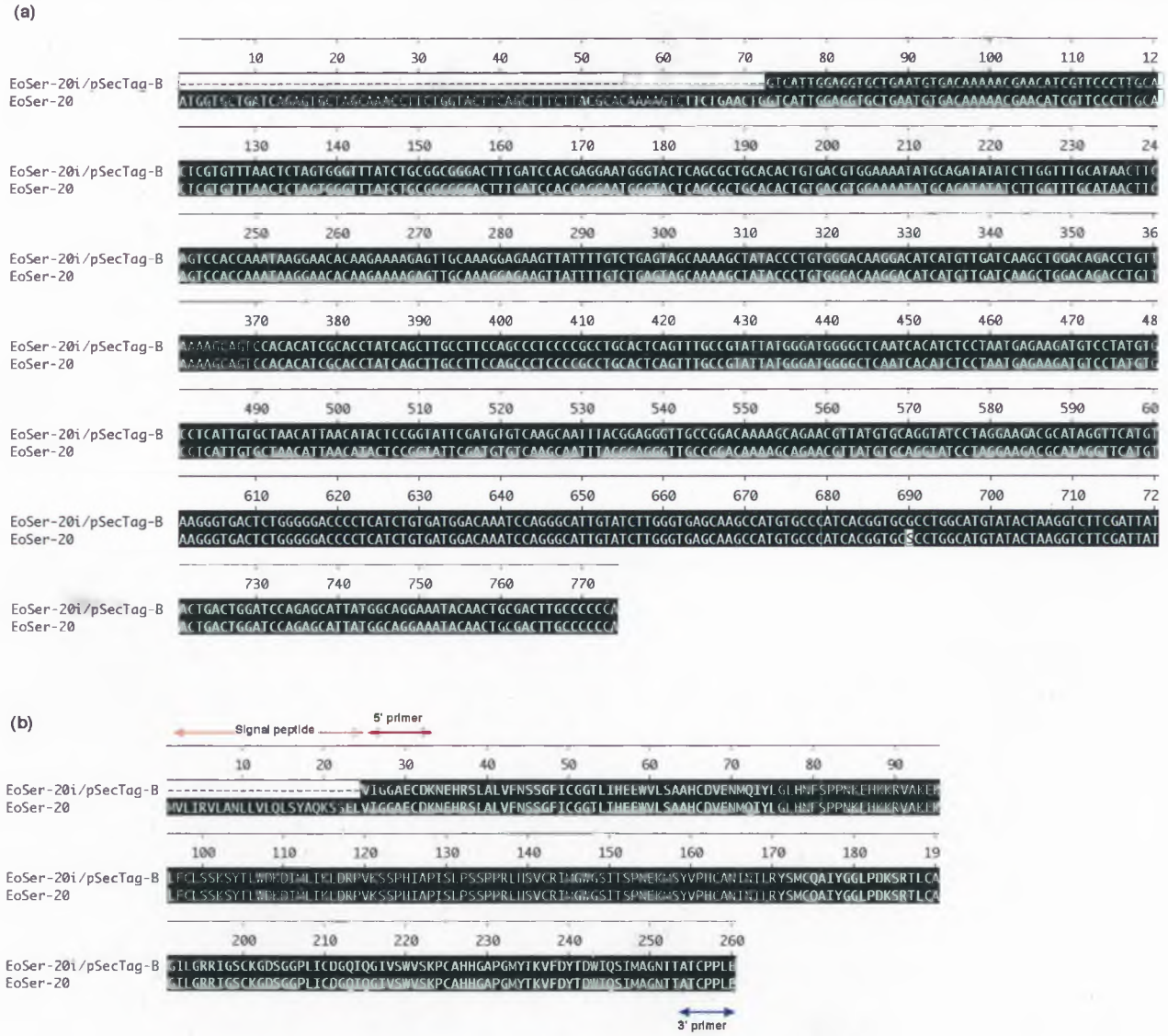


Fig.7.13. (a) Alignment of neucleotides and (b) deduced amino acid of EoSer-20i /pSecTag-B to EoSer20. Sites of PCR primers used to amplify the serine protease ORF are indicated.

7.6. Discussion

The remarkable progress of DNA vaccination from the initial observation in 1992 that plasmids could be used to express exogenous DNA in mammalian cells and stimulate immune responses (Tang et al., 1992) to the current clinical trials (Liu, 2003) has largely been concerned with inducing protective cellular immune responses against intracellular bacteria, viruses and parasites. Some experimental DNA vaccines are proving to be as, in some cases, more effective than clinical vaccine formulations (Donnelly et al., 1995; Tascon et al., 1996).

Harrison et al., (2000) demonstrated for the first time that DNA immunisation is a promising technique for antivenom production because it represents a system of generating toxin-specific antibodies. The primary objective of this study was therefore to (i) use DNA immunisation to generate specific antibodies to the major haemostsis-disruptive toxins in the venom of *E. ocellatus* and (ii) to define the route of administration favouring maximal induction of specific antibodies.

PCR amplification was successful in amplifying the carboxyl-disintegrin and cysteine-rich domain [approximately 700bp] of the *EoMP-6* prothrombin activator cDNA (Hasson et al., 2003). The PCR product was sub-cloned into TOPO vector and inserted in frame the mammalian vector pSecTag-B.

Prior to DNA immunisation, the EoDc-2/pSecTag-B construct was used to transfect mammalian fibroblast cells, COS-7. This assay confirmed that EoDC/pSecTag-B was transcriptionally and translationally valid as illustrated by the detection of the 28kD EoDC-2 protein in SDS-PAGE and immunoblotting. The EoDC-2/pSecTag-B-transfected

COS-7 cells also confirmed that the EoDC-2 protein was secreted into the culture supernatant confirming the correct operation of the signal peptide sequence (Fig.7.5a and Fig.7.5b). The next validation step was the confirmation that EoDC-2/pSecTag-B was successfully recovered from the Tefzel tubing 'shots'. These assays were performed to ensure that all possible errors that may interfere with DNA immunisation, particularly by the Gene Gun, were eliminated.

Different routes of DNA immunisation were evaluated. BALB/c mice received five immunisations of three shots (3μg) for Gene Gun DNA delivery, or 100μg for either ID or IM DNA delivery at each immunisation. The first three DNA immunisations were performed at two week intervals and the final immunisations were given at monthly intervals thereafter. Sera were collected from the immunised mice [by tail bleed] two weeks post immunisation.

The results of the EoDC-2/pSecTag-B DNA immunisation clearly demonstrated that Gene Gun immunisation was superior to ID immunisation in terms of efficiency of seroconversion. The inability of the mice immunised with EoDC-2/pSecTag-B by IM to raise anti-EoDC-2 antibodies was unexpected. The plasmid used was from the same stock used to unsuccessfully immunize mice by the GG and ID routes, indicating that the plasmid was not at fault. This suggests that the route of delivery was the problem. Harrison et al. (2000) observed that mice immunised with JD9 DNA [the DC domain of an analogous SVMP from the venom of *B. jararaca* by IM raised markedly weaker antibody titres than mice immunised by Gene Gun. Together, these results indicate that IM DNA immunisation is not appropriate for raising antibodies.

I have observed that EoDC-2/pSecTag-B immunisation elicited a highly heterogenous response in mice (data not shown) [i.e., some mice responded while others did not]. DNA immunisation studies have previously provided evidence for differential transfection efficiencies in individual mice as reflected by variable levels of expression of the encoded protein (Wolf et al., 1990; Acsadi et al., 1991). The variation in the antibody response of individual mice within each group may reflect different rates of epidermal cell transfection following GG and ID immunisations.

Results of the time course of both GG and ID immunisation with EoDC-2/pSecTag-B DNA antibody titres after the second immunisation showed barely detectable deficiencies from the background. Thereafter, antibody titres doubled with each immunisation in both the GG- and ID-immunised mice. This suggests that time intervals between boosts and/or DNA dosage needs to be investigated further with a view to improving the antibody titre. If time had permitted, I would have performed the latter experiments as well as investigating the antibody isotypic responses generated by the various routes of immunisation.

SVMPs from venoms of phylogenetically related vipers show extensive molecular sequence and structural similarities (Bjarnson and Fox, 1995; Nishida et al., 1995; Paine 1992; Bjarnson and Fox, 1994; Harrison et al., 2003). One of the main theoretical attractions of the DNA immunisation approach to generating toxin-specific antivenoms is that the extensive sequence and structural conservation of venom toxins can be exploited to generate antivenoms capable of neutralizing the venoms of a wide variety of snakes (Harrison, 2004). Conventional antivenoms are effective if used against the snake venom used for immunisation (Lalloo and Theakston, 2003). The phylogenetic limits to the

cross-reactivity of the EoDC-2 antibodies raised by GG and ID were examined by probing immunoblots of venoms of a variety of *Echis* vipers (Fig.7.11a and Fig.7.11b). The EoDC-2-specific antibodies reacted to the 50-60 kDa molecules in the homologous *E. ocellatus* venom with an intensity matched by its reactivity with analogous bands in venoms of *Echis* vipers of various African origins. The immunological reactivity of the anti-EoDC-2 sera to components in *Echis* vipers from Iran (*E. pyramidum*) and Pakistan (*E. sochureki*) was considerably weaker than that of the *E. ocellatus* and the other African viper.

In conclusion GG delivery of EoDc-2 DNA induced titres of total IgG greater than those achieved by the ID or IM delivery routes. It has been suggested that direct transfection of epidermal dendritic cells results in their migration to draining lymph nodes where antigen presentation to the immune system occurs (Robinson and Torres, 1997). The lower IgG titres obtained by ID injection suggest that this delivery route is markedly less efficient than GG in activating dendritic cells.

Binding of the EoDC-2 antibody to the EoDC-2 domain of *E. ocellatus* would be expected to interfere with the interaction of this domain to the $\alpha_2\beta_1$-integrins on platelets and thereby contribute to neutralization of venom-induced haemorrhage. Alternatively, reports that the disintegrin-cysteine rich domain modulates the substrate specificity of venom MDCs (Kamiguti et al., 2003), and possibly their catalytic function (Jeon et al., 1999), suggest that EoDC-2- specific antibody has the potential to inhibit the enzymatic degradation of sub-endothelium by the *E. ocellatus* MP domain. It is conceivable that EoDC-2 antibody may operate in both modalities to inhibit venom haemorrhagic activity.

Regrettably, there was not enough time to test the venom-neutralizing efficacy of the EoDC-2-specific antibody. This antibody would have been subjected to an *in vitro* Minimum Haemorrhagic Dose (Theakston and Reid, 1983) assay that determines the ability of an antibody to neutralize anti-haemorrhage efficacy of the antibody raised by the *B. jararaca* JD9 DNA construct (Harrison et al., 2000). Also because the JD9 domain was very similar in terms of sequence and structure to EoDC, this suggests that the EDC-2 antibody should possess equal therapeutic efficiency against the toxins responsible for the haemorrhagic effect of *E. ocellatus* venom.

The main problem in this part of this study has been the lack of seroconversion achieved following immunisation with DNA encoding *E. ocellatus* CTL and serine proteases and the lack of time available for investigating factor(s) responsible. It is unlikely that for the pSecTag-B plasmid was responsible because this was the plasmid used for the successful EoDC-2 immunisations. It is possible that the expressed proteins were either toxic to the transfected murine cells, thus preventing production of sufficient protein to stimulate an immune response. Alternatively, the expressed proteins were simply poor immunogens, despite the numerous antigenic motifs predicted for these proteins [see Jameson-Wolf algorithm profiles of these proteins in Chapters 4 and 5]. Given more time, I would have performed RT-PCR of tissues 'shot' by GG to assess transcription rates for the immunisation constructs and also probed frozen sections of the skin with *E. ocellatus* venom-specific antibody to determine the translational efficacy of DNA immunisation with the CTL and serine protease constructs.

Finally this is the first time ant-EoDC2 to be generated against the *E. ocellatus* venom prothrombin activator-like metalloprotease. Further characterisation of this antibody is

now required to gain insight into its neutralization efficacy in order to have significant contribution and potential to improve the treatment of systemic effects that exerted by the saw scaled viper *E. ocellatus* envenoming.

Chapter 8

Antibody zymography: a novel in vitro assay to determine the protease-neutralizing potential of specific antibodies and snake antivenoms.

8.1. Introduction

Before the development of improved vaccines and monoclonal antibodies (Kohler and Milstein 1975), antiserum was the therapy of choice for a number of diseases including tetanus, rabies and diptheria. Full realization of the therapeutic potential of monoclonal antibodies (mAbs) was recently achieved by molecular "humanization" of mAbs and linkage of mAbs to target-killing agents such as toxins, radioisotopes, cytokines and enzymes which has enabled commercialization of many mAb-based therapeutics against infectious and non-infectious diseases (Brekke and Sandlie, 2003; Trikha et al, 2002). One of the difficulties in the field has been, and will continue to be, the selection from a large population, of the specific antibody with the required function.

As shown earlier I have isolated numerous DNA sequences encoding some of the most medically-important *E. ocellatus* venom toxins (Bharati et al, 2003; Harrison et al, 2003b; Hasson et al, 2003) as immunizing reagents to generate specific antibodies. Chapter 7 describes the generation of toxin specific antibodies by DNA immunisation. However, assessing the toxin-neutralizing efficacy of such toxin-specific antibodies is problematic. The venom-neutralizing efficacy of conventional antivenoms is routinely determined using *in vivo* lethality (LD_{50}), haemorrhagic (MHD) median effective dose (ED_{50}) estimation and necrosis by the minimum necrotising dose (MND) assays in mice and the intravenous injection (i.v. LD_{50}) also in mice (WHO, 1981; Theakston and Reid, 1983; Laing et al., 1992; 1995). The lethality assays, in general, determine the amount of antivenom which, when injected intravenously together with a defined lethal venom (or pool venoms) challenge dose in mice, results in the survival of half the animals within the group after 24 hours. However, these assays are inappropriate for testing the toxin-

neutralizing function of specific antibodies generated to many venom toxins which, on their own, do not typically result in death, haemorrhage or necrosis (e.g., PLA_2, CTL, serine proteases, hyaluronidase, L-amino acid oxidases).

Moreover, the demand for lower development costs, which are essential if the product is to be purchased by hospitals and Ministries of Health for use in high snake bite risk areas in the rural tropics, together with stringent UK Home Office regulations for humane animal experimentation, have encouraged a new approach to the design of antivenom testing (Sells et al., 1997; 1998).

The modified zymography technique described here, developed to assess the potential of antibodies to neutralize snake venom toxins, enables efficient identification of antibodies that inhibit protease function. Previously Bee et al, (2001) demonstrated that gelatin zymography (SDS-PAGE incorporating gelatin) is an efficient means of identifying proteolytic enzymes in venoms. Because gelatin is denatured collagen, measurement of venom gelatinolytic activity appears to be a reasonable, if not infallible, *in vitro* method for estimating venom haemorrhagic activity. In this study I and other members of the Alistair Reid Venom Research Unit, have explored whether the gelatin-zymography technique could be adapted to assess the ability of antibodies to neutralize these enzymes. We report, for the first time, that the addition of antivenom into the matrix of the zymogram neutralizes the function of venom gelatinolytic proteases. A manuscript describing the work is currently under review for publication in the journal of Immunological Methods.

8.2. Materials and methods

8.2.1. Snake venoms

Venoms were extracted and lyophilised from snakes maintained in the herpetarium, Liverpool School of Tropical Medicine.

(i) Five venom samples from *E.ocellatus* occurring in distinct geographical areas [Fanshin, Langtang, Zamko, Saminako and Kaltungo] in Nigeria were used for SDS-PAGE.

(ii) Two different sets of venoms were used for the antibody zymography assays. The first consisted of ten viper species; *Bothrops jararaca* [Bj] and *B. atrox* [Ba] from Brazil, *Bitis arietans* [Bita] from Ghana, *Crotalus atrox* [Ca], *C. viridis helleri* [Cvh] and *Agkistrodon contortrix laticinctus* [Acl] from USA, *Protobothrops flavoviridis* [Pf] from Japan, *Echis ocellatus* [Eo] from Nigeria, *E. pyramidum leakeyi* [Epl] from Kenya, and *Cerastes cerastes cerastes* [Ccc] from Egypt. The second set consisted of venoms from twelve *Echis* viper species; *E. multisquamatus* [Em] from Turkmenistan, *E. pyramidum* [Ep-SA] and *E. coloratus* [Ec-SA] from Saudia Arabia, *E. pyramidum* [Ep-I] from Iran, *E. pyramidum* [Ep-O] from Oman, *E. pyramidum leakeyi* [Epl] from Kenya, *E. ocellatus* [Eo-N] from Nigeria, *E. sochureki* [Es] from Pakistan, *E. leucogaster* [El] from Mali, *E. pyramidum* [Ep-T] from Tunisia, *E. ocellatus* [Eo-G] from Ghana and *E. coloratus* [Ec-Is] from Israel.

8.2.2 SDS-PAGE

SDS-PAGE was performed as described in Chapter two.

8.2.3 Antivenoms

Two antivenoms were used in this analysis. EchiTAb^{TM} (Micropharm Ltd, Wales) is an effective antivenom developed in sheep immunised with venom from *E. ocellatus* (Laing et al, 1995; Meyer et al., 1997) consisting of ovine, papain-cleaved Fab fragments of IgG. The SAIMR *Echis* antivenom, developed in horses immunised with *E. ocellatus* venom (South African Institute for Medical Research, SAIMR) consists of equine, pepsin-cleaved $F(ab')_2$ IgG fragments (Theakston, 1997). I have used Fab fragment antibody from non-immunised sheep (MicroPharm, Ltd) and immunoglobulin (Ig)-depleted foetal calf serum as controls. The starting concentration for the antivenoms and control sera was 120mg/ml and PBS was used to prepare dilutions.

8.2.4. Gelatin-zymography

The gelatin-zymograms were constructed using published protocols (Feitosa et al., 1998). Fifteen percent SDS-PAGE (3.75 ml of 40% polyacrylamide, 2.5 ml Tris-pH 6.8, 2.5 ml distilled water, 150 µl of SDS, 50µl of 10% ammonium persulphate and 10µl TEMED) gels were co-polymerized with 2.5 ml molten gelatin (Sigma) at a final concentration of 2.5mg/ml in a Mini Protean 3 apparatus (BioRAD). Venoms were reconstituted with cold PBS (4°C) to a final concentration of 5mg/ml and mixed with an equal volume of protein loading buffer lacking β-mercaptoethanol (2%SDS, 62mM TrisHCl, pH 6.8, 0.1 % bromophenol blue and 10% glycerol without boiling). Ten microliter of each venom was added to the gelatin zymogram and separated by electrophoresis at 115 volts for 90 minutes. To re-nature venom proteolytic enzymes, SDS was removed from the gel by three 10 minute washes in 2.5% Triton-X 100 (BDH) solution. The gels were then

incubated overnight at 37°C in zymogram reactivating buffer (50mM Tris-HCl pH 8.0, 5 mM $CaCl_2$, 10 ng NaN_3), stained with 5% Coomassie Blue R-250 (BDH) for 15 minutes and destained with a 30% methanol, 7% acetic acid and 63% water solution. Clear zones of substrate lysis against a blue background indicated the presence of gelatinolytic venom enzymes.

8.2.5. Antibody-zymography

To assess the protease-neutralizing efficacy of antivenom, simply substituted water for antivenom or control antibody preparations in the preparation of the zymograms, without further modification of the gel constituents or protocol. The total volume of the mini gel was 5.6 ml. Therefore to achieve the required dilution of antibody preparations of 1:10 (12 mg/ml antivenom), 1:100 (1.2 mg/ml antivenom) and 1:1000 (0.12 mg/ml antivenom), I respectively substituted 560 µl, 56 µl and 5.6 µl of water with antibody. The total volume of water or water/antibody was 2.5 ml.

8.2.6. Antibody SDS-PAGE control gels

To investigate the extent to which antibody in gels inhibited the electrophoretic migration of venom proteins, I constructed and performed antibody-zymograms as above, but without gelatin. The venom protein profiles were conventionally stained with Coomassie blue.

8.3 Results.

The following two sections [8.3.1-8.3.2] describe SDS-PAGE and protease-activity profiling of *E. ocellatus* venom samples from snakes resident in five distinct regions of Nigeria.

8.3.1 Protein composition of E. ocellatus venom taken from species located in different areas of Nigeria.

This section demonstrates the proteome profile of *E. ocellatus* venom from five different regions in Nigeria. Although samples were loaded with the same concentration (5mg/ml) and showed similarities in the number of protein bands, the intensity of their protein composition varied somewhat. This was apparent in venoms particularly from Zamko and Saminako.

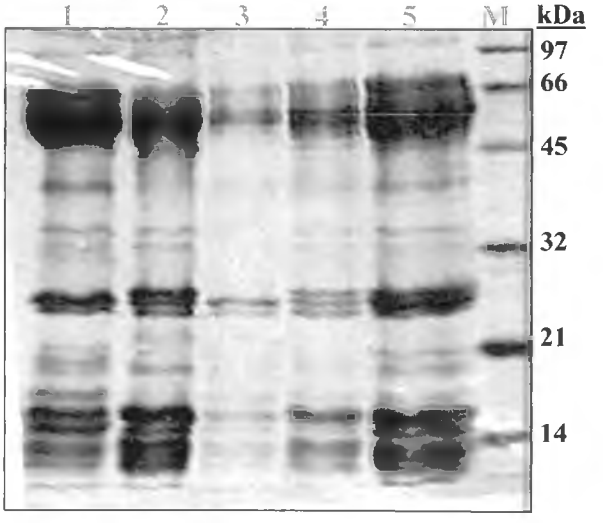


Fig.8.1. SDS-PAGE of *Echis ocellatus* venom samples from five different regions in Nigeria. *E. ocellatus* venom samples [Lane 1-5, referred to samples obtained from Fanshin (1), Langtang (2), Zamko (3), Saminako (4) and Kaltungo (5)]. 5mg/ml were electrophoresed in 15% polyacrylamide gel under reducing conditions. The gel was stained with Coomassie Blue R-250 and destained. Low molecular weight markers (Bio-RAD) shown in Lane 1 were phosphorylase b (97kDa), bovine serum albumin (66kDa), ovalbumin (45kDa), carbonic anhydrase (32 kDa), soyabean trypsin inhibitor (21kDa) and lysozyme (14kDa). After electrophoresis, the gel was stained in 5% Coomassie blue for 15 minutes and destained with methanol: acetic acid: water (30: 7: 63%).

8.3.2. Zymography profile of the five samples of E. ocellatus venoms

Determination of gelatinolytic profiles of *E. ocellatus* venom samples from the five geographical areas are shown in Fig.8.2 using gelatin substrate. The gelatinolytic activities of all venom samples generally were very similar except for the Kaltungo venom sample which shows that it possesses low molecular weight gelatinolytic activities.

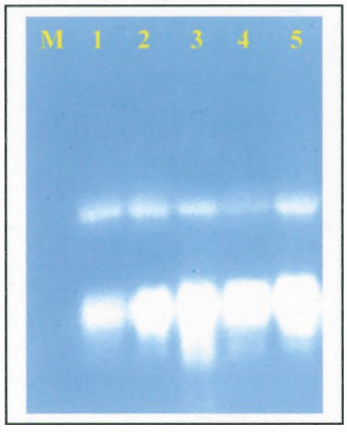


Fig.8.2. ***Gelatinolytic activity of E. ocellatus venom samples from the five geographical regions.*** Lanes 1-5 represent Langtang, Zamko, Kaltungo, Saminako and Fanshin, respectively. Samples (5 mg/ml) were electrophoresed into the zymogram 15% PAGE gels at 115 constant volts. After electrophoresis the gels were washed in 2.5% Triton X-100 for 30 min to remove SDS and then the gels were incubated in an activating buffer (50mM Tris-HCl [pH 8.0], 5 mM $CaCl_2$, 10ng NaN_3) at 37°C for 18h, which then stained in 5% Coomassie blue for 15 minutes and destained with methanol: acetic acid: water (30: 7: 63%).

8.3.3. Comparison of the profile of E. ocellatus venom with venoms from other Echis species.

The *E. ocellatus* venom samples from Kaltungo were readily available from our hepaterium. The Kaltungo samples were a reasonable representative of the protein composition (Fig.8.1) and protease-activity (Fig.8.2) of *E. ocellatus* venom and thus, were selected for all future experiments performed in this chapter.

Comparison of the *E. ocellatus* venom protein profile with venoms from other *Echis* species demonstrated a generally dominant composition of 50-60 kDa, 25-30 kDa and 10-20 kDa molecules with minor variants between the species (Fig.8.3). Further investigation should determine whether these similarities and differences correlate with the functional effects of the venoms.

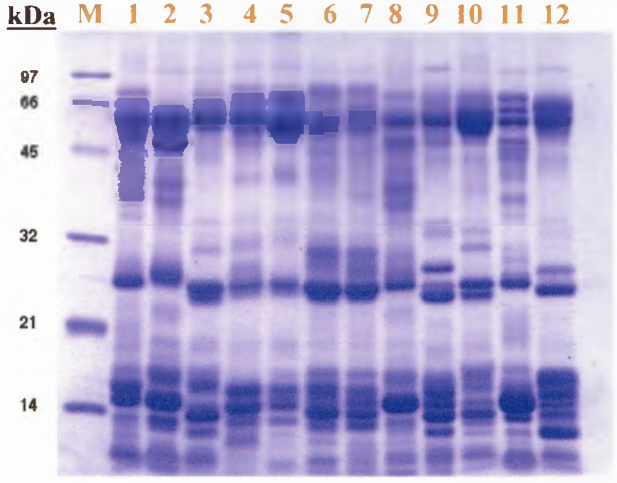


Fig.8.3. Comparison of proteome variation of *E. ocellatus* venom sample (Kaltungo) with those of the snake venoms of other *Echis* species. Lane 1-12 as stated in section 8.2.1 represent: [Em] from Turkmenistan, [Ep-SA], [Ec-SA] from Saudia Arabia, [Ep-I] from Iran, [Ep-O] from Oman, [Epl] from Kenya, [Eo-N] from Nigeria, [Es] from Pakistan, [El] from Mali, [Ep-T] from Tunisia, [Eo-G] from Ghana and [Ec-Is] from Israel. Samples (5 mg/ml) were electrophoresed into 15% SDS-PAGE gel at 115 constant voltage. After electrophoresis, the gel was stained with 5% Coomassie blue for 15 minutes and destained with methanol: acetic acid: water (30: 7: 63%).

8.3.4. Antibody zymography

8.3.4.1. *Conventional gelatin zymography.*

All the viper venoms contained gelatinolytic proteases as illustrated in the conventional gelatin-zymograms (Fig.8.4a, Fig.8.5a). However, significant variation in gelatinolytic

activity was evident across all the viper genera (Fig.8.4a) and even within species of the same genus (Fig.8.5a). Several samples from both sets of venoms had only a single proteolytic band, whereas others contained two or more gelatinolytic proteases of distinct molecular weight. With some exceptions, the *Echis* venoms contained fewer high molecular weight gelatinolytic proteases than venoms from other species as shown in (Fig.8.4a). While the gelatinolytic activity of each batch of venom was consistent within each experiment, there were batch-batch differences in venoms from some species (eg, *E. p. leakeyi* (Epl) in Fig.8.4a and Fig.8.4b). Venoms from snakes of the same species can exhibit age-related functional changes (Theakston and Reid, 1978), which may account for the above batch-batch gelatinolytic profile differences.

8.3.4.2. Antibody gelatin zymography.

The addition of antibodies to the matrix of the gelatin zymograms achieved the objective of illustrating the protease-neutralizing ability of antibodies and identifying the molecular weight of the target protease. The gelatinolytic activities of the first set of viper venoms (Fig.8.4a) were abolished by the presence of the highest concentration of antivenom (1:10) in the zymogram (Fig.8.4b) and inhibited in a dose-dependent fashion by inclusion of Fab EchiTAb^{TM} at 1:100 and 1:1000 concentrations (Fig.8.4c and d). Zymograms incorporating 1:10 concentrations of either control [normal] sheep Fab (Fig.8.4e) showed proteolytic profiles similar to that of the conventional zymogram (Fig.8.4a), demonstrating that the protease-neutralizing ability of EchiTAb^{TM} was effected by specific Fab immunoglobulin molecules.

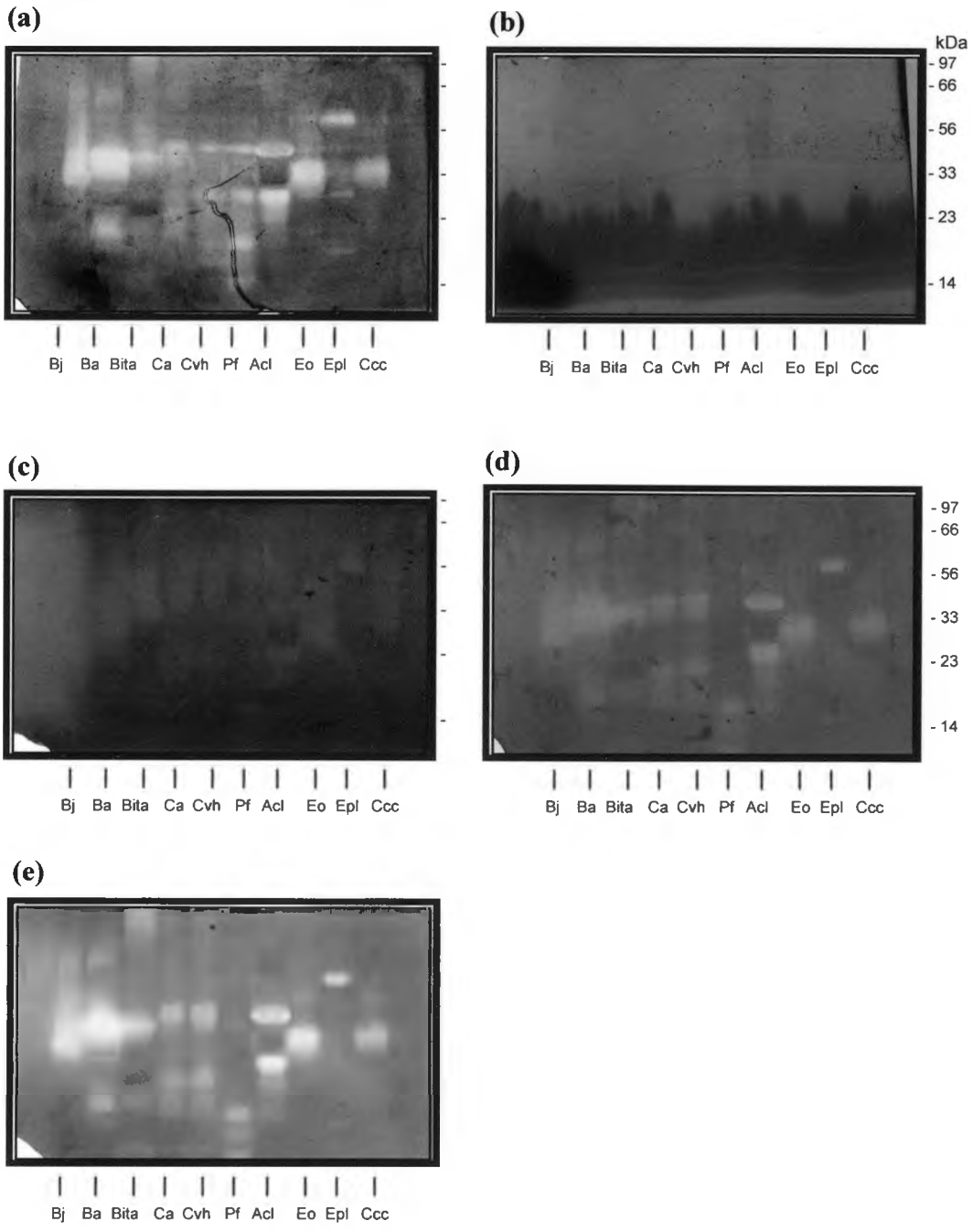


Fig.8.4. Neutralisation of viper venom gelatinolytic proteases by Fab EchiTAb™ antivenom. Fifty µg of venom from *Bothrops jararaca* [Bj], *B. atrox* [Ba], *Bitis arietans* [Bita], *Crotalus atrox* [Ca], *C. viridis helleri* [Cvh], *Protobothrops flavoviridis* [Pf], *Agkistrodon contortrix laticinctus* [Acl], *Echis ocellatus* [Eo], *E. pyramidum leakeyi* [Epl] and *Cerastes cerastes cerastes* [Ccc] were added to (**a**) conventional gelatin-zymgrams and antibody-zymograms (AZ) containing EchiTAb™ antivenom at 1:10 (**b**), 1:100 (**c**), 1:1000 (**d**) dilutions and a 1:10 dilution of control (non-immunised sheep) ovine Fab (**e**).

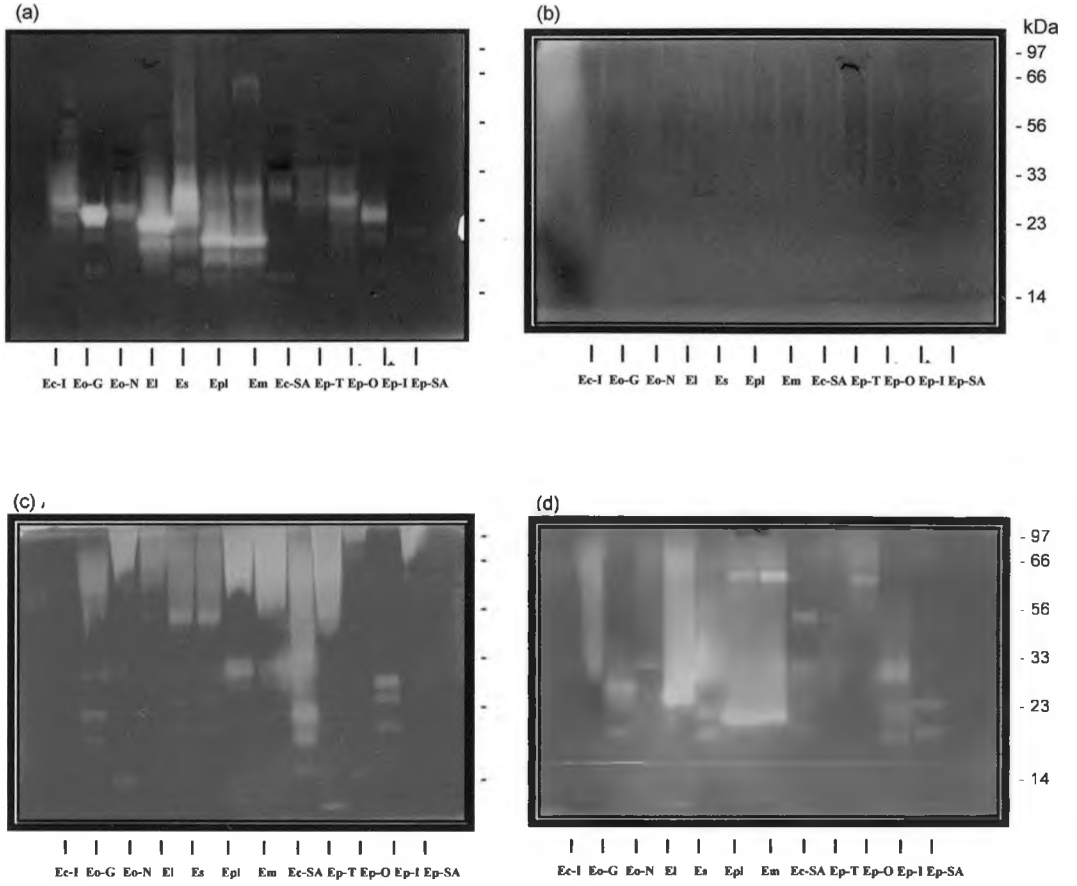


Fig.8.5. Neutralisation of viper venom gelatinolytic proteases by $F(ab')_2$ SAIMR antivenom. Fifty μg of venom from *E. coloratus* [Ec-Is], *E. ocellatus* from Ghana [Eo-G] and Nigeria [Eo-N], *E. leucogaster* [El], *E. sochureki*[Es], *E. pyramidum leakeyi* [Epl], *E. multisquamatus* [Em], *E. coloratus* [Ec-SA] and *E. pyramidum* from Tunisia [Ep-T], Oman [Ep-O], Israel [Ep-I] and Saudi Arabia [Ep-SA] were added to (**a**) a conventional gelatin-zymgrams and antibody-zymograms (AZ) containing $F(ab')_2$ SAIMR antivenom at 1:10 (**b**), 1:100 (**c**), 1:1000 (**d**) dilutions.

The ability of the SAIMR *Echis* equine $F(ab')_2$ antivenom to neutralize the gelatinolytic proteases in venoms of the various *Echis* vipers was similarly dose-dependent (Fig.8.5b, c and d). Unfortunately, I was unable to obtain $F(ab')_2$ from non-immunised horses to run this appropriate control. Some venom proteases in the SAIMR antivenom zymograms appeared to be more subjected to a delayed migration (smearing Figures 2c and d) than gels containing the EchiTAb^{TM} antivenom and we attribute this observation to the larger fragment size of the $F(ab')_2$ in the SAIMR antivenom.

8.3.4.5. Antibody SDS-PAGE

The possibility that inclusion of antivenom in the gel matrix resulted in indiscriminate precipitation of the venom proteins was a concern and difficult to assess because staining of gelatin made visualization of the venom proteins difficult. To address this, I have constructed and performed antibody-zymograms as above, but without gelatin (Fig.8.6). The venom proteins were very difficult to detect in the AZ gel (Fig.8.6b) incorporating EchiTAb^{TM} at 1:10 concentration because of the blanket staining of the IgG Fab fragments but they were readily seen in AZ gels incorporating EchiTAb^{TM} at 1:100 and 1:1000 (Fig.8.6c and d, respectively). The migration profiles of the venom proteins in the latter two gels were nearly identical to the AZ gels without antivenom (Fig.8.6a). These results indicate that, at antivenom concentrations which permitted detection of the venom proteins, inclusion of antivenom into the AZ gel did not significantly effect venom protein electrophoretic mobility.

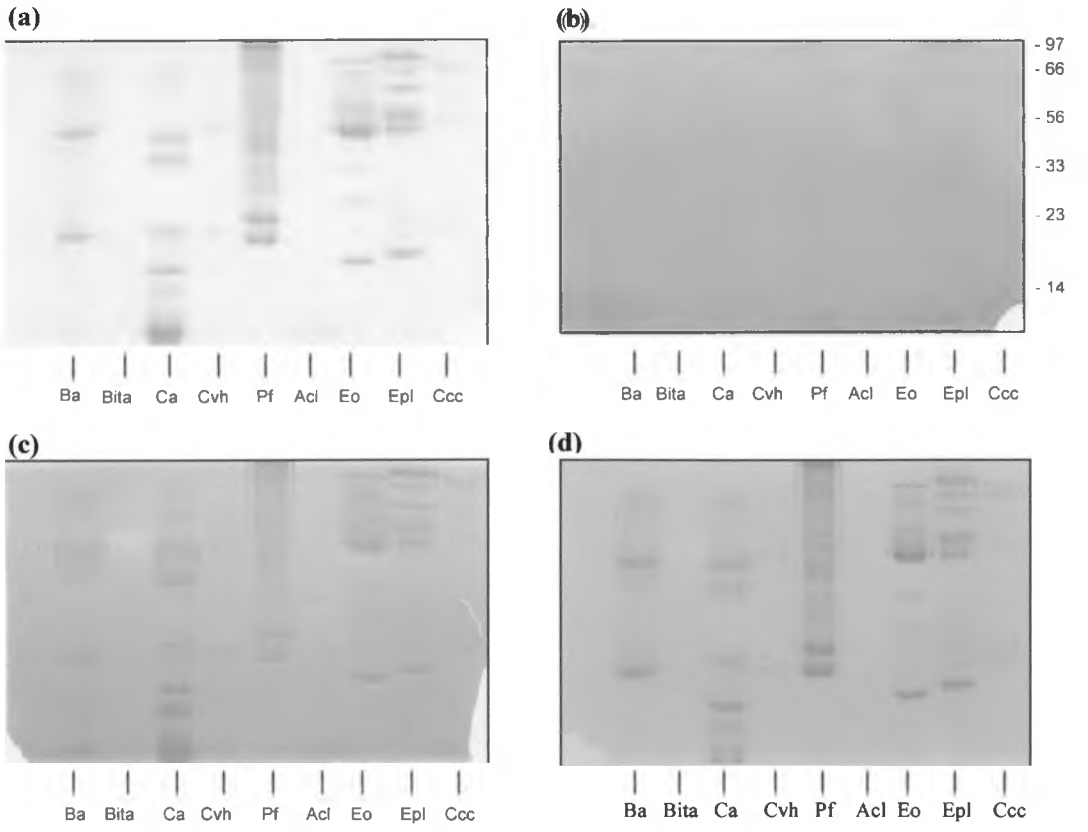


Fig.8.6. Fab immunoglobulin fragments do not inhibit the electrophoretic migration of venom proteins. Fifty μg of venom from *B. atrox* [Ba], *Bitis arietans* [Bita], *Crotalus atrox* [Ca], *C. viridis helleri* [Cvh], *Protobothrops flavoviridis* [Pf], *Agkistrodon contortrix laticinctus* [Acl], *Echis ocellatus* [Eo], *E. pyramidum leakeyi* [Epl] and *Cerastes cerastes cerastes* [Ccc] were added to (**a**) an antibody-zymogram constructed and prepared without gelatin (AZ-gelatin) and to AZ-gelatin gels containing EchiTAb^{TM} antivenom at 1:10 (**b**), 1:100 (**c**) and 1:1000 (**d**) dilutions.

8.4. Discussion

In the first attempt to identify antibodies specific to gelatinolytic venom proteins, I have subjected venom/gelatin-zymograms to conventional immunoblotting procedures and incubated the nitrocellulose filters with antivenoms using protocols similar to those described by Poppelmann et al., (2002). This adaptation was however quickly discontinued because of the poor visual quality of the results and because it provided no useful data on the protease-neutralizing ability of the antibodies.

I next adopted conventional methods to determine the preclinical efficacy of antivenom. Thus, venom and equine antivenom were incubated together at 37°C for 30 minutes and then the mixture was subjected to gelatin-zymography. Unfortunately this assay was not satisfactory because of the extensive distortion in the higher molecular weight range of the gel, which I presumed to be due to the intact IgG. I did not proceed further with this protocol because of additional concerns over the possibility that IgG/protease complexes in the starting mixture would be separated during electrophoresis and thus restore the gelatinolytic activity of the protease.

Since the first description of gelatin-zymography (Heussen and Dowdle, 1980), there have been several modifications including incorporation of substrates such as starch (Martinez et al, 2000), fibrin (Choi et al, 2001), fibrinogen (Komori et al 1994), casein (Raser, 1995) and milk powder (Poppelmann et al, 2002) to detect and isolate proteases from biological materials. Immunoblotting of zymograms has also been used to detect proteases in complex culture supernatants (Poppelmann et al, 2002). Reverse zymography, which incorporates proteases and protease substrates in the gel matrix, has been used to identify

endogenous inhibitors of mammalian collagenase and stromelysin (Herron et al, 1986) and snake venom inhibitors of venom gelatinolytic proteases (Bee et al, 2001). My present data demonstrating that the protease-neutralizing function of specific antibodies is retained when antibody is incorporated into the zymogram gel matrix, further extends the versatility of this technique. I have named this technique "antibody zymography".

The incorporation of viper antivenoms, comprising either Fab or $F(ab')_2$ fragments of IgG from sheep (Fig.8.4b, c, and d) or horses (Fig.8.5e, b, c and d) immunised with viper venoms, into gelatin-zymograms demonstrated a concentration-dependent neutralization of viper venom proteases. The contrasting ineffectiveness of Fab from non-immunised sheep (Fig.8.4e) and Ig-depleted foetal calf serum (Fig.8.5e) in this assay demonstrated that antivenom-neutralization of venom proteases was affected by specific immunoglobulin moieties. I had not anticipated that antivenoms generated against venom from a single *Echis* species would prove capable of neutralizing venom proteases from such a wide spectrum of phylogenetically diverse vipers. In mitigation of this apparent lack of specificity of the technique, it is important to note that the amino acid sequence and structure of venom proteases are highly conserved amongst the vipers (Bjarnason and Fox, 1994; Moura da Silva et al., 1995; Kini and Evans, 1987; Oyama and Takahashi, 2002) and that antivenoms (Kornalik and Taborska, 1989) and specific antivenom toxin antibodies (Harrison et al., 2003; Tanjoni et al., 2003) exhibit equal ability to bind to venom proteins from diverse viper species.

A restriction of the antibody zymogram technique is that it is preferable to incorporate purified IgG components rather than whole anti sera into the gel matrix. Thus, I obtained

identical protease-inhibiting results (not shown) from venom gelatin-zymograms incorporating sera from venom toxin DNA-immunised mice and normal mice. This result is consistent with that of Escalante et al. (2004) and strongly indicates that there are protease-inhibitory elements including α_2-macroglobulin in murine sera that mask the inhibitory activities of protease-specific antibodies. The lack of constitutive protease inhibitors in foetal calf serum (Fig.8.5e) indicate, however, that this is not likely to be universal amongst mammalian sera and is consistent with the observation that some mammals, such as opossum, hedgehog and mongoose, contain inhibitors of venom proteases that confer significant resistance to the pathology of viper envenoming (Rocha et al, 2002).

The size of the immunoglobulin molecule incorporated into the zymogram is another restriction of the antibody zymogram method. Results (data not shown) from zymograms incorporating equine antivenoms consisting of intact IgG showed extensive inhibition of the electrophoretic migration of venom gelatinolytic proteases and distorted the upper region of the zymogram. It is possible that this anomaly could be reduced by substitution of glycine with tricine in the construction of the SDS-PAGE (Martinez-Moya et al., 2002). Furthermore, the smaller the IgG fragments, the greater the likelihood that they will migrate under electrophoretic pressure because they are less tightly enmeshed within the zymogram matrix. I believe that this explains the reproducibile bichromic image of the Fab EchiTAb^{TM} antivenom (Fig.8.4b) as opposed to the monochromic image of the $F(ab')_2$ SAIMR antivenom (Fig.8.5b) at 1:10 concentrations. I have postulated that the bichromic image of the Fab antivenom disappears at 1:100 and 1:1000 concentrations (Fig.8.4c and d

respectively) because the diluted IgG fragments comprise less of the Coomassie Blue-stained matrix than at the 1:10 concentration.

Despite these limitations, the antibody zymogram is likely to prove useful for assessing the ability of specific antibodies to neutralize the *in vitro* functions of venom zinc metalloproteases and serine proteases. Work needs to be done in future to determine whether the antibody zymogram can be further modified into an ELISA format, along the lines of the gelatin degradation ELISA which has been utilised previously (Bee et al, 2001). If successful this will increase the high throughput potential of the technique. It is also possible that antibody zymography could reduce the numbers of mice required for current preclinical efficacy assessment of viper antivenoms.

Chapter 9

General discussion

9.1. General discussion

The overall objective of my PhD project was to generate antibodies by DNA immunisation against the most haemostasis-disruptive molecules in *E. ocellatus* venom. These were identified, as discussed in Chapter 1, as SVMPs, PLA_2s, CTLs and serine proteases. Because cDNAs encoding these molecules from *E. ocellatus* venom were not present in the genetic databases, it was necessary for me to create an *E. ocellatus* venom gland cDNA library to isolate toxin-encoding cDNA.

An *E. ocellatus* venom gland cDNA library was therefore constructed and demonstrated [by random isolation of cDNA] to contain sequences of sizes ranging from 1.8 to 0.4 KB. DNA encoding the target toxins were expected to fall within this range. The absence of specific antibody to the target *E. ocellatus* toxins discounted an antibody-screening approach to isolate toxin-encoding cDNAs. Therefore, a DNA screening approach was needed. A conventional DNA-hybridization screening of the library was impossible because of the lack of specific toxin-encoding DNA. The only viable technique was therefore PCR amplification. The next scientific challenge was therefore to design PCR primers to enable the isolation of cDNAs encoding the target molecules. I approached this by examining numerous DNA sequences encoding the target toxins from a wide variety of vipers as possible. It was soon apparent that, despite extreme phylogenetic and geographical origin differences between the vipers, the toxin sequences contained domains that were highly conserved. Since these domains included the extreme 5' signal peptide sequences and, usually, the extreme 3' terminals, it was obvious that I could exploit this phylogenetic sequence conservation to achieve the project objectives. Accordingly, I designed consensus 5' and 3' PCR primers for PLA_2, CTL and serine proteases. A slightly

different approach was taken for PCR amplification of SVMPs because (i) the large propeptide domain of SVMPs is cleaved to produce the nascent protein and (ii) the SVMP-specific DNA immunisation construct was always intended to express only the C-terminal DC domain.

This PCR approach to isolating *E. ocellatus* toxin encoding cDNA was highly successful and resulted in a significant contribution to the literature and genetic databases concerning SVMPs (Harrison et al., 2003), PLA_2 (Bharati et al., 2003), CTL (Harrison et al., 2003) and serine proteases (unpublished). The main contributions of these studies, as described in detail in the previous chapters, are summarized bellow.

E. ocellatus *PLA_2-encoding cDNA.* In Chapter 3 I described the isolation of novel cDNAs encoding twenty colonies ($EoPLA_2$ 1-20) of 416 nucleotides that corresponded to an open reading frame that predicts a polypeptide of 138 amino acids and a molecular weight of 15.7kDa. The isolated clones were identified (BLAST) as belonging to the sub-group II of the PLA_2 superfamily. The observed sequence discrepancies of the $EoPLA_2$ cDNAs revealed six distinct variants out of the twenty clones mentioned above. The deduced amino acid sequences of these cDNAs encoded proteins with high overall sequence similarity to the viper group II PLA_2 protein family. The work described in this chapter formed part of a publications by our group (Bahrati et al., 2003).

E. ocellatus *CTL-encoding cDNA.* In Chapter 4 I reported the cloning of novel cDNA sequences encoding seven groups of the haemostasis-disruptive C-type lectin (CTL) proteins from the venom glands of *E. ocellatus*. All these CTL sequences consisted of 441 nucleotides that were predicted to encode open reading frame proteins of 148 amino acids

(17.2 kDa). All EoCTL sequences showed a greater sequence similarity to the β than α CTL subunits in venoms of related Asian and American vipers. The work in this chapter formed part of a publication by our group (Harrison et al., 2003)

E. ocellatus serine protease-encoding cDNA. The utilization of PCR amplification of *E. ocellatus* venom gland cDNA with the viper serine protease-specific primers was successful and produced 14 cDNAs sequences that were identified (BLAST) as belonging to the serine protease enzyme family. All *EoSP* cDNAs were of similar total length (approximately 0.80 kb) and encoded 260 amino acids with a predicted molecular weight of 28.5 kDa. Using a surface probability algorithm, I was able to assign the 14 *E. ocellatus* serine protease cDNAs into four main variants [*EoSP-01*, *EoSP-03*, *EoSP-07* and *EoSP-17*]. Sequence similarities of the EoSP variants were 62-80% with the kinin-releasing and fibrinogen-clotting serine protease (KN-BJ) from venom of *B. jararaca* (Serrano et al., 1998).

E. ocellatus SVMP-encoding cDNA. I have described the isolation of *E. ocellatus* venom gland cDNAs encoding a protein of 514 amino acids that showed 91% sequence similarity to Ecarin, a prothrombin-activating metalloproteinase from the venom of the East African viper, *E. pyramidum leakeyi,* that induces severe consumption coagulopathy. The high degree of sequence conservation of *EoMP-6* with other prothrombin-activating and haemorrhagic metalloproteinases of related vipers extended to the conservation of structural domains predicted to have high immunogenic potential. The work in this chapter has been published (Hasson et al., 2003)

The platelet-aggregating disintegrin venom toxins were not included in the target list because I considered that the sequence similarity of disintegrin to the DC domain of the SVMP was so extensive that an antibody against the DC domain would be predicted to neutralize both disintegrin and SVMP function. There are a number of other important viper toxins [as described in Chapter 1] that were not included in the target list such as L amino acid oxidase, hyaluronidase and bradykinin-like molecules. The shortage of sequence data for these additional viper toxins, which may or may not be present in *E. ocellatus* venom, coupled with the lack of available time meant that I could not incorporate these molecules into my PhD objectives.

My next task was to prepare DNA immunisation constructs from the cDNAs I isolated that encoded *E. ocellatus* SVMP, CTL and serine protease toxins. There was insufficient time to process the *E. ocellatus* PLA_2 cDNA into an immunisation construct. To achieve this, I manipulated the cDNAs to remove the native signal peptide from the EoCTL and Eoserine protease sequences because a murine signal peptide was incorporated within the pSecTag-B plasmid. For the reasons described above and in Chapter 7, I removed the partial propeptide and metalloprotease-catalytic domains from *EoMP-6* to create an immunisation construct designed to express only the DC domain. For reasons that are not obvious, mice immunised with the EoCTL and Eoserine protease constructs failed to seroconvert. In contrast, mice immunised with the EoDC DNA construct responded with high titres of IgG. My results showed that the route of DNA delivery was very important in terms of the efficiency of seroconversion. Intramuscular injection of DNA failed to stimulate detectable antibody responses, intradermal injections of EoDC DNA induced modest IgG responses suggesting that the skin is a more favoured tissue for DNA injection

than muscle. However, Gene Gun immunisation was shown to induce far superior IgG titres. This result agrees with the literature (Harrison et al., 2000) as describe in extensive details in Chapter 1.

The cellular responses of the DNA immunised mice was not examined in this study because the ultimate intent was to generate antibodies for passive serotherapy and not for vaccine purposes.

The results presented in Chapter 7 also showed that the EoDC-specific antibody reacted with analogous molecules in venoms of a variety of *Echis* species. This result was predicted from the antigenic index profile study performed in Chapter 6. If immunoblot reactivity is an accurate measure of toxin neutralization [which is yet to be empirically determined], then these results suggest that the structural conservation of venom toxins from phylogenetically-distinct vipers can be exploited by our toxin-specific antivenom approach to generate antivenoms ideally of remarkable potential. The treatment of snake bite by conventional antivenoms requires the administration of antivenoms raised from the same venom as that which envenomed the patient (Lalloo and Theakston, 2003). Other potential advantages of the toxin-specific DNA immunisation approach to antivenom include: cost – the more polyspecific an antivenom the less the production cost. Production – the production of DNA is cheaper than any other immunizing material-including snake venom. Safety – does not involve extraction from highly dangerous snakes. However, attractive the potential of this approach to antivenom production is, there are several major obstacles to overcome. These include (i) the limitation of the PCR approach, (ii) deciding upon the range of toxins required for inclusion in the DNA

immunisation to achieve maximal therapeutic effect (iii) how will the volumes of the respective toxin-specific antibodies comprising the final antivenom be determined (iv) the seroconversion effectiveness of sheep or horses, animals more appropriate to antivenom production than mice, to DNA immunisation are likely to be lower than mice. Therefore, considerable work will need to be conducted to identify the optimal plasmid constructs and immunisation protocols for generating high titre toxin-specific antibodies in sheep and horses.

Another problem specific to the toxin-specific DNA immunisation approach to antivenom production is the assessment of the efficacy of each antibody species. All of the current antivenom efficacy assays involve the assessment of the ability of an antivenom to neutralize highly complex physiological effects of whole venom. These effects require synergistic activities of many venom toxins that are usually not exerted by a single toxin. Therefore, the above assays are not suited and not appropriate for adequate preclinical tests on toxin-specific DNA immunisation-derived antibodies. The exception is the SVMPs that are capable of eliciting haemorrhage in the absence of other molecules. For this reason the MHD assay has been successfully used to determine the haemorrhagic effect of SVMPs (Howes et al., 2003) and the neutralizing efficacy of SVMP-specific antibody (Harrison et al., 2000). However, the MHD and other *in vivo* assays would not be appropriate for assessing the toxin-neutralizing-efficacy of antibodies generated to CTLs, serine proteases, disintegrins, PLA_2 and so on. To investigate a solution to this problem, I adapted an existing technique [zymography] that identifies proteins with proteolytic activity, by adding procedures to allow the identification of antibodies capable of neutralizing that proteolytic activity. I reasoned that (i) many of the most potent viper

toxins are enzymes; (ii) that zymogram substrates are available for most of these venom toxins and (iii) that incorporating antibodies into the zymogram matrix would be likely to prevent enzyme function. The results presented in Chapter 8 largely ratify these predictions and indicate that while the new technique, called antibody zymography, has many useful applications, it also has limitations. The limitation with the greatest bearing on the present project is that antibody to be analysed must be a Fab or $F(ab')_2$ derivative of the intent IgG. This observation meant that I could not submit my EoDC-specific antibodies to the test, and is true for most antibodies our group generates in mice, because the volume of antibody generated from DNA-immunised mice is insufficient for the assay after removal of the Fc domain. It also means that our group will need to produce toxin-specific antibodies in rabbits [or other animals yielding larger volumes of sera] if we wish to utilize this new test. The test does however, have many applications outside of the venom field, example testing the functional utility of monoclonal antibodies. A manuscript describing this work is being reviewed for publication in the Journal of Immunological Methods.

Finally the results obtained in this study were significant and may contribute in representing a valuable addition to the venom gland transcriptome of the saw scaled viper *E. ocellatus* and revealed the toxins composition of the *E. ocellatus* viper to be as complex as that of the better characterised New World vipers. However, given the experience obtained in this project I would advocate preliminary experiments to determine whether the generated antibody against the carboxyl-disintegrin cysteine-rich domain, of the metalloprotease-like prothrombin activator, would contribute in neutralizing the effects of haemorrhgins in venom of *E. ocellatus* as well as other *Echis* species.

Appendix A

A.1 Chemicals and bioreagents

- General laboratory chemicals and reagents were purchased from BDH Ltd., and were Analar grade.

- Other chemicals were supplied as follows:

- Sigma Chemical Co. Ltd., Poole, UK

  Ammonium persulphate, ampicillin (sodium salt), kanamycine sulphate, tetracycline hydrochloride, chloramphenicol, calcium chloride, polyethylene glycol, X-Gal, IPTG.

- Life Technologies, Paisley, Scotland

  Selected agar, Select peptone, Select yeast extract

- Severn Biotech, Kidderminster, UK

  40%, 19:1 acrylamide: bisacrylamide solution

- Flowgen, Sittingbourne, UK

  NuSieve molecular grade agarose

- Sartorius, Epsom, UK

  Syringe Filters, 0.22μm

- Boehringer Mannheim Ltd, East Sussex, UK

  Restriction enzymes, DNA polymerase, DNA ligase, *Taq* polymerase, EDTA, nucleotide triphosphates, Whatman 3MM filter paper, Nitrocellulose

- Promega, Southampton, UK

  Restricton enzymes, DNA molecular weights markers.

- BIORAD, UK

  Low molecular weight protein markers.

- PIERCE, Illinois, US

  Coomassie Blue, Ponsue S reagents for determination of protein concentration.

- Sigma-Genosys or Sigma-Aldrich Ltd., U.K

  Custom oligonuceotides for PCR and DNA sequencing

Enzymes reactions were performed as per suppliers' instructions.
All solutions, where appropriate, were made up using double glass distilled, deionised water (ddH_2O)

- DNA Markres: Wild type λ DNA digested with *Hind* III and *EcoR*I to give DNA fragments of Size (Promega, Madison W1, USA): 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 2.000, 1,500, 1,000, 750, 500, 250 bp.

- Protein Markers: Low molecular weight (BIORAD) sizes: 97, 66, 44, 33, 23, 14 kDa. A vial containing lyophilised protein markers was re-constituted in 5 ml sample buffer (reduce buffer. see section A.5.).

A.2. Equipment

A2.1. Centrifuge and rotor details

- Eppendorf Anderman centrifuge, 1.5ml tube fixed angle housing. Maximum speed 13,000rpm.
- Sorval RC5BSS-34 rotor: Fixed angle 34o, 6 buckets, 46,000 RCF at maximum speed of 20,000 rpm.

A2.2. Other Equipment

- PCR Gene Cycler BioRad, Hercules, CA, USA.

- Transillumintor	320nm UV transilluminator TM36 supplied by Ultra Violet Products, Cambridge, UK.
- DNA sequencing machine	Beckman Coulter CEQ™2000 XL DNA Analysis System.
- ELISA plate reader:	BIORAD 450 Microplate reader

A.3. Biological Material

A3.1. Escherichia coli strains

Strain	*Genotype*	*Reference*
TOP10F′	{*lac*Iq Tn*10* (TetR)} *mcr*A .(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*.M15 .*lac*X74 *rec*A1 *ara*D139 .(*ara-leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitogen
XL1-Blue	*endA*1, *gyrA*96, *hsdR*17, *lac*⁻, *recA*1, *relA*1, *supE*44, *thi*-1,[F' *lacI*^{q}Z▲*M15*, *proAB*, *Tn*10]. Note: Tn10 confers resistance to tetracycline.	CLONTECH

E. coli strains were streaked on appropriate agar plates and maintained for short periods, not exceeding 3 to four weeks, at 4°C. Bacterial stocks were subjected to long-term storage at -20°C in LB containing 50% (v/v ratio) glycerol

A3.2. Animals

Mouse strain	Source
Balb/c	Biomedical Services, University of Liverpool

A.4. Reagent required for cDNA synthesis (CLONTECH

- First strand synthesis (10μM)
5'-AAG-CAG-TGG-TAT-CAA-CGC-AGA-GTG-GCC-ATT-ACG-GCC-GGG-3'

- CDC III/3' PCR Primer (10μM)
5'-ATT-CTA-GAG-GCC-GAG-GCG-GCC-GAC-ATG-d$(T)_{30}$N$_{-1}$N-3'
(N=A, G, C, or T; N-1= A, G, or C).

- 5X First-strand buffer
  Consist of the following:
  - 250 mM Tris (pH 8.3)
  - 30 mM MgCl2
  - 375 mM KCl

- Control Poly A+ RNA
  This is a human Placenta; 1.0μg/μl)

- *Sfi* I digestion
  -20 units/μl

- 10X *Sfi* I Buffer
- cDNA purification
  - CHROMA SPIN-400 Columns
  - 1X Fractionation Column Buffer

- Vector Ligation
  - λTriplEx2 (*Sfi I* A and B arms) 0.5μg/μl
  - T4 DNA ligase 400 units/μl

- 10X DNA Ligation Buffer
  Consist of the following:
  - 500 mM Tris-HCl (pH7.8)
  - 100 mM $MgCl_2$
  - 100 mM DTT
  - 0.5mg/ml BSA.

A.5. Reagents for bacteria culture

- Deionised H_2O (Milli-Q-filtered, not DEPC-treated)

- Transduction and tittering of λ phage in *E. coli*
  - 10 μl, 10 mM MgSO4
  - 1l LB, prepared for phage transductions or plaque tittering.

- Maltose stock solution 2%
  - 20g Maltose
  - 100 ml of deionised H_2O
  - Filter sterilize and store at 4°C.

- Luria Bertani broth medium
    - 31 g of ready mixed LB broth Lenox containing:
    - 10 g tryptone
    - 5 g yeast extract
    - 5 g NaCl
    - Final volume was made up to 1 l with deionised water and autoclaved.

- Luria Bertani (LB) agar plate
    - 31g of LB-Agar Lennox [containing 10g Trypton-B, 5g Yest Extract-B, 5g NaCl]
    - 15g Agar-B
    - 1 l de-ionised distilled water. Pour plates were stored at 4°C.

- SOC medium

    This was supplied by invtrogen, UK.
    - 20 g Bacto trypton
    - 5 g Bacto yeast extract
    - 0.6 g NaCl
    - 0.5 g KCl
    - 10 mM $MgCl_2$
    - 10 mM $MgSO_4$
    - 20 mM Glucose
    - Final volume was made up to 1 liter.

- Ampicillin (50mg/ml)
    - 0.5 g ampicilin powder (Boehringer Mannheim)
    - 10 ml de-ionised water, filtered sterilized, aliquoted and frozen at –20°C.

- Kanamycin (50mg/ml)
    - 0.5 g of kanamycin powder (sigma, UK)
    - 10 ml de-ionised water, filtered sterilized aliquoted and stored at 4°C.

- LB/MgSO4 soft-top agar
    - 1 l LB broth

- 10 ml (1M) MgSO4 (10mM final concentration)
- 7.2 g agar.
- Autoclaved and stored at 4°C.

- 10X Lambda dilution (SM) buffer.
  - prepared as shown in the table below.

	Final concentration	*To prepare 1L of solution*
NaCl	1.0M	58.3g
$MgSO4.7H_2O$	0.1M	24.65g
Tris-HCl (pH 7.5)	0.35M	350.0ml of 1M
Add H_2O to a final volume of 1L. autoclave and store at 4°C		

- 1X SM buffer
  - 100 ml,10x SM buffer (above)
  - 5ml 2%, Gelatin (0.01% final concentration). [The addition of gelatin stabilizes the library titer for long-term storage]
  - final volume was made up to 1 l with distilled water.
  - Autoclave and stored at 4°C.

- Blue/White screening of plasmid in *E Coli.*

  - IPTG (100mM in H_2O), Isopropyl β-D-thiogalactopyranoside.
  Filtered sterilize and stored at 4C

  - X-Gal (100mM)
  - Dissolve in dimethylformamide (DFM) and stored at –20°C.

A.6. Reagents for PCR

- Primers

  Primers for PCR were supplied by Sigma-Aldrich, UK. Stock primers solutions of 10*p*M were prepared and frozen at –20°C.

- *Taq* polymerase

  Supplied by Life Technologies, USA, in 5u/μl.

- Magnesium chloride ($MgCl_2$) solution

  Supplied by Roche, UK, in 25mM.

- PCR buffer, 10X.

  This was supplied by Life Technologies, USA , consisted of:

  - 20 mM Tris-HCl, pH 8.3
  - 50 mM KCl
  - 1.5 mM $MgCl_2$
  - 0.001% Gelatin.

- dNTP mix (Roche, UK) 2.5 mM

  -Each dNTP (dATP, dCTP, dGTP, dTTP) was supplied in 100mM concentration. A stock of dNTPs solution was prepared by combining the four dNTPs in equal volumes (125 µl each dNTP) 500µl of de-ionized water was added to bring the final concentration to 2.5 mM.

- TAE electrophoresis buffer

  1X working solution was prepared as follows,

  - 48.4 g Tris-base
  -11.42 ml Glacial acetic
  -7.44 g Na2EDTA.$2H_2O$
  - Final volume was made to 10 l.

- DNA Loading Buffer [6X SLOB]

  - 20% (w/v) FicolL 400
  - 0.1M Na2 EDTA, pH 8.0
  - 1.0% (w/v) SDS
  - 0.25% (w/v) Bromophenol blue
  - 0.25% (w/v) Xylene Cyanol

- DNA MW Marker (Promega, Madison W1, USA)

  - 1Kb Ladder was prepared by adding 100µl DNA ladder in 500µl 6x SLOB.

A.7. Cloning reagents.

- Restriction enzymes and incubation buffers.

The various restriction enzymes and their incubation buffer, which were used in the project, were supplied by promega, Germany.

The restriction enzymes *BamH* I and *Xho*I were used for DNA digestion and were provided in 10 u/µl concentration.

- SuRE/Cut buffer B

Consisted of

- 10 mM Tris-HCl
- 10 mM NaCl
- 5 mM $MgCl_2$
- 1 mM: 2mecaptoethanol pH8.0 (at 37°C) as supplied and used as a compatible buffer for digestion with *BamH I* and *Xho I*.

- T4 DNA ligase

This was provided as 1unit/l and used to join DNAs with sticky ends.

- Ligase reaction buffer (5x)

- Consisted of
- 250 mM Tris-HCl, pH7.6
- 50 mM ATP
- 5mM DDT
- 25% [w/v] polyethylene glycol-8000.
- The buffer was stored with T4 DNA ligase at –20°C.

A.8: Reagents for DNA and RNA extraction and analysis.

- Mini-spin prep kit, Qiagen, (Hilden, Germany)

- Qiagen cell re-suspension buffer (P1): 50 mM Tris-HCL, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A). Qiagen cell lysis buffer (P2): 200 mM NaOH, 1% SDS. Qiagen neutralization buffer. Qiagen neutralization buffer (P3): 750 mM

NaCl; 50 mM MOPS, pH7.0 15% ethanol; 0.15% Triton X-100. Qiagen washing buffer (PB) and Qiagen secondary washing buffer (PE).

- Qiagen plasmid Maxi kit solutions Qiagen, (Hilden, Germany)
  - Qiagen re-suspension buffer (P1): 50 mM Tris-HCL, pH 8.0; 10 mM EDTA; 100µg/ml RNase A). Qiagen lysis buffer (P2): 200 mM NaOH, 1% SDS. Qiagen neutralization buffer, (P3): 3 mM potassium acetate, pH5.5. Qiagen equilibration buffer, (QBT): 750 mM NaCl; 50 mM MOPS, pH7.0 15% ethanol; 0.15% Triton X-100. Qiagen washing buffer (QC): 1.0 M NaCl; 50 mM MOPS, pH 7.0; 15% ethanol. Qiagen elution buffer (QF): 1.25 M NaCl; 50 mM Tris-HCl, pH 8.5; 15% ethanol.

- Prep-A-Gene DNA purification kit
  - Binding buffer. Consist of; 6 M Sodium Perchlorate, 50 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0).
  - Washing buffer. Consist of 800mM NaCl; 40 mM Tris (pH 7.5); 4 mM EDTA (pH 7.5). Final concentration after addition of a 1x volume 100% ethanol: 400 mM NaCl; 20 mM Tris (pH 7.5); 2 mM EDTA (pH7.5); 50%ethanol (v/v).
  - Matrix was used according to manufacturer instructions and resuspended in binding buffer.

- Aqua Pure RNA isolation kit (Bio-RAD, USA)
  - RNA Lysis Solution
  - Protein/DNA Precipitation Solution.
  - RNA Hydration Solution.

- 10x 3-(N-morpholino)- propane sulphuric acid (MOPS) running buffer
  - 1x MOPS was prepared from the stock solution containing the following;
  -83.6 g (0.4M) MOPS, pH 7.0
  - 200 ml (0.5M) sodium acetate
  - 20 ml (0.5M) EDTA
  - Final volume was made up to 1l with deionised water [MOPS was stored should be stored in the dark at 4°C, as it is light sensitive].

- Ammonium acetate (0.5M)/ 10mg ethidium bromide
    - 0.5 M ammonium acetate
    - 0.5 μg/ml ethidium bromide
    - 0.5 M ammonium acetate or 10 mM sodium phosphate (pH 7.0)/1.1 M formaldehyde with and without 10 μg/ml acridine orange.

- 0.05 M NaOH/1.5 M NaCl
    - 5ml (10M) NaOH
    - 300ml (5M) NaCl
    - Final volume was made up to 1 l with deionised water.

- 0.5M Tris-Cl (pH 7.4)/1.5M NaCl
    - 500 ml (1M) Tris-Cl
    - 300 ml (5M) NaCl
    -Final volume was made up to 1l with deionised water.

- 20x SSC
    -175 g NaCl
    -88 g Trisodiumcitrate.$2H_2O$
    - pH was adjusted to 7.0 with 1M HCl
    - Final volume 1l with deionised water

- 2x SSC was prepared from the stock solution of 20x,
    - 100 ml of 20xSSC
    - 900 ml deionised water.

- 2x SSC/0.1% SDS
    - 100ml 20x SSC
    - 1g SDS
    - Final volume was made up to 1 l with deionised water.

- 0.2x SSC/0.1% SDS

-10ml 20x SSC

-1g SDS

- Final volume was made up to 1 l with deionised water

- 0.1x SSC/0.1% SDS

-5 ml 20x SSC

-1g SDS

- Final volume was made up to 1 l with deionised water

- Formamide Prehybridisation/Hybridization (FPH) solution

-5x SSC

-5x Denhadrant solution

-50% (v/v) Formamide

-1% (w/v) SDS

- 100µl/ml denatured salmon sperm DNA

- Formaldehyde loading buffer

The stock solution was 12.3 M (37%) formaldehyde, pH>4.0. containing the following:

- 1 mM EDTA, pH 8.0
- 0.25% (w/v) Bromophenol blue
- 0.25% (w/v) Xylene cyanol
- 50% (w/v) Glycerol

- Sample loading buffer (RNA-SLB) was ready made and its component as follows;

- 36 µl (12.3M) Formaldehyde
- 20 µl 10x MOPS
- 100 µl Formamide

* 40 µl Formaldehyde loading buffer [*was added after the above components were incubated for 15 minutes at 53°C]

A.9. General Stocks, Buffers, reagents

- Phosphate buffered saline (PBS)

  This was prepared by dissolving

  - 8 g sodium chloride (NaCl)
  - 0.2g potassium chloride (KCl)
  - 1.44 disdoium hydrogen orthophosphate (Na_2HPO_4)
  - 0.24g potassium dihydrogen orthophosphate (KH_2PO_4)
  - 800ml distilled water
  - pH was adjusted to 7.3 with HCl.
  - Final volume was made up to 1 l with distilled water.

- Sodium acetate pH 5.0, 3M

  - 24.6 g sodium acetate ($C_2H_3O_2Na$)
  - 80 ml distilled water.
  - pH was adjusted to 5.0 with glacial acetic acid ($C_2H_2O_2$)
  - Final volume was made up to 100 ml with distilled water.

- Calcium Chloride ($CaCl_2$) 1M stock.

  - 21.9g $CaCl_2.6H_2O$
  - 100 ml deionised water.

[Lower concentrations of ($CaCl_2$) were prepared from this stock].

- DEPC treated solution

  0.2ml DEPC was added to 100ml of solution and mixed vigorously to get DEPC into solution and then incubated at RT for 12 hours. DEPC was removed from solutions by autoclaving it. [Note: DEPC is carcinogen so gloves has to be worn.]

- Tris-HCl (TE) buffer.

10mM Tris-HCl, pH8.0; 1mM EDTA, pH8.0

- Isopropyl-b-D-thiogalactopyranoside [IPTG], 100mM

  -125 mg of IPTG

  - 10.05 ml deionised water.

The solution was sterilized by filtration (0.22μm) and frozen at –20°C

- Phenol:chloroform:Iso-amyl acohol

Combined ratio 25:24:1 (v:v:v ration) and kept at 4°C.

- Chloroform: Isoamyl alcohol

Combined ratio 24:1 and kept at 4°C

- Ethyenediamincetrateacetic acid (EDTA), 0.5M Stock solution.
  - 9.3g of EDTA
  - 50 ml deionised water.

Lower concentrations were prepared from this stock.

A.10. Reagents for protein electrophoresis

- Acrylamide (40%)

Used as required.

- Sodium dodecyl sulphate (10%)
  - 10 g SDS
  - 100 ml distilled water.

- Tris/HCL (1.5M); pH 8.8 buffer
  - 27.25 g Tris-base
  - 80 ml deionised water.
  - pH was adjusted to 8.8 with HCl
  - Final volume of 150 ml was made up with deionised water and stored at 4°C.

- Tris/HCl (0.5M); pH 6.8 buffer
  - 6.1 g Tris-base
  - 60 ml deionized water.
  - pH was adjusted to 6.8 with HCl
  - Final volume of 100 ml was made up with deionised water and stored at 4°C.

- Ammonium persulphate (APS), 10%
    - 1 g APS
    - 10 ml deionised water
    - Stored at 4°C for two weeks

- Protein loading buffer [SDS reducing buffer, 6xPLOB]
    - 1ml of 0.5M Tris/HCL; pH6.8,
    - 0.8ml glycerol,
    - 1.6 ml 10% [w/v] SDS
    - 0.4 ml 2-b-mercaptoethanol
    - 0.05% [w/v] bromophenol blue 0.2 ml
    - Final volume was made up to 8 ml.

- Protein loading buffer [SDS non-reducing buffer]
    - 4mls of 1M Tris/HCL; pH6.8
    - 5 ml glycerol
    - 1.5g SDS. [SDS was added to Tris buffer and stirred for 15mins on hot plate 30-40°C].
    - 300 ml bromophenol blue solution
    - Final volume was made up to 20ml

- Ethidium bromide [highly mutagenic, handle with care].

    Stock solution (0.5mg/ml),
    - 50 mg ethidium bromide
    - 100 ml-deionised H_2O.
    - Solution was stored at 4°C. [Note ethidium bromide is a carcinogen]

- Tetramethylethylenediamine (TEMED):
    - Was used as required.

-

Coomassie blue R250 stain (0.5%)

- 5 g coomassie blue stain
- 1 l [40% methanol and 10% acetic acid fixative solution].

- Ponceau S, 2%
  - 0.2g Ponceau stain powder
  - 100ml 3% TCA (Tri-chloroacetic acid)
  - Stored at room temperature.

- SDS Polyacrylamide gel (SDS-PAGE) 12 and 15%.

	Quantity of materials to prepare SDS with different percentage	
Materials	***12%***	***15%***
- 40% Acrylamide-BIS	**3.75ml**	**3.75ml**
- TRIS pH8.8	**2.50ml**	**2.5ml**
- DH_2O	**4.50ml**	**3.75ml**
- 10% SDS	**100µl**	**100µl**
- 10% APS	**60µl**	**60µl**
- TEMED	**07µl**	**07µl**

- Stacking gel preparation

Materials	***Quantity***
- 40% Acrylamide –BIS	**0.7ml**
- TRIS Ph6.8	**2ml**
- H_20	**5ml**
- 10% APS	**60ml**
- TEMED	**10ml**

- Protein electrophoresis solution (5X) stock solution.

Running buffer (1X) was prepared by adding 200ml of 5X buffer [Table below] to 800ml distilled water.

Material	*Quantity*
- **Tris Base**	**120g**
- **Glycine**	**375g**
- **SDS**	**40g**
- **H_2O**	**Up to 2 Litter**

- Destain buffer.

Destain buffer was prepared as shown in the following table:

Materials	*Quantity*
- **Methanol**	**200 ml**
- **Glacial Acetic Acid**	**750 ml**
- **ddH_2O**	**Up to 10 Litter**

- Gels for DNA electrophoresis

0.7% and 1.1% agarose gels were used throughout this work and prepared as follows;

- 0.7 g or 1.1 g agarose
- 100ml 1x TAE buffer
- heated up [Microwave, until dissolved]
- 15µg ethidium bromide. [This was added when solution was cooled down to about 50°C]

- Transfere buffer for Western blot.

-3 g Tris-base
- 14.4 g glycine

- 200ml methanol,
- Final volume was made up to 1 l with distilled water.

- Western blot washing buffer, TBST

1x Washing buffer was prepared as shown in the table below.

Materials	*Quantity*
- Tween-20	**10ml**
- 2M Tris pH8.5	**50ml**
- 5M NaCl	**300ml**
- H_2O	**Up to 10 liters**

- Western blot blocking solution.

5% [w/v] Skimmed milk was used as a blocking solution.

- 5 g skimmed milk powder
- 100 ml 1x TBS washing buffer.

- Western blot developer solution

- 50 mg of 3, 3'-Diaminobenzidin (DAB)
- 100 ml 1XPBS [mixed well]
- 25 µl Hydrogen peroxide (H_2O_2). [Addition of H_2O_2 was added when nitrocellulose membrane was ready to be submerged in the developer solution.]

- Iso-butanol saturated-Water

N-butanol mixed with water in 50:50 (v/v) ratio.

- Triton X-100

Used as required. In this study 2.5 ml of Tritonx-100 mixed well in 97.5ml of deionised water.

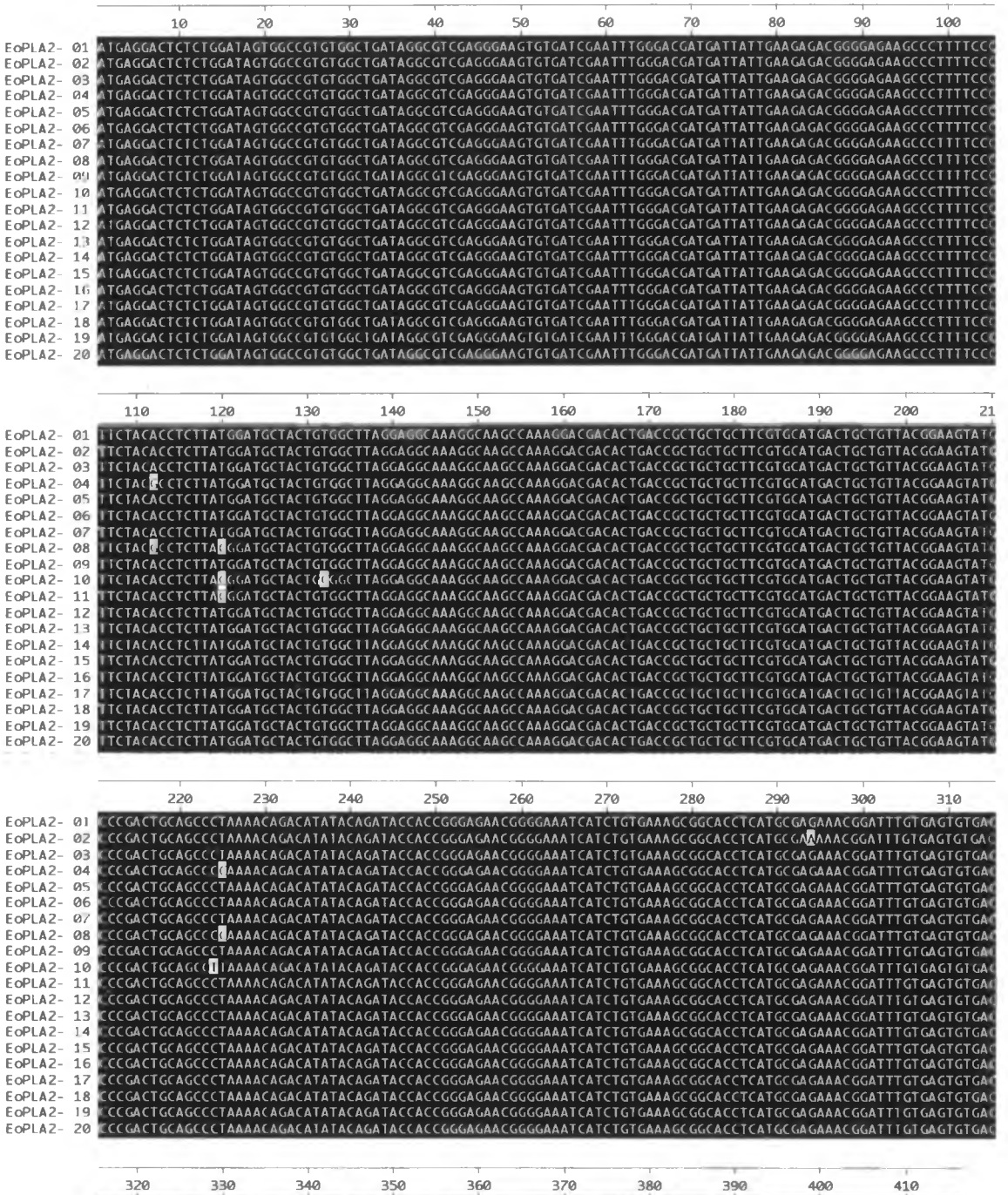
- Non-reducing loading sample buffer.

Prepared by combining the following

- 4ml (1M) Tris-HCl buffer pH6.8
- 1.5 g SDS
- 5 ml Glycerol
- 300 µl Bromophenol Blue solution
- Final volume was made up to 20 ml with deionised water.

Appendix B

(a)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EoPLA2- 03 ATGAGGACTCTCTGGATAGTGGCCGTGTGGCTGATAGGCGTCGAGGGAAGTGTGATCGAATTTGGGACGATGATTATTGAAGAGACGGGGAGAAGCCCTTTTCCC
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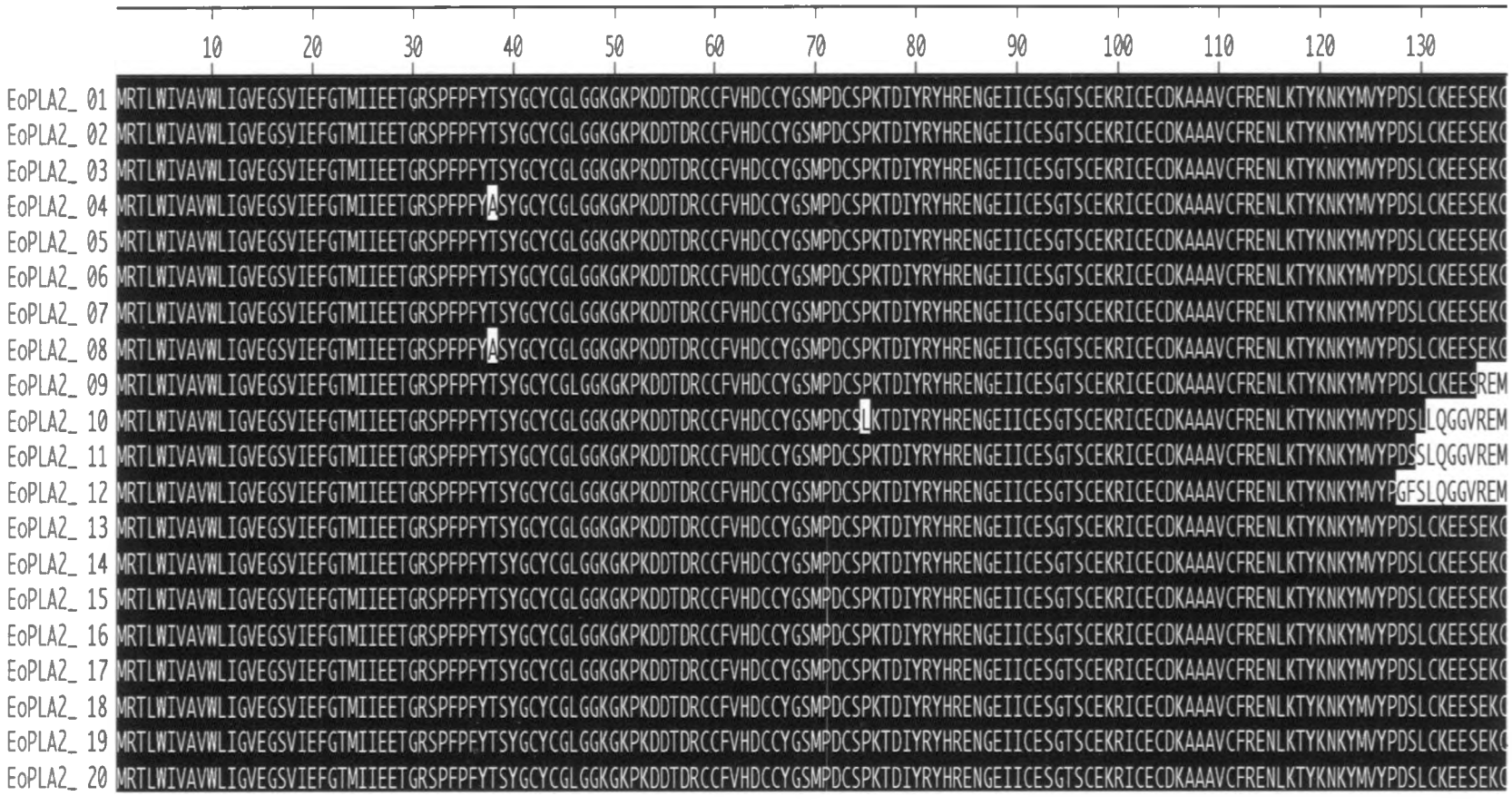
EoPLA2- 16 CCCGACTGCAGCCCTAAAACAGACATATACAGATACCACCGGGAGAACGGGGAAATCATCTGTGAAAGCGGCACCTCATGCGAGAAACGGATTTGTGAGTGTGAC
EoPLA2- 17 CCCGACTGCAGCCCTAAAACAGACATATACAGATACCACCGGGAGAACGGGGAAATCATCTGTGAAAGCGGCACCTCATGCGAGAAACGGATTTGTGAGTGTGAC
EoPLA2- 18 CCCGACTGCAGCCCTAAAACAGACATATACAGATACCACCGGGAGAACGGGGAAATCATCTGTGAAAGCGGCACCTCATGCGAGAAACGGATTTGTGAGTGTGAC
EoPLA2- 19 CCCGACTGCAGCCCTAAAACAGACATATACAGATACCACCGGGAGAACGGGGAAATCATCTGTGAAAGCGGCACCTCATGCGAGAAACGGATTTGTGAGTGTGAC
EoPLA2- 20 CCCGACTGCAGCCCTAAAACAGACATATACAGATACCACCGGGAGAACGGGGAAATCATCTGTGAAAGCGGCACCTCATGCGAGAAACGGATTTGTGAGTGTGAC
320 330 340 350 360 370 380 390 400 410
EoPLA2- 01 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 02 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 03 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 04 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 05 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 06 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 07 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 08 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 09 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTCGAGAGAAATGC
EoPLA2- 10 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCCGGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 11 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCCGGATTGCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 12 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCCGGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 13 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 14 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 15 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 16 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 17 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 18 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 19 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC
EoPLA2- 20 AAGGCCGCGGCAGTCTGCTTCCGAGAGAATCTGAAGACATACAAAAACAAATATATGGTTTACCC-GGATTCTCTTTGCAAGGAGGAGTC-AGAGAAATGC

(b)



Appendix B. (a) The nucleotide and (b) deduced amino acid sequences of the twenty EoPLA2 cDNAs isolated from the *E. ocellatus* venom gland.

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Cloning of a prothrombin activator-like metalloproteinase from the West African saw-scaled viper, *Echis ocellatus*

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Abstract

Systemic envenoming by the saw-scaled viper, *Echis ocellatus*, is responsible for more deaths than any other snake in West Africa. Despite its medical importance, there have been few investigations into the toxin composition of the venom of this viper. Here we describe the isolation of *E. ocellatus* venom gland cDNAs encoding a protein of 514 amino acids that showed 91% sequence similarity to Ecarin, a prothrombin-activating metalloproteinase from the venom of the East African viper, *E. pyramidum leakeyi*, that induces severe consumption coagulopathy. Structural similarities between the *E. ocellatus* metalloproteinase and analogues in venoms of related vipers suggest that antibodies raised to phylogenetically conserved *E. ocellatus* metalloproteinase domains may have potential for cross-specific and cross-generic neutralisation of analogous venom toxins.

Keywords: *Echis ocellatus*; cDNA cloning; Prothrombin activator

The saw-scaled viper *Echis ocellatus* is the most abundant (Trape et al., 2001) and medically important viper species in West Africa (Warrell and Arnett, 1976). In Nigeria, *E. ocellatus* is responsible for 95% of all envenoming by snakes (Meyer et al., 1997), causing several hundred deaths annually (Pugh and Theakston, 1980; Chippaux, 2002). In some areas, victims of *E. ocellatus* envenoming occupy 70% of hospital beds (Revault, 1996). Local effects of *Echis* viper envenoming include pain, swelling, blistering and haemorrhage which, in severe cases, can lead to necrosis, permanent disfigurement and even amputation of the affected limb (Warrell et al., 1977). Systemic effects include potentially lethal consumption coagulopathy, haemorrhage and hypovolaemic shock (Warrell et al., 1977). Despite the medical importance of *E. ocellatus*, few studies have been conducted to identify the venom toxins responsible for these effects. In a recent effort to more clearly define the venom gland transcriptome of *E. ocellatus* we have isolated DNA sequences encoding phospholipase A_2 (PLA_2) and C-type lectin (CTL) isoforms from an *E. ocellatus* venom gland cDNA library (Bharati et al., 2003; Harrison et al., 2003a). Studies on the venoms of related vipers have shown (i) that group II PLA_2s exert a variety of anti-coagulant, platelet aggregation-inhibitory, cytotoxic and, in some American pit vipers, neurotoxic effects (Kini and Evans, 1990; Markland, 1998) and (ii) that CTLs contribute to blood incoagulability by their distinct effects on the coagulation cascade and platelet aggregation (Wisner et al., 2002). In conjunction with colleagues who chromatographically isolated a haemorrhagic *E. ocellatus* venom metalloproteinase (Howes et al., 2003), we next sought to isolate DNA sequences encoding *E. ocellatus* venom metalloproteinases.

Snake venom metalloproteinases (SVMPs) are members of the reprolysin family of zinc-containing metalloproteinases (Bjarnason and Fox, 1995) and are grouped into four classes (I–IV) based on their structural composition. The group I SVMPs comprise a catalytic domain that is

Abbreviations: SVMPs, snake venom metallproteinases; PLA_2, phospholipase A_2; CTL, C-type lectin; PCR, polymerase chain reaction.

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sequentially and C-terminally extended with a RGD-containing disintegrin domain (group II), a cysteine-rich domain (group III) and a C-type lectin domain (group IV). The group III SVMPs typically possess greater haemorrhagic activity than the other groups (Gutierrez and Rucavado, 2000); these were therefore the target of this study.

We exploited the extensive sequence similarity of the group III viper SVMPs (Bjarnason and Fox, 1994; Paine et al., 1994; Selistre de Araujo and Ownby, 1995) to design polymerase chain reaction (PCR) primers complimentary to highly conserved regions within the N-terminal propeptide domain (VEDHCY) and at the C-terminus (CVDVNTAY). *E. ocellatus* (origin: Kaltango, Nigeria) venom gland cDNA was subjected to PCR with the 5′ (GGATCC-ATG-GTT-GAG-GAT-CAC-TGC-TAT; incorporating a methionine start codon and a *Bam*HI site) and 3′ (CTCGAG-TCA-GTA-ATC-ATC-CAG-GTC-ATC-ACC-CCT; incorporating a stop codon and a *Xho*I site) PCR primers using a thermal cycler (Gene Cycler, BioRad California, USA) programmed for an initial denaturing (95 °C, 6 min) and annealing (70 °C, 1 min) step, followed by 35 cycles of extension (74 °C), denaturing (95 °C) and annealing (63 °C) steps of 1 min each and terminating with an extension step (74 °C) of 7 min. Amplicons of the expected size (1.5 Kb) were purified from an agarose gel, ligated into a TA cloning vector (pCR-2.1-TOPO; Invitrogen, Groning, Netherlands) and used to transform chemically competent *Escherichia coli* (*TOP 10F*; Invitrogen). Plasmid DNA from several colonies was sequenced using a Beckman Coulter CEQTM 2000 XL DNA analysis instrument. The DNA sequences of four clones were virtually identical (98% sequence similarity) and the nucleotide and deduced amino acid sequence (encoding a 57 KDa protein) of a representative cDNA (termed *EoMP-6*, GenBank accession number: AY261531) is described in Fig. 1.

BLAST (Altschul et al., 1997) searches of the genetic databases revealed the deduced amino acid sequence of *EoMP-6* to be 91% and 59% similar to the prothrombin-activating SVMPs of *E. pyramidum leakyi* (Ecarin, A55796 (Nishida et al., 1995)) and *Bothrops erythromelas* (Berythractivase, AAL47169 (Silva et al., 2003)), respectively. *EoMP-6* also showed high levels of sequence similarity to haemorrhagic SVMPs from *Bothrops jararaca* (Jararhagin, 59%, P30431 (Paine et al., 1992)), *Agkistrodon contortrix laticinctus* (Aclh, 58%, AAL47169 (Selistre de Araujo and Ownby, 1995)), *Crotalus atrox* (Catrocollastatin, 59%, S55270 (Zhou et al., 1995)), *Protobothrops flavoviridis* (Hr1a, 58%, BAB92013 (Kishimoto and Takahashi, 2002)) and *E. pyramidum leakeyi* (Ech-I, 54%, S48160 and Ech-II, 56%, S48169, (Paine et al., 1994). The inferred amino acid sequence of *EoMP-6* was aligned to the above sequences using the CLUSTAL W program (Thompson et al., 1994) with PAM 250 residue weight matrix (Fig. 2).

EoMP-6 has the same modular organization as SVMPs from related vipers with a highly conserved pro-peptide domain containing the cysteine switch motif (PKMCGVT) thought to prevent proteolytic activity of the zymogen (Silva et al., 2003; Siigur et al., 1996). The *EoMP-6* proteolytic domain (MP) is typically less conserved except for those residues of structural (CI/VM methionine turn and all cysteine residues) or catalytic (MXXEXXHXXGXXH zinc-binding motif) significance. The met-turn is thought to promote hydrophobic interactions within the protein structure thereby ensuring the structural integrity of the zinc-binding site (Selistre de Araujo and Ownby, 1995). The zinc-binding site (Gomis-Ruth et al., 1993) is the active site responsible for the haemorrhagic activiy of these proteases (Bjarnason and Fox, 1994). In common with Ecarin and Berythractivase, but in contrast to the other haemorrhagic SVMPs, *EoMP-6* encoded a protein with three consensus *N*-glycosylation sites (*NXS/T*) at position 160, 194 and 225. While the functional significance of this observation remains unclear it suggests a closer structural, and perhaps functional, alignment of *EoMP-6* to the prothrombin-activating SVMPs. The disintegrin domain of *EoMP-6* contains a DCD motif that aligns to the $\alpha_2\beta_1$ collagen receptor-binding ECD motif of Jararhagin (Kamiguti, 1997). It is unclear whether the D for E substitution in *EoMP-6* has functional significance. The C-terminal cysteine-rich domain of *EoMP-6* showed a high level of sequence similarity to this region in other SVMPs, including Atrolysin A in venom of *C. atrox* (Jia et al., 2000) and specific sequence motifs in Jararhagin (HQC-DAC{317–334} and VKC-PGQ{362–377}) which have been attributed with platelet-inhibitory function by binding to $\alpha_2\beta_1$ integrin (Kamiguti et al., 2003).

This study is part of a project to isolate DNA sequences encoding the most clinically significant *E. ocellatus* toxins prior to their use as DNA immunization constructs to develop a panel of toxin-specific antibodies with venom-neutralising potential (Bharati et al., 2003; Harrison et al., 2000, 2002, 2003a, 2003b). To guide the design of the DNA immunization constructs and to evaluate the potential inter-specific and inter-generic immunoreactivity of each toxin-specific antibody (thereby estimating the geographic limits of their potential clinical usefulness), we have used a protein structure-predicting algorithm (Jameson and Wolf, 1988) to (i) identify domains of strong antigenic potential in the toxin gene product and (ii) determine whether these domains are conserved in analogous venom toxin gene products of related vipers. Because the propeptide domain of SVMPs is post-translationally cleaved from the mature venom protein (Bjarnason and Fox, 1994), this domain was excluded from the analysis of the predicted *EoMP-6* gene product (Fig. 3).

The peaks in the *EoMP-6* antigenic index profile (Fig. 3) indicate the numerous domains predicted to have a surface location and potential for antibody induction. The thin vertical lines (A–M) illustrate that many of the antigenic residues of *EoMP-6* are shared by SVMPs of related vipers

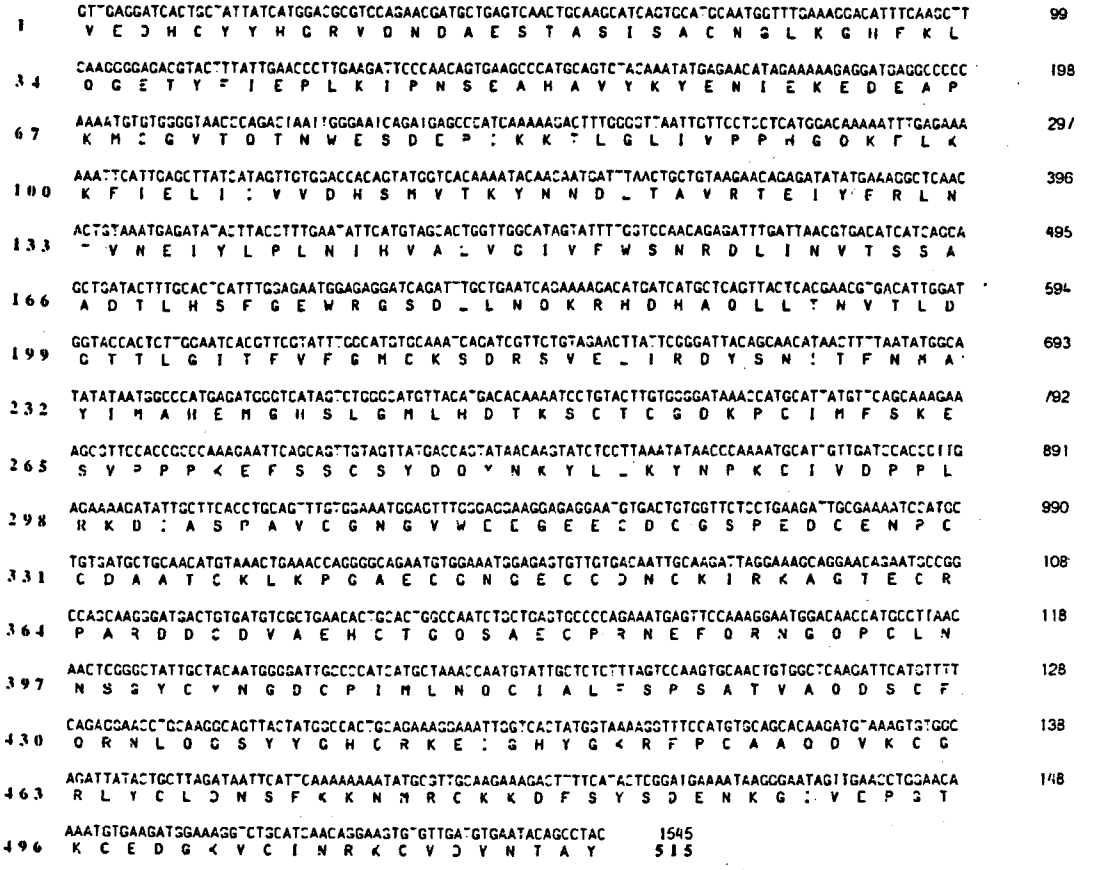

Fig. 1. The nucleotide and deduced amino acid sequence of *EoMP-6*. The numerals on the right and left indicate the amino acid and nucleotide sequences, respectively.

and that antibodies raised by *EoMP-6* DNA immunisation are likely to possess considerable cross-reactivity. As expected the antigenic profile of *EoMP-6* was very similar to that of Ecarin and, to a lesser extent, to Ech-I and Ech-II from *E. p. leakeyi* venom. The latter viper is a phylogenetically close relative of *E. ocellatus* (Lenk et al., 2001) with an East African distribution. Line D corresponded to the catalytic site (position 234–246, Fig. 2) and appeared to be highly immunogenic in all the SVMPs. Line H corresponded to the DCD motif in the cysteine rich domain (position 368–370, Fig. 2). Line K (position 373–378) corresponded to the platelet-inhibitory VKC domain of Jararhagin (Kamiguti et al., 2003). It is tempting to predict that antibodies generated against clinically significant domains in *EoMP-6* might competitively inhibit the function of these domains in venom toxins of related vipers. The binding of antibodies to the other antigenically conserved domains without an ascribed function are also predicted to disrupt protein function by steric hindrance. The veracity of these speculations need to be confirmed experimentally; a focus of our current activity. Furthermore, the truncated peptide sequence (KTLDSFGEWR) derived from a purified haemorrhagic fraction of *E. ocellatus* venom (Howes et al., 2003) differed from that *EoMP-6* (DTLHSFGEWR, position 167–176, Fig. 2). Together, these studies suggest a multimeric composition of *E. ocellatus* SVMPs and indicate the need for a more global analysis of the *E. ocellatus* venom gland transcriptome.

In conclusion, we have isolated for the first time, cDNA encoding a prothrombin-activating metalloproteinase of clinical importance from venom glands of *E. ocellatus*. The high degree of sequence conservation of *EoMP-6* with other prothrombin-activating and haemorrhagic metalloproteinases of related vipers extended to the conservation of structural domains predicted to have high immunogenic potential. These observations lead us to speculate that antibodies generated to these domains may have clinical potential in the treatment of *E. ocellatus* envenoming. Also, since SVMPs show a high degree of sequence and structural

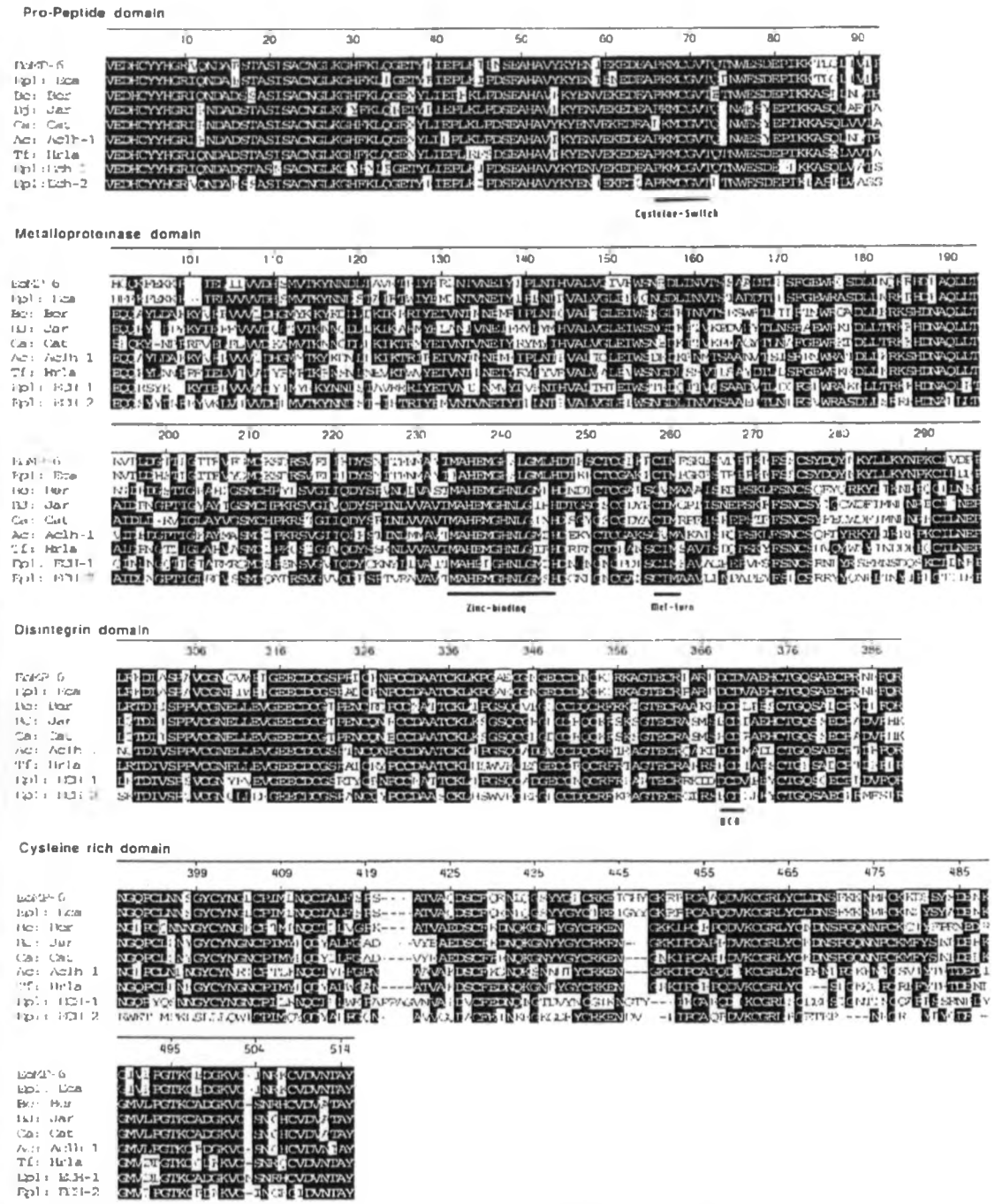

Fig. 2. Alignment of the predicted amino acid sequence of *EoMP-6* with SVMPs of related vipers. The alignment is divided into the four structure/function domains of SVMPs. Epl:Eca—Ecarin from *E.p. leakeyi* venom; Be:Ber—Berythractivase from *B. erythromelas* venom; Bj:jar—Jararhagin from *B. jararaca* venom; Ca:cat-Catrocollastatin from *C. atrox* venom; Ac:Aclh—SVMP from *A. contortrix laticinctus* venom; Tf:Hr1a—SVMP from venom of *P. flavoviridis* (previously termed *Trimesurus flavoviridis*); Epl:Ech-1 and Epl:Ech-2—SVMPs from *E.p. leakeyi* venom. Gaps have been inserted to optimise the sequence alignment. Residues identical to *EoMP-6* are shaded in black. Motifs of structural and catalytic significance are indicated.

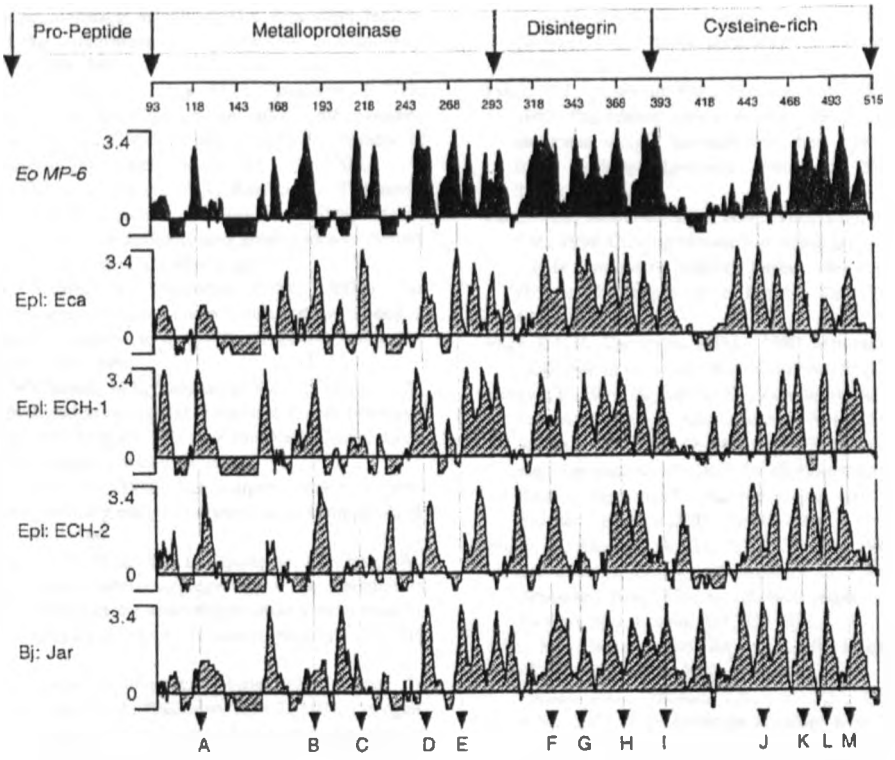

Fig. 3. Jameson and Wolf antigenic profiles of *EoMP-6* and SVMPs from related vipers. The structural organisation of SVMPs is depicted in the uppermost box. The large arrows distinguish the four main domains of the intact zymogen and indicate the amino acid sequences shown on the horizontal scale. The pro-peptide domains have been excluded from this analysis. The vertical scales represent comparative antigenic values. The thin vertical lines (A–M) are a subjective assignation of antigenic domains that exhibit the greatest phylogenetic conservation. Lines D, H and K correspond to the catalytic zinc binding, DCD and platelet-inhibitory motifs, respectively, as described in the text. Epl:Eca, Epl:Ech-1 and Epl:Ech-2—Ecarin and two other SVMPs from *E.p. leakeyi* venom; Bj:jar—Jararhagin from *B. jararaca* venom.

similarity, the possibility exists that rationally designed toxin-specific antibodies may possess potential for the treatment of envenoming by related vipers.

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Molecular cloning of phospholipases A_2 from venom glands of *Echis* carpet vipers

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Abstract

Venom toxin-specific antibodies offer a more rational treatment of snake envenoming than conventional antivenom. Here, we describe novel cDNAs encoding phospholipase A_2 (PLA_2) isoforms from venom gland RNA of *Echis pyramidum leakeyi* (Epl), *Echis sochureki* (Es) and *Echis ocellatus* (Eo). The deduced amino acid sequences of these cDNAs encoded proteins with high overall sequence identity to the viper group II PLA_2 protein family, including the 14 cysteine residues capable of forming seven disulphide bonds that characterize this group of PLA_2 enzymes. Comparison of the PLA_2 sequences from *Echis* with those from related vipers failed to make significant geographic, taxonomic or PLA_2-function distinctions between these *Echis* PLA_2 isoforms. However, their deduced hydrophilicity profiles revealed a conserved tertiary structure that we will exploit, by epidermal DNA immunization, to generate PLA_2-neutralizing antibodies with polyspecific potential.

Keywords: Phospholipases A_2; *Echis pyramidum leakeyi*; *Echis sochureki*; *Echis ocellatus*; cDNA cloning

1. Introduction

The *Echis* genus of carpet vipers constitutes one of the most medically important groups of snakes and is responsible for the majority of snakebite deaths and morbidity across Africa and the Indian subcontinent (Warrell and Arnett, 1976). The extreme haemorrhagic and coagulopathic manifestations of *Echis* envenoming are effected by zinc metalloproteinases that cause bleeding by degradation of the vascular subendothelium and a variety of other toxins that operate in distinct ways to prevent blood from clotting (e.g. disintegrins, prothrombin activators and factor IX/X activators; (Kamiguti et al., 1998)). It is well established that group II phospholipase A_2 (PLA_2) enzymes in venoms of related vipers in Asia, Europe, the Middle East and the Americas contribute to venom-induced pathology through a broad spectrum of toxic activities, including neurotoxicity (Habermann and Breithaupt, 1978), oedema-inducing activity (Vishwanath et al., 1987), anticoagulant activity (Boffa et al., 1976), haemorrhagic activity (Vishwanath et al., 1985; Yamaguchi et al., 2001) and activities that cause initiation or inhibition of platelet aggregation (Kini and Evans, 1990).

Comparatively little is known about the contribution of PLA_2 activity to the pathology of *Echis* viper envenoming. Kemparaju et al. (1994) isolated a 14 kDa basic (pI 7.2–7.6) PLA_2 (EC-IV-PLA_2) from Indian saw-scaled viper (*Echis carinatus*) venom that produced neurotoxicity and oedema in mice but had no direct haemolytic, myotoxic, cytotoxic or anticoagulant activities. Kemparaju et al. (1999) isolated a 16 kDa acidic (pI 4.2–4.8) PLA_2 (EC-I-PLA_2) from *E. carinatus* venom that was non-lethal to mice and devoid of neurotoxicity, myotoxicity, anticoagulant activity and cytotoxicity, but induced mild oedema in the foot-pads of mice. The purified PLA_2 inhibited ADP, collagen and adrenaline-induced aggregation of human platelets, that was both dose and time-dependent. Desmond et al. (1991) purified and partially sequenced the primary structure of two

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16 kDa PLA_2's (Ec1 and Ec2) from the venom of Kenyan *Echis pyamidum leakeyi* that exhibited sequence similarity with other toxic PLA_2's, but their role in the toxicity of the venom was not established. Polgar et al. (1996) purified and characterized an enzymatically active Ser-49 basic (pI 7.9) PLA_2, Ecarpholin S, from the venom of *Echis sochureki*. A PLA_2 complexed with another venom protein has been purified and crystallized from Indian *E. carinatus* venom (Nagpal et al., 1999). Only two *Echis* PLA_2 DNA sequences have been submitted to the genetic databases (*E. coloratus* AF253049/50; direct submission; Kordis, 2001).

A major research initiative of our laboratory is to generate venom toxin-specific antibodies by DNA immunization, as a means of developing rational immunotherapy (Harrison et al., 2000, 2002), particularly for vipers of greatest medical importance in Africa. We have included PLA_2s in this strategy, despite the paucity of data on their clinical importance in *Echis* envenoming, because it seemed imprudent to ignore a group of bioactive molecules with potential to disrupt haemostasis at several distinct levels and that comprise between 6 and 13% of the total venom protein of *E. p. leakeyi* (Desmond et al., 1991). Here, we report the isolation of three novel PLA_2-encoding cDNAs from *Echis* species with distinct residential boundaries in West Africa (*Echis ocellatus*), East Africa (*E. p. leakeyi*) and the Indian subcontinent (*E. sochureki*). The conserved secondary structure of all the PLA_2 isomers, across species and genus boundaries, augers well for the generation of neutralizing antibody with polyspecific cover.

2. Materials and methods

2.1. Animals, vector and cells

Adult carpet vipers, *E. p. leakeyi* (Kenya), *E. sochureki* (Pakistan) and *E. ocellatus* (Nigeria) used in this study were maintained in the herpetarium, Liverpool School of Tropical Medicine, Liverpool, UK. The TA cloning vector (pCR 2.1-TOPO) and chemically competent *E. coli* cells (TOP 10F′) were purchased from Invitrogen, Groningen, The Netherlands.

2.2. Isolation of total RNA and construction of cDNA library

Venom glands were dissected from three sacrificed snakes of each species (three days after venom extraction), homogenized under liquid N_2 and total RNA extracted using guanidinium thiocyanate–phenol–chloroform (Chomczynski and Sacchi, 1987) following standard protocols (Sambrook et al., 1989). Lambda phage cDNA libraries for the snakes were constructed by RT-PCR using the SMART cDNA library construction kit (CLONTECH, CA, USA) and Gigapack III Gold Packaging Extract (Stratagene, CA, USA) following the manufacturer's instructions.

2.3. Isolation and analysis of PLA_2 toxin cDNAs

A Polymerase Chain Reaction (PCR) strategy (Israel, 1993) was used to screen the cDNA libraries. A sense primer (5′-GGA-TCC-ATG-AGG-ACT-CTC-TGG-ATA-3′) and an antisense primer (5′-CTC-GAG-TCA-TCA-GCA-TTT-CTC-TGA-CTC-CTC-3′) complimentary to highly conserved amino (M-R-T-L-W-I) and carboxyl (E-E-S-E-K-C) regions of published group II PLA_2 DNA sequences of related viper species were designed and synthesized (Sigma-Genosys Ltd, UK). Two stop codons (TGA) and restriction endonuclease sites for *Bam* H1 and *Xho* 1 were included in the 5′ and 3′-primers, respectively, to facilitate future subcloning into mammalian expression plasmids.

PCR was performed using a thermal cycler (Gene Cycler, BioRad Hercules, CA, USA) programmed for an initial denaturation (95 °C × 6 min) and annealing (55 °C × 1 min) step, followed by 35 cycles (1 min duration each) of extension (74 °C), denaturation (94 °C) and annealing (55 °C). A final extension step (7 min) at 72 °C was also included in the program to ensure that all the products were double stranded. The PCR-amplified products were subcloned into the TA cloning vector, pCR 2.1-TOPO (Invitrogen), and used to transform chemically competent *E. coli* cells (TOP 10F′) under ampicillin selection, following the manufacturer's instructions. Plasmid DNA was extracted using a commercially available kit (Qiagen, Hilden, Germany) and digested with *Bam* H1 and *Xho* 1 at 37 °C to select plasmid containing inserts of the predicted size for DNA sequencing. DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method (Sanger et al., 1977) in a Beckman Coulter CEQ™ 2000 XL DNA Analysis System. The predicated amino acid sequences were compared to sequences in the GenBank, PDB, SwissProt, PIR and PRF databases using the BLAST program (Altschul et al., 1997).

The amino acid sequences of the three *Echis* cDNAs were aligned to PLA_2 isoform sequences from related *Viperidae* species: *E. coloratus* (AF253049), *Vipera ammodytes* (AF253048), *V. palaestinae* (U60017), *Trimeresurus flavoviridis* (D10720), *Calloselasma rhodostoma* (AF104067), *Bothrops asper* (AF109911), *B. jararacussu* (X76289) and *Crotalus atrox* (AF269131) using Lasergene software (DNASTAR, Madison, USA) and a phylogenetic tree constructed for the latter sequences and for peptide sequences of native PLA_2 from *E. p. leakeyi* (EplPLA$_2$-Ec1, (Desmond et al., 1991)) and *E. sochureki* (Ecarpholin S, P48650) using the CLUSTAL W program (Thompson et al., 1994) with PAM 250 residue weight matrix. The predicted isoelectric point of each PLA_2 isoform examined here was

determined using Lasergene software (Protean, DNASTAR).

3. Results and discussion

PCR amplification of *E. p. leakeyi*, *E. sochureki* and *E. ocellatus* venom gland libraries produced several cDNA sequences for each species (designated Epl, Es or Eo, respectively) that were identified (BLAST) as belonging to the PLA_2 enzyme family. Here, we show that single cDNA sequences for each snake species $EplPLA_2$-5, (Genbank submission-AF539920), $EsPLA_2$-4, (Genbank submission-AF539919) and $EoPLA_2$-5 (Genbank submission AF539921) were highly similar at the DNA and predicted amino acid levels (Fig. 1a and b). $EplPLA_2$-5 and $EsPLA_2$-4 (417 nucleotides) encoded an open reading frame of 139 amino acids (15.9 kDa) and $EoPLA_2$-5 (414 nucleotides) encoded a protein of 138 amino acids (15.7 kDa). Consistent with the secretory nature of venom PLA_2 proteins, the initiating 16 amino acid residues of all three *Echis* PLA_2 cDNAs encoded a conserved signal peptide domain (hatched arrow). The location of the less-conserved anticoagulant domain identified by Kini and Evans (1987) lies between residues 68–83 (solid arrow). Despite the geographical separation of *E. p. leakeyi* and *E. sochureki* species, the predicted amino acid sequences of $EplPLA_2$-5 and $EsPLA_2$-4 show only two amino acid substitutions, the first within the signal peptide domain at position 12 ($EplPLA_2$-5:Met; $EsPLA_2$-4:Val) and the second at position 62 ($EplPLA_2$-5:Met; $EsPLA_2$-4:Leu). These amino acid substitutions are, however, unlikely to affect the tertiary structure because the positions of the 14 cysteine residues are completely conserved in both molecules and likely reflect a common lineage (Moura-da-Silva et al., 1995).

The predicted amino acid sequences encoded by $EplPLA_2$-5, $EsPLA_2$-4 and $EoPLA_2$-5 were aligned with the PLA_2 sequences of vipers showing the highest similarity (Fig. 2a) during the BLAST search. The near-identical $EplPLA_2$-5 and $EsPLA_2$-4 showed a high percentage sequence identity with PLA_2 sequences from *E. coloratus* (72%; 71%) *V. palaestinae* (71%; 70%), *V. ammodytes* (69%; 69%), *T. flavoviridis* (60%; 61%), *B. jararacussu* (60%; 61%) and *C. rhodostoma* (58%; 59%). A phylogenetic tree of the above sequences and peptide sequences of

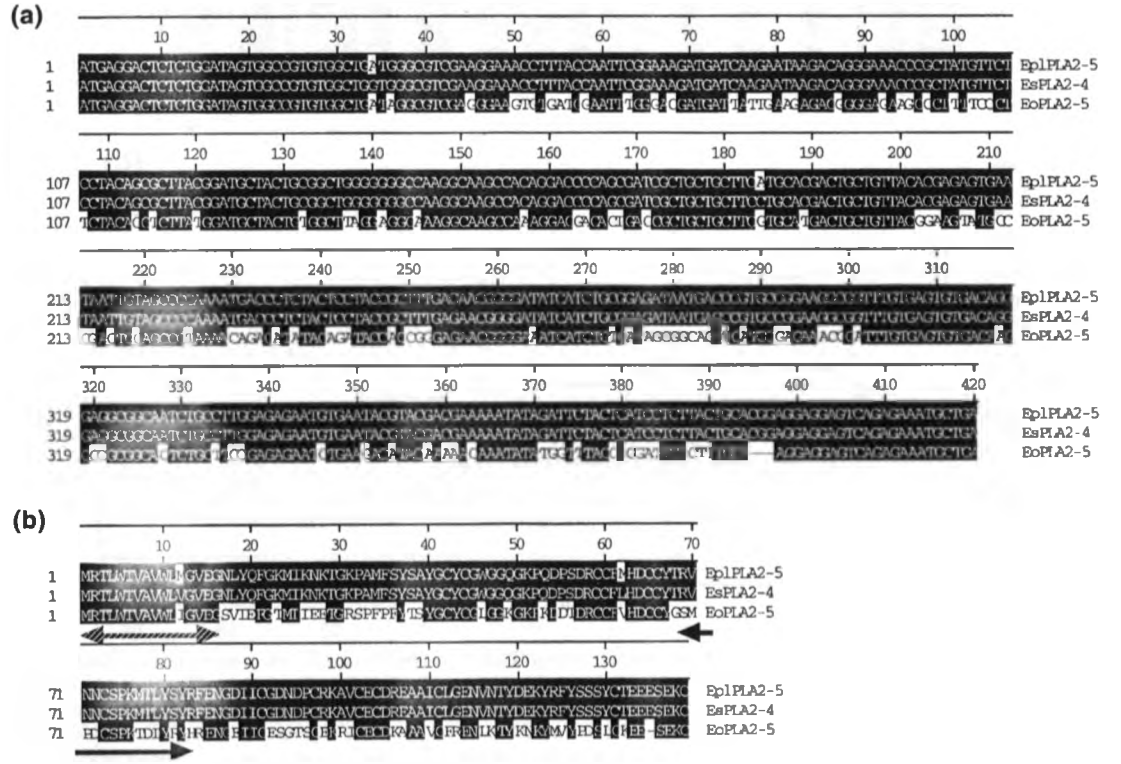

Fig. 1. (a) Complete cDNA and (b) deduced amino acid sequences of PLA_2's from *E. p. leakeyi* ($EplPLA_2$-5), *E. sochureki* ($EsPLA_2$-4) and *E. ocellatus* ($EoPLA_2$-5). The signal peptide (hatched arrow) and anticoagulant domain (solid arrow) are indicated.

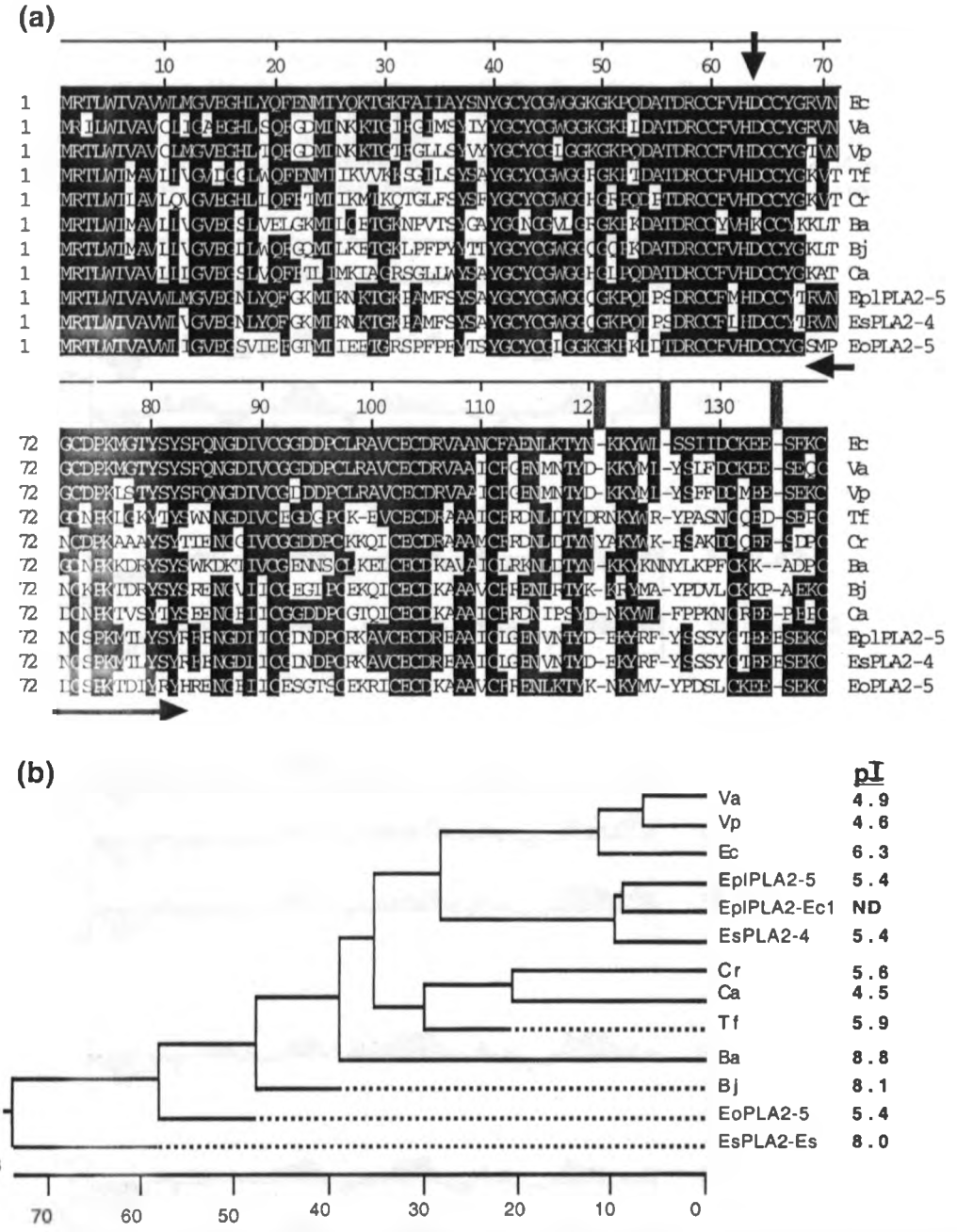

Fig. 2. Analysis of the amino acid sequences of EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5 with PAL$_2$'s from related *Viperidae* snake species by (a) sequence identity (residues shaded in black match *E. coloratus*; the vertical and horizontal arrows indicate the conserved calcium-binding aspartate residue and anticoagulant domain, respectively) and (b) phylogeny and predicted isoelectric point (pI). The tree was constructed from deduced amino acid sequences of group II snake venom PLA$_2$'s shown in Fig. 2a and include Ecarpholin S (EsPLA$_2$-Es) and EplPLA$_2$-Ecl. The scale beneath the tree measures the distance between sequences (in millions of years). Ec: *E. coloratus*; Va: *V. ammodytes*; Vp: *V. palaestinae*; Tf: *T. flavoviridis*; Cr: *C. rhodostoma*; Ba: *B asper*; Bj: *B. jararacussu*; Ca: *C. atrox*; EplPLA$_2$-Ecl, Desmond et al. (1991); EsPLA$_2$-Es: *E. sochureki* Ecarpholin S; ND: not determined.

native PLA_2 from *E. p. leakeyi* ($EplPLA_2$-Ecl, (Desmond et al., 1991) and *E. sochureki* (Ecarpholin S, P48650) was constructed (Fig. 2b) using the CLUSTAL W program with PAM250 residue weight matrix. The amino acid sequence of $EplPLA_2$-5 cDNA matched with near identity (Fig. 2b) to the sequence of a native PLA_2 from *E. p. leakeyi* venom ($EplPLA_2$-Ecl) suggesting a direct genomic relationship. In contrast, the predicted amino acid sequence of $EsPLA_2$-4 showed only 42% identity (Fig. 2b) to the peptide sequence of a native PLA_2, Ecarpholin S from *E. sochureki* (Polgar et al., 1996). This clearly indicates that $EsPLA_2$-4 encodes a distinct *E. sochureki* PLA_2 isoform. The amino acid sequence encoded by $EoPLA_2$-5 showed comparatively lower overall identity to viper PLA_2 isoforms than $EplPLA_2$-5 and $EsPLA_2$-4: *B. jararacussu* (65%), *C. atrox* (54%), *T. flavoviridis* (50%), *V. ammodytes* (51%), *E.*

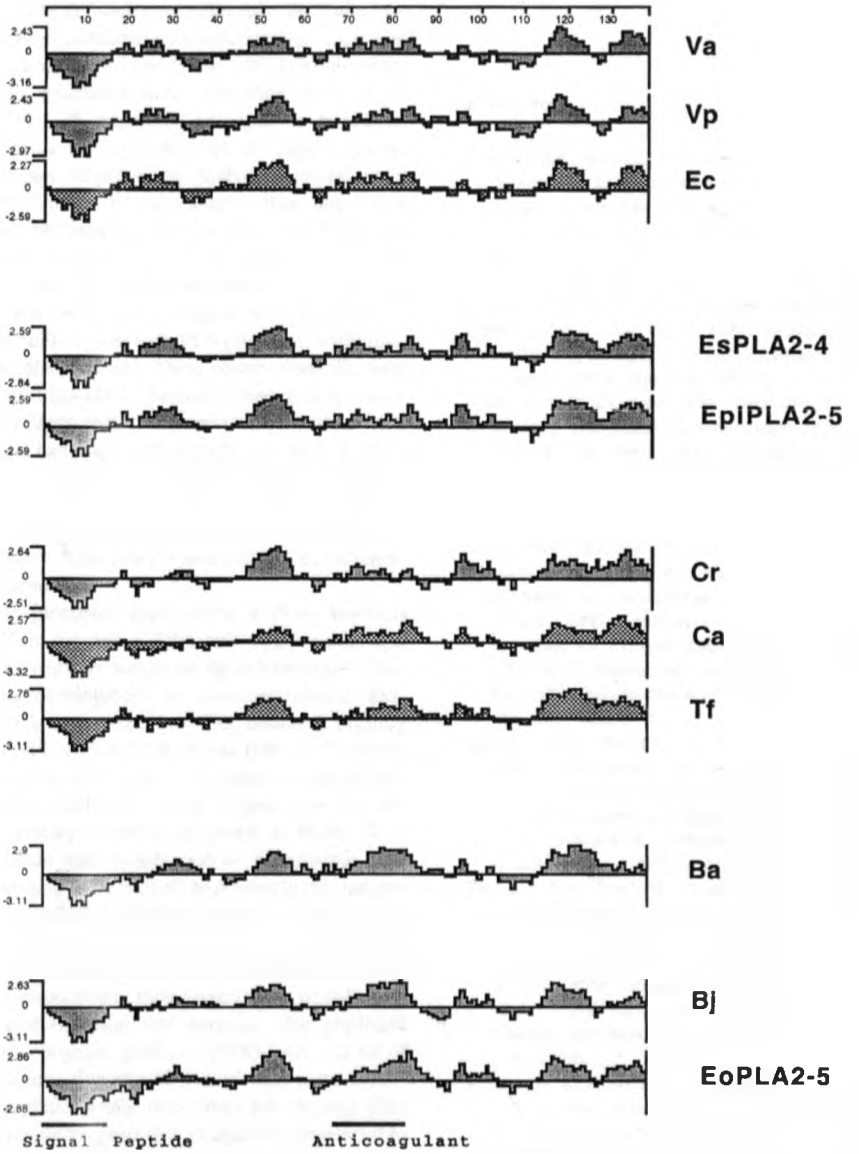

Fig. 3. Kyte–Doolittle hydrophilicity profile of viper PLA_2 isoforms used in Fig. 2a. The top horizontal scale represents the number of amino acid residues. The vertical scale represents the hydrophilic (+) and hydrophobic (−) domains, respectively. The conserved signal peptide and anticoagulant domains are indicated.

coloratus (50%), *B. asper* (50%), *V. palaestinae* (50%) and *C. rhodostoma* (48%).

In an effort to assign potential functional attributes to EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5, their deduced amino acid sequences were examined (i) for the expression of lysine or aspartate residue at position 64 (49 in sequences lacking the signal peptide) known to confer calcium-mediated catalytic activity, (ii) for the number of lysine residues within the anticoagulant domain and (iii) to identify sequences with a basic isoelectric point (pI). The latter two attributes have been correlated with anticoagulant activity of native PLA$_2$ enzymes (Kini and Evans, 1987). All the viper PLA$_2$ isoforms examined here, including EplPLA$_2$-5, EsPLA$_2$-4, EoPLA$_2$-5, expressed an aspartate residue at position 64 (with the exception of *B. asper*; lysine) indicating that they all possessed catalytic potential. Only *B. asper* PLA$_2$ isoform contained more than one lysine residue in the anticoagulant domain (Fig. 2a). Only the PLA$_2$ isoforms from *B. asper*, *B. jararacussu*, and Ecarpholin S from *E. sochureki* showed a basic pI (Fig. 2b). These indications suggest that EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5 encode PLA$_2$ isoforms with little, if any, anticoagulant activity. These observations illustrate the problems of using cDNA-derived amino acid sequence data to predict functional characteristics of proteins that exist as multiple isoforms, each with the potential to effect distinct functions. Current research is focused on linking sequence data for the existing (and remaining) PLA$_2$ cDNAs extracted from *E. p. leakeyi*, *E. sochureki* and *E. ocellatus* to isoforms recovered from two dimensional gel electrophoresis of whole venom.

The diverse functional capabilities of PLA$_2$ isoforms whose amino acid sequences differ only slightly does not, however, have a negative impact on the achievement of our objectives—the development of toxin-neutralizing antibodies by DNA immunization. The deduced primary structures of EplPLA$_2$-5, EsPLA$_2$-4 and EoPLA$_2$-5 include the requisite, highly conserved, 14 cysteine residues that form the seven disulphide bonds responsible for the characteristic tertiary structure of group II PLA$_2$. It is therefore predicted that, transfection of mammalian cells with PLA$_2$-encoding DNA is likely to present to the immune system molecules that faithfully represent native venom PLA$_2$. The hydrophilic profile (Fig. 3) (Kyte and Doolittle, 1982) of all the PLA$_2$ molecules showed remarkable conservation, irrespective of their geographical distribution, pI, taxonomic designation and toxicity. The predicted Jameson–Wolf antigenic profiles (DNASTAR, USA) of these PLA$_2$ isoforms aligned with near identity to the Kyte–Doolittle hydrophilic profile plots (data not shown). This observation strongly suggests that an antibody developed to an amino acid domain that (i) has a high antigenic index and (ii) is conserved across snake species has potential for viper polyspecific neutralization of viper venom PLA$_2$ pathology.

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Novel sequences encoding venom C-type lectins are conserved in phylogenetically and geographically distinct *Echis* and *Bitis* viper species

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Abstract

Envenoming by *Echis* saw scaled vipers and *Bitis arietans* puff adders is the leading cause of death and morbidity in Africa due to snake bite. Despite their medical importance, the composition and constituent functionality of venoms from these vipers remains poorly understood. Here, we report the cloning of cDNA sequences encoding seven clusters or isoforms of the haemostasis-disruptive C-type lectin (CTL) proteins from the venom glands of *Echis ocellatus*, *E. pyramidum leakeyi*, *E. carinatus sochureki* and *B. arietans*. All these CTL sequences encoded the cysteine scaffold that defines the carbohydrate-recognition domain of mammalian CTLs. All but one of the *Echis* and *Bitis* CTL sequences showed greater sequence similarity to the β than α CTL subunits in venoms of related Asian and American vipers. Four of the new CTL clusters showed marked inter-cluster sequence conservation across all four viper species which were significantly different from that of previously published viper CTLs. The other three *Echis* and *Bitis* CTL clusters showed varying degrees of sequence similarity to published viper venom CTLs. Because viper venom CTLs exhibit a high degree of sequence similarity and yet exert profoundly different effects on the mammalian haemostatic system, no attempt was made to assign functionality to the new *Echis* and *Bitis* CTLs on the basis of sequence alone. The extraordinary level of inter-specific and inter-generic sequence conservation exhibited by the *Echis* and *Bitis* CTLs leads us to speculate that antibodies to representative molecules should neutralise the biological function of this important group of venom toxins in vipers that are distributed throughout Africa, the Middle East and the Indian subcontinent.

Keywords: C-type lectins; *Echis ocellatus*; *Echis pyramidum leakeyi*; *Echis carinatus sochureki*; *Bitis arietans*; Sequence conservation

1. Introduction

Snake bites cause over 20,000 deaths annually in Africa (Chippaux, 2002). The majority of these deaths result from systemic envenoming by *Echis ocellatus* (West Africa), *E. pyramidum leakeyi* (East Africa) saw scaled vipers and *Bitis arietans* puff adders (pan-African distribution). Survivors of envenoming also suffer long-term disability, scarring and occasionally amputation as a consequence of local venom-induced damage to tissues extending from the bite site (Warrell, 1996). Despite their medical importance, there has been surprisingly little research performed on the composition and constituent functionality of venoms from these snakes. The systemic and local pathological effects of venoms from the African *Echis* and *Bitis* vipers show many parallels with venoms from Asian and American pit vipers. The extensive literature on the latter vipers indicates that of the 100 or more constituents of viper venoms, a relatively small number of venom toxins are responsible for the pathological effects of envenoming. A group of zinc-containing metalloproteinases degrade the vascular subendothelium to cause haemorrhage and, by virtue of a carboxyl disintegrin-like domain, interfere with the aggregation of platelets that would normally serve to repair the damage and thereby arrest bleeding (Kamiguti et al., 1996; Bjarnason and Fox, 1994). Similar metalloproteinases with prothrombin- and factor X-activating effects (Nishida et al., 1995) and thrombin-like serine proteases (Pirkle and Theodor, 1990) promote consumptive coagulation (Hutton and Warrell, 1993). This venom arsenal is reinforced by other toxins,

Abbreviations: CTL, C-type lectins; PLA_2, phospholipase A_2; spp, species in plural.

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including RGD-containing disintegrins, phospholipases A_2 (PLA_2) and C-type lectin (CTL) proteins (Markland, 1998) that prevent blood clotting by interference with platelet aggregation and distinct elements of the coagulation cascade.

One of our main research objectives is to develop toxin-specific antivenom to treat envenoming by the African *Echis* and *Bitis* vipers. This requires a more complete definition of the venom proteome of these vipers. We and others have previously cloned sequences encoding venom haemorrhagic (Paine et al., 1994) and prothrombin-activating (Nishida et al., 1995; Yamada et al., 1996) zinc metalloproteinases of *E.p. leakeyi*. These cDNAs exhibited high sequence similarity to analogous molecules in related Asian and American vipers. More recently, we isolated cDNAs encoding PLA_2 enzymes of *E. ocellatus*, *E.p. leakeyi* and *E.c. sochureki* and demonstrated that they also showed a high level of sequence conservation (Bharati et al., in press).

Here we report the cloning of *E. ocellatus*, *E.p. leakeyi*, *E.c. sochureki* and *B. arietans* cDNAs (termed *Eo*, *Epl*, *Es* and *Ba*, respectively) that encode several isoforms of the CTL proteins. In venoms of related vipers, these venom toxins exert a variety of haemostasis-disruptive functions including binding the IX and X coagulation factors to inhibit the clotting cascade and agonistic and antagonistic effects on platelet aggregation (Wisner et al., 2002). *E.c. sochureki* (Indian subcontinent) was included to evaluate whether the sequence conservation of viper venom toxins would extend to the phylogenetically close but geographically distant Asian viper. The *Echis* and *Bitis* viper CTL cDNAs isolated here showed considerable sequence identity with each other and to analogues of related vipers, including retention of specific amino acid substitutions used to distinguish CTL isomers.

2. Materials and methods

2.1. Animals

Adult *E.p. leakeyi* (Kenya), *E.c. sochureki* (Pakistan) and *E. ocellatus* (Nigeria) carpet vipers and *B. arietans* puff adders (Ghana and Saudi Arabia) used in this study were maintained in the herpetarium, Liverpool School of Tropical Medicine, Liverpool, UK.

2.2. Extraction of total venom gland RNA and construction of cDNA libraries

Venom glands were dissected from three snakes of each *Echis* species and from two *B. arietans* snakes. The vipers were sacrificed 3 days after venom extraction when toxin gene transcription rates are at a peak. Glands were homogenized under liquid N_2 and total RNA extracted using guanidinium thiocyanate-phenol-chloroform as described previously (Bharati et al., in press). Lambda phage cDNA libraries for *E. sochureki*, *E. ocellatus* and *B. arietans* were constructed by RT-PCR using the SMART cDNA library construction kit (Clontech, California, USA). The lambda ZapII vector was used to construct the *E.p. leakeyi* cDNA library (Stratagene, California, USA). The lambda phage of the four snake species was packaged using Gigapack III Gold Packaging Extract (Stratagene) and boiled for 5 min prior to being used as targets of polymerase chain reaction (PCR) amplification.

2.3. Isolation and analysis of cDNA sequences

A PCR strategy was used to isolate sequences encoding CTLs from the cDNA libraries. A sense primer (5′-GGA-TCC-ATG-GGG-CGA-TTC-ATC-TTC-3′) and an anti-sense primer (5′-CTC-GAG-CTA-TGC-CGG-GCT-CTT-GCA-GAC-GAA-3′) complimentary to highly conserved amino-terminal signal peptide (M-G-R-F-I-F) and to the less conserved carboxy-terminal (F-V-C-K-S-P-A) domains of published CTL DNA sequences of related viper species were synthesized commercially (Sigma-Genosys, UK). A TAG stop codon was inserted in the 3′ primer and *Bam*H1 and *Xho*1 restriction endonuclease sites (bold) were included in the 5′ and 3′ primers, respectively, to facilitate future subcloning.

PCR was performed using an initial denaturation (95 °C×6 min) and annealing (55 °C×1 min) step, followed by 35 cycles (1 min each) of extension (74 °C), denaturation (94 °C) and annealing (55 °C) and a terminal extension step (7 min) at 72 °C in a thermal cycler (Gene Cycler, BioRad, Hercules, CA, USA). The inclusion of water-only controls with each PCR reaction allowed us to monitor and prevent cross-over contamination. The amplicons were subcloned into the TA cloning vector, pCR 2.1-TOPO, (Invitrogen, Groningen, The Netherlands) and used to transform chemically competent *E. coli* cells (TOP10F', Invitrogen) under ampicillin selection. Plasmid DNA was extracted (Mini-spin prep kit, Qiagen, Hilden, Germany) and digested with *Bam*H1 and *Xho*1 at 37 °C to select plasmids containing inserts of the predicted size for DNA sequencing.

DNA sequencing was carried out by the dideoxy-nucleotide chain-termination method in a Beckman Coulter CEQ™ 2000 XL DNA Analysis System. To confirm that the cDNA sequences encoded CTLs, the predicted amino acid sequences were subjected to BLAST searches of the GenBank, PDB, SwissProt, PIR and PRF databases. All the cDNAs exhibited significant sequence homology to CTLs of related vipers. The CLUSTAL W program (Thompson et al., 1994) with PAM 250 residue weight matrix was used to align deduced amino acid sequences representing each *Echis* spp and *B. arietans* CTL isoform with analogues in venoms from related *Viperidae* species: ECLV IX/X bp β (AAB36402) from *E. leucogaster* (Chen and Tsai, 1996), Echicetin β (P81996) from *E. carinatus* (Peng et al., 1994), Bitiscetin α (JC5058) and β (JC5059) subunits from *B. arietans* (Matsui et al., 1997), CHH-B α (P81508) and β (P81509) subunits from *Crotalus horridus horridus*

(Andrews et al., 1996), Convulxin β (CAA76182) from *C. durissus terrificus* (Leduc and Bon, 1998), Alboaggregin α subunit 4 (P81114) from *Trimeresurus albolabris* (Kowalska et al., 1998), α (D83331) and β (D83332) IX/X bp subunits from *Protobothrops flavoviridis* (Matsuzaki et al., 1996), Flavocetin-A β subunit (AAN72437) also from *P. flavoviridis* (Shin et al., 2000), Aggretin (JC7105) from *Calloselasma rhodostoma* (Chung et al., 1999), α (AF176420) and β (BAB20441) subunits from *Deinagkistrodon acutus* (direct submissions) and, finally, α (AF190827) and β (AF197915) subunits from *Gloydius halys* (direct submissions). The phylogenetic trees constructed from the above alignments were generated by a neighbour-joining (Saitou and Nei, 1987) algorithm in Lasergene software (DNASTAR, USA). The predicted antigenic profile (Jameson and Wolf, 1988) of the published and new *Echis* and *B. arietans* CTL isoforms analysed here was determined using Protean software (DNASTAR).

3. Results

3.1. Isolation of cDNAs encoding E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans CTLs

PCR screening of the *Echis* spp and *B. arietans* venom gland cDNA libraries resulted in a total of 11 *E.p. leakeyi* (*Epl*), 14 *E.c. sochureki* (*Es*), 9 *E. ocellatus* (*Eo*) and 11 *B. arietans* (*Ba*) cDNAs whose sequences matched (BLAST searches) those of published CTLs. Where two or more identical sequences were obtained from any one of these libraries, a single representative cDNA was used for subsequent analysis. The cDNAs consisted of 441–447 nucleotides (data not shown) and were predicted to encode open reading frame proteins of 147–149 amino acids (16.9–17.5 kDa). Accession numbers assigned to the new *Echis* and *B. arietans* CTL sequences are as follows: *Es CTL*-1 (AY254331), *Es CTL*-3 (AY254332), *Es CTL*-8 (AY254334), *Es CTL*-9 (AY254336), *Epl CTL*-1 (AY254337), *Epl CTL*-4 (AY254338), *Epl CTL*-5 (AY254339), *Epl CTL*-7 (AY254340), *Eo CTL*-1 (AY254330), *Eo CTL*-2 (AY254333), *Eo CTL*-27 (AY254335), *Ba CTL*-1 (AY254325), *Ba CTL*-2 (AY254326), *Ba CTL*-5 (AY254327), *Ba CTL*-6 (AY254328) and *Ba CTL*-8 (AY254329).

3.2. The CTLs of E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans exhibit extensive inter-specific and inter-generic sequence similarity or identity

Alignment of the predicted amino acid sequences of the 16 species-specific cDNAs encoding CTLs from the *Echis* spp and *B. arietans* (Fig. 1) revealed extensive sequence similarities. Phylogenetic tree analysis (Fig. 2) was used to catego-

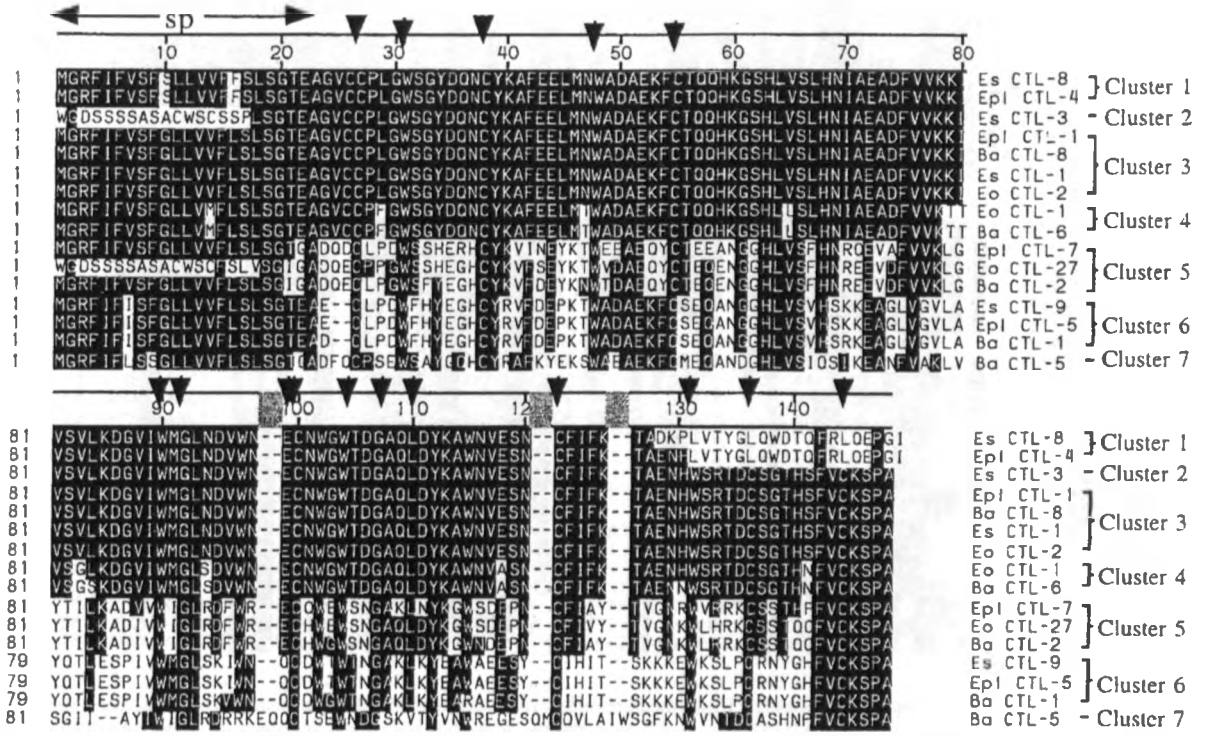

Fig. 1. Alignment and grouping of the *E. ocellatus, E.p. leakeyi, E.c. sochureki* and *B. arietans* CTL deduced amino acid sequences. The horizontal arrows depict the signal peptide (sp) domain (residues 1–23). The vertical arrows identify amino acid residues referred to in the text that are associated with the carbohydrate recognition domain of mammalian CTL proteins. Residues shaded in black are identical to *Epl CTL*-1.

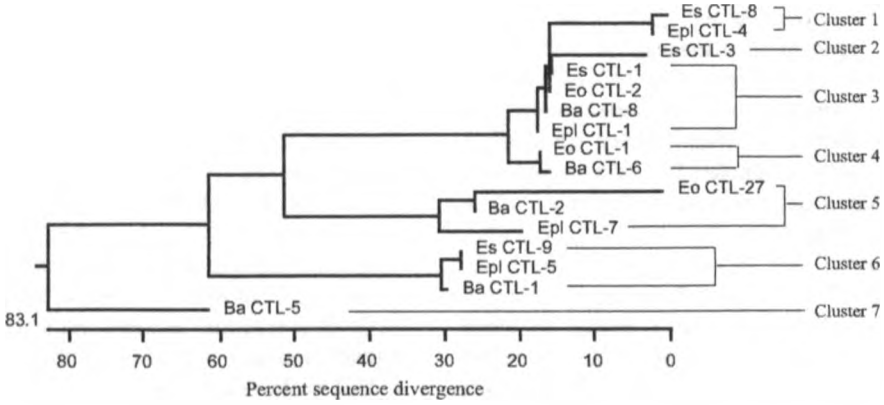

Fig. 2. Phylogenetic tree analysis of the *E. ocellatus, E.p. leakeyi, E.c. sochureki* and *B. arietans* CTL deduced amino acid sequences. This neighbour-joining analysis was used to categorise the sequences into seven distinct clusters. Branch lengths (dark horizontal lines) are drawn to scale and represent the percent amino acid divergence for each sequence.

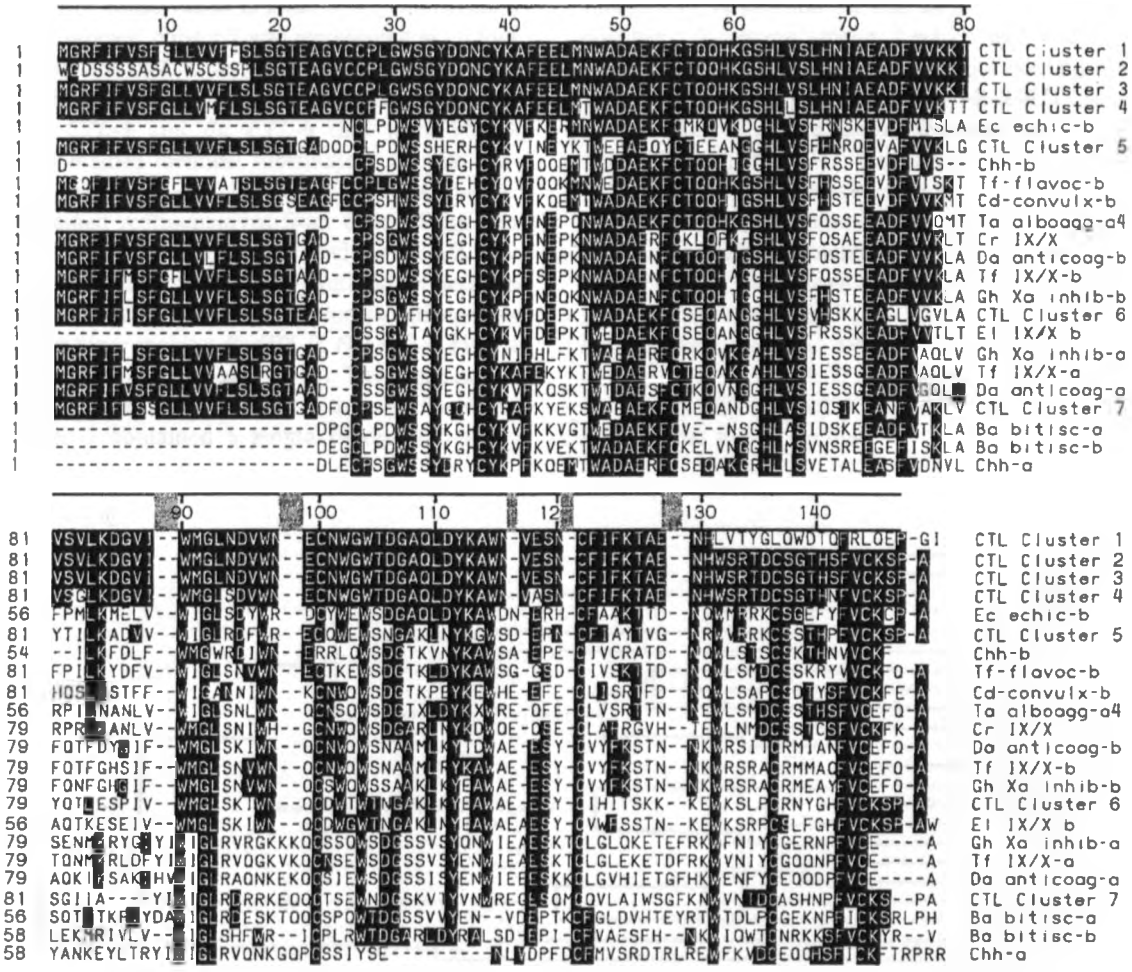

Fig. 3. Amino acid sequence alignment of the *E. ocellatus, E.p. leakeyi, E.c. sochureki* and *B. arietans* CTL clusters with venom CTLs from related vipers. Ec echic-b=echicetin β from *E. carinatus*; Chh-a and -b=α and β CTL subunits from *C.h. horridus*; Tf-flavoc-b=β CTL subunit from *P. flavoviridis*; TfIX/X-a and -b=α and β subunits of the IX/X binding protein of *P. flavoviridis*; Cd-convulx-b=β subunit of convulxin from *C. durissus terrificus*; Ta-alboagg-a4=alboaggregin α subunit 4 from *T. albolabris*; Cr IX/X=aggretin, a factor IX/X binding protein from *C. rhodostoma*; Da anticoag-a and -b=α and β subunits of the anticoagulant CTL from *D. acutus*; Gh Xa inhib-a and -b=α and β subunits of the factor Xa inhibiting CTL from *G. halys*; El IX/X-b=β subunit of ECLV, the IX/X binding protein of *E. leucogaster*; Ba bitisc-a and -b=α and β subunits of bitiscetin from *B. arietans*. Residues shaded in black are identical to *Epl CTL*-1 of cluster 3.

rise the 16 CTL sequences into seven distinct clusters, based solely on sequence alignment. Cluster 1 contained *Es CTL*-8 and *Epl CTL*-4 which were 98.1% identical (differing by three residues) and, had they not contained a distinct carboxyl-terminus, would have retained this level of sequence identity to sequences in cluster 3. Cluster 2 was represented by a single cDNA, *Es CTL*-3, which did not contain the methionine start codon and its amino terminal sequence differed markedly from the signal peptide sequence (residues 1–23) conserved in most of the other cDNAs. Intriguingly, *Eo CTL*-27 had a similar amino terminal sequence to *Es CTL*-3 but the down stream sequence encoded a distinct mature protein. The distinct 5′ sequence composition of *Es CTL*-3 and *Eo CTL*-27 was not considered the result of cloning artefacts since four other *Es CTL* cDNAs were found with identical sequences.

Immediately downstream of the signal peptide domain, the sequences within each CTL cluster showed a remarkable degree of sequence conservation in most of the viper species examined. For instance, cluster 3 was represented in all four viper species by *Epl CTL*-1, *Ba CTL*-8, *Es CTL*-1 and *Eo CTL*-2, which were completely identical. Cluster 4 contained *Eo CTL*-1 and *Ba CTL*-6 that were 98.7% identical and contained identical substitutions of nine residues evenly distributed along the length of the sequence. Cluster 5 contained representatives from three of the four viper species, *Epl CTL*-7, *Eo CTL*-27 and *Ba CTL*-2, that showed a comparatively low sequence similarity of between 75% and 84%. Cluster 6 also contained representatives from three of the four viper species: *Es CTL*-9, *Epl CTL*-5 (100% sequence identity) and *Ba CTL*-1 (96.8%). The sequences in this group were less than 60% similar to those in the above groups. Cluster 7 contained a single representative sequence, *Ba CTL*-5, that showed less than 50% sequence similarity to all the preceding cDNAs.

The deduced amino acid sequences of the *Echis* spp and *B. arietans* CTLs possessed the cysteine scaffold that defines the carbohydrate-recognition domain (CRD) of mammalian CTLs (Drickamer, 1988) that bind sugars in a Ca^{2+}-dependent manner. Disulphide bridges formed between C27 and C38, C55 and C144 and between C122 and C136 (arrows in Fig. 1) were conserved in all the *Echis* spp and *B. arietans* CTL sequences, except in those of cluster 1 where the carboxyl cysteine residues were both substituted with leucine residues. The CRD-invariant residues W31, W48, W90-X-G92, W104-X-X-G107 and W131 (Wisner et al., 2002) were all represented in the *Echis* and *B. arietans* CTL clusters, except cluster 1 which showed an L for W substitution at position 131. In contrast to the conserved mammalian CRD sequences, all the above viper venom CTL clusters showed a G for W substitution at position 92 and an L for P substitution at position 110, except cluster 7 which had a V at position 110. Also, the viper sequences did not contain the E-P-N and Q-P-D triplets associated with CRD binding by mammalian CTLs to mannose and galactose, respectively. The *Echis* and *Bitis* CTL sequences appeared incapable of binding calcium, a feature common to venom CTLs that bind to coagulation factors IX and X (Atoda et al., 2002). Thus, unlike CTL subunits of *P. flavoviridis* (Shin et al., 2000) and *E. leucogaster* (Atoda et al., 2002) that contain the requisite S-66, E-68, E-72 and E-146 for calcium binding (Mizuno et al., 1997), all the *Echis* and *Bitis* CTL clusters, except cluster 7, showed an H for E-68 and an S for E-146 substitution. Cluster 1 sequences, however, retained the E-146 residue.

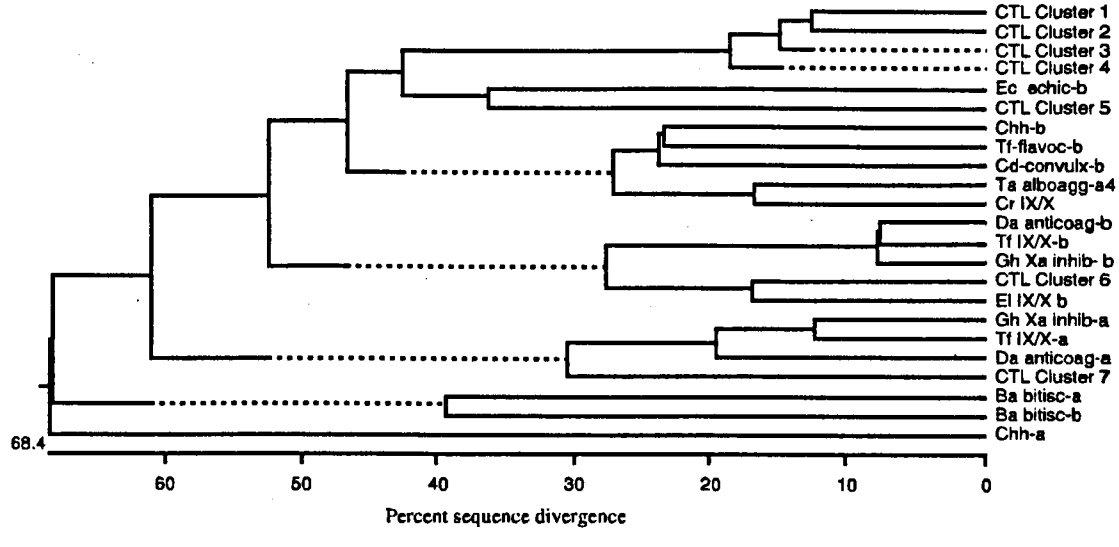

Fig. 4. Phylogenetic tree (neighbour-joining) analysis of the *E. ocellatus*, *E.p. leakeyi*, *E.c. sochureki* and *B. arietans* CTL clusters and venom CTLs from related vipers. Branch lengths are drawn to scale and represent the percent amino acid divergence for each cluster or sequence.

3.3. Sequence alignment of the E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans CTL clusters with analogues in related viper species

Viper venom CTL sequences in the genetic databases were compared with the *Echis* spp and *B. arietans* CTL clusters (Fig. 3) by BLAST. Clusters 1–4 represent novel, highly similar, CTL isomers with less than 65% sequence similarity to analogues in related viper species. Phylogenetic tree analysis showed that clusters 1–4 were most closely associated with β-subunit CTLs (Fig. 4). Cluster 5 was assigned to the same clade as the β-subunit of echicetin from *E. carinatus* and generally was aligned with other CTL β-subunits. Cluster 6 showed the greatest sequence similarity (62%) to the CTL β-subunit of the West African *E. leucogaster* viper. Of all the *Echis* spp and *B. arietans* CTL clusters, only cluster 7 seemed to represent an α-CTL subunit sequence which showed the highest sequence similarity (59%) to the α-subunit of the Xa-binding CTL of the Asian *G. halys* viper. None of the clusters showed more than 50% sequence similarity (data not shown) to the partial peptide sequences for the α- and β-subunits of the EM 16, a CTL purified from the venom of the Asian *E. multisquamatus* viper (Marcinkiewicz et al., 2000). Similarly, the α- and β-subunits of bitiscetin from *B. arietans* venom showed no greater than 42% sequence similarity to any of the *Echis* spp or *B. arietans* CTL sequences.

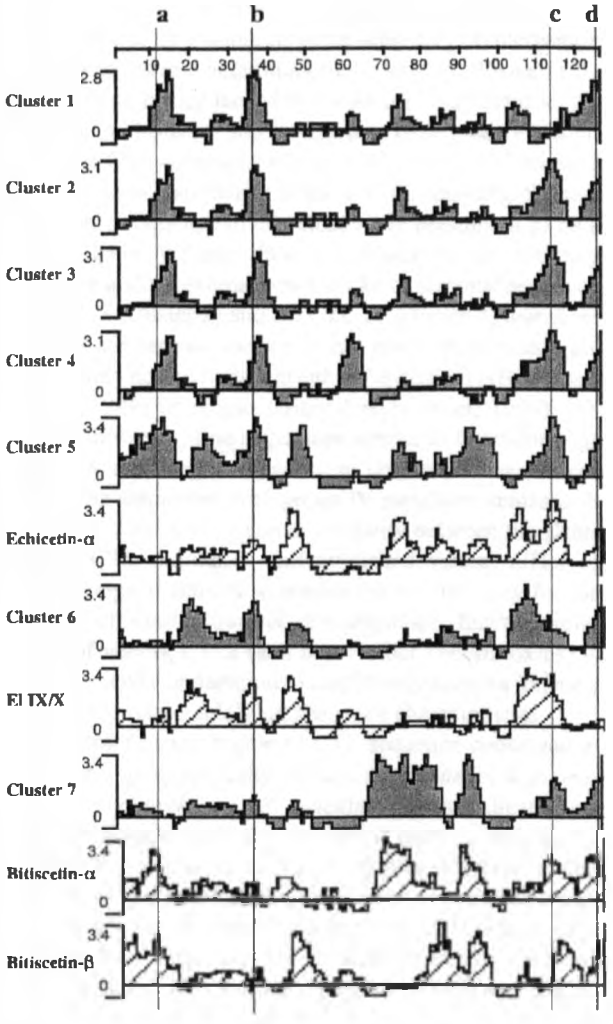

Fig. 5. Jameson–Wolf antigenic profiles of the *E. ocellatus*, *E.p. leakeyi*, *E.c. sochureki* and *B. arietans* CTL clusters and all known CTLs from related African vipers. The top horizontal scale represents the number of amino acid residues (the 23 signal peptide residues are not presented here). The vertical scales represent comparative antigenic values. The four thin vertical lines (a–d) are a subjective assignation of antigenic domains that exhibit the greatest phylogenetic representation.

3.4. Predicted antigenic profiles of the E.p. leakeyi, E.c. sochureki, E. ocellatus and B. arietans CTL clusters

Since the main focus of our research is to develop toxin-neutralising antibodies by immunisation with DNA encoding specific toxins in venoms of the most medically important African vipers (Harrison et al., 2000, 2002), we next compared the algorithm-predicted immunogenicity of the *Echis* spp and *B. arietans* CTL cluster cDNA sequences with those of all the published CTLs from vipers of African origin (Fig. 5). The deduced signal peptide domains of the *Echis* spp and *B. arietans* CTL were not represented here because they would be cleaved from the native proteins. The thin vertical lines are a subjective identification of the most immunogenic CTL domains that show the greatest phylogenetic conservation. Line (a) approximately corresponded to the residues C-Y-K (38–40; Fig. 3) and was common to clusters 1–5. The highly conserved domain between residues 48 and 55 (W-A-D-A-E-K-F-C) was not predicted to be immunogenic. Line (b) corresponded to the residues around H-L-V-S (63–66) and was common to clusters 1–6 and the *E. leucogaster* CTL that bind coagulation factors IX/X. Lines (c) (residues around C136) and (d) (V-C-K at positions 144–146) appeared to represent immunogenic domains common to all the new and published African viper venom CTL sequences.

4. Discussion

The *Echis* and *Bitis* CTL sequences described here contained all the cysteine residues and most of the associated amino acid motifs that define the CRD domain and overall architecture of the mammalian and viper CTL sequences (Drickamer, 1988). These results represent a significant addition to the venom gland transcriptome of the *Echis* saw scaled vipers and *Bitis arietans* and reveal the CTL composition of these vipers to be as complex as that of the better characterised New World vipers (Wisner et al., 2002). The latter review illustrates that viper venom CTLs possess a range of pharmacological functions that disrupt haemostasis by inhibiting platelet aggregation through interaction with several platelet receptors, including von Willebrand factor domains (e.g., Botrocetin; Bitiscetin), the glycoprotein receptors GPIa (e.g., Aggretin), GPIb

(e.g., Echicetin, Flavocetin, CHH-A and B) and GPVI (e.g., Convulxin). Venom CTLs also activate the coagulation cascade through cleavage of factor X (e.g., RVV-X), factor IX (e.g., IX activator) and factor II (e.g., Carinactivase). Conversely, other venom CTLs inhibit coagulation by binding to factors IX and X (e.g., IX/X binding proteins from venoms of *P. flavoviridis* and *E. leucogaster*).

The *Echis* and *Bitis* sequences grouped in CTL clusters 1–4 appear to be distinct but highly similar CTL subunits that are novel to the serpents genetic database. In common with CTL β-subunits of flavocetin-A and convulxin from venoms of *P. flavoviridis* and *C. d. terrificus*, respectively (Shin et al., 2000; Leduc and Bon, 1998), CTL clusters 1–4 contained a double cysteine motif at positions 26 and 27 that have been invoked to differentiate β- from α-subunits of CTLs (Shin et al., 2000). The N-terminal additional C26 of β-CTL subunits is thought to form a disulphide bridge with a C-terminal cysteine residue of α-subunits permitting the polymerisation of these peptides into heterodimeric molecules (Wisner et al., 2002). Although they lacked the additional C26 residue, the sequences in clusters 5 and 6 were also most closely associated with β-CTL subunits. Only *Ba CTL*-5 (cluster 7) showed a closer sequence similarity to the α-CTL subunits. Since it was the only sequence in this cluster, we presume it to be a low copy gene. We infer from the absence of an initiating methionine and non-signal peptide-like N-terminal sequence of *Es CTL*-3 (cluster 3) and *Eo CTL*-27 (cluster 5) that these sequences are derivatives from other genes, presumably the group IV metalloproteinases at either the carboxyl (Bjarnason and Fox, 1994) or amino terminal ends (Kini, 1996). We could not, however, find sequences similar to these domains in group IV metalloproteinases or in venom CTL sequences known to be associated with group IV metalloproteinases. It is tempting to use the sequence similarity between the *Echis* and *Bitis* CTL sequences and published venom CTLs of known biological activity to predict the functionality for the new CTL clusters. However, the sequence–function relationship of venom CTLs (and many other venom toxins) is still insufficiently understood to confidently assign a function to a molecule based solely on sequence characteristics.

The extraordinary degree of CTL sequence conservation between the geographically distant *E. ocellatus*, *E.p. leakeyi*, *E.c. sochureki* and *B. arietans* vipers indicates the biological importance of the multimeric nature of this group of venom toxins. Here, and as frequently observed in the snake venom literature (Deshimaru et al., 1996), the DNA sequences encoding these *Echis* and *Bitis* CTLs showed a higher level of sequence conservation than the deduced amino acid sequences (data not shown). Accelerated evolution of snake venom toxins has been mooted to confer an evolutionary advantage by increasing the range of prey susceptible to envenoming. The identical inter-specific and inter-generic sequences for cluster 3 CTLs and near identity of sequences in other clusters suggest that accelerated venom toxin evolution is regulated by a sophisticated control mechanism, rather than a system that simply accepts degenerative substitutions. While this is likely to be a fruitful area of research, it was not the purpose of this study.

Our objective was to evaluate the range of CTL molecules likely to be represented in venoms of *E. ocellatus*, *E.p. leakeyi*, *E. sochureki* and *B. arietans* vipers in order to guide the design of DNA-immunisation constructs required to generate toxin-neutralising antibodies. We have shown that DNA immunisation is an efficient means of generating specific antibodies with potent venom toxin-neutralising activity (Harrison et al., 2000, 2002) and that the toxin-specific antibodies exhibit immunological reactivity to venoms from a wide range of viper species (Harrison et al., 2003). Our utilisation of a low-stringency PCR approach was successful in amplifying several distinct CTL isoforms from the venom glands of these vipers. The predicted antigenic profiles of the *Echis* and *Bitis* CTLs illustrated in Fig. 5 indicate that neutralisation of this complex group of toxins will require antibodies with several specificities. Thus, while an antibody raised by immunisation with cluster 3 DNA is likely to be effective against the gene products of clusters 2, 3 and 4, additional antibodies will be required to neutralise the other clusters. Also, because of the medical importance of echicetin (Peng et al., 1994) and bitiscetin (Hamako et al., 1996), we have initiated experiments to isolate cDNAs encoding these molecules from the respective libraries. Therefore, to be confident of neutralising the function of this group of venom toxins, we speculate the requirement of between six and eight CTL antibody specificities. While this may seem excessive, our results indicate that such a panel of antibodies would neutralise this important group of functionally diverse toxins in venoms of the most important African vipers across their geographic range. Given (i) the basal status of the *Echis* genus in viperine lineages (Lenk et al., 2001) and (ii) the high degree of inter-specific and inter-generic CTL sequence conservation observed here, it is possible that the venom CTL-neutralising effectiveness of such a panel of antibodies may extend to other African vipers and also to phylogenetically related vipers in Asia.

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