Research Article

Serine Protease Variants Encoded by *Echis ocellatus* Venom Gland cDNA: Cloning and Sequencing Analysis

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Envenoming by *Echis* saw-scaled viper is the leading cause of death and morbidity in Africa due to snake bite. Despite its medical importance, there have been few investigations into the toxin composition of the venom of this viper. Here, we report the cloning of cDNA sequences encoding four groups or isoforms of the haemostasis-disruptive Serine protease proteins (SPs) from the venom glands of *Echis ocellatus*. All these SP sequences encoded the cysteine residues scaffold that form the 6-disulphide bonds responsible for the characteristic tertiary structure of venom serine proteases. All the *Echis ocellatus EoSP* groups showed varying degrees of sequence similarity to published viper venom SPs. However, these groups also showed marked intercluster sequence conservation across them which were significantly different from that of previously published viper SPs. Because viper venom SPs 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 ocellatus EoSPs* on the basis of sequence alone. The extraordinary level of interspecific and intergeneric sequence conservation exhibited by the *Echis ocellatus EoSPs* and analogous serine proteases from other viper species leads us to speculate that antibodies to representative molecules should neutralise (that we will exploit, by epidermal DNA immunization) the biological function of this important group of venom toxins in vipers that are distributed throughout Africa, the Middle East, and the Indian subcontinent.

1. Introduction

Envenoming resulting from snake bites is an important public health hazard in many regions, particularly in tropical and subtropical countries [1, 2]. The saw-scaled viper *Echis ocellatus* is the most abundant [3] and medically important viper species in West Africa [4]. Envenoming by saw-scaled viper (*Viperidae: Echis*) species is thought to be responsible for more snakebite deaths worldwide than any other snake genus [5]. In northern Nigeria, *E. ocellatus* is responsible for 95% of all envenoming by snakes [6], causing several hundred deaths annually [7]. The precise incidence of snakebite is difficult to determine and is often grossly underestimated, but in some areas of the Nigerian savannas, victims of *E. ocellatus* envenoming may occupy more than

10% of hospital beds [8]. In the Benue valley of Nigeria, for example, the estimated incidence is 497 per 100 000 population per year with 10%–20% untreated mortality [9]. 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 [10]. Systemic effects include potentially lethal consumption coagulopathy, haemorrhage and hypovolaemic shock [10].

Snake venoms contain a great variety of toxic proteases [11, 12]. Many of these components are proteases, for example, metalloproteases [13], serine proteases [14], phospholipases A_2 [15] and C-type lectins [16] 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 [12, 17–19]. 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 [19–25]. Approximately 100 snake venom toxins have been identified as "thrombin-like" enzymes activating the blood coagulation factor [26]. These "thrombin-like" proteases hydrolyze fibrinogen specifically and release either fibrinopeptide A or B or both [27] 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 [28].

Another group of serine proteases of *Batroxobin, Crotalase,* and Ancrod venoms affect other substrates, for example, plasminogen [27] by cleaving fibrinogen in manner distinct from that of thrombin. Other venom serine proteases function like mammalian kallikrein (or kininogenase) releasing bradykinin from kininogen [29–31] and are called "kallikrein-like" proteases [29], an example of this is halystase [32], 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 venoms of *A. caliginosus, C. atrox, C. viridis, and Trimeresurus mucrosquamatus* [29, 30, 32–34]. The latter showed both a strong β-fibrinogenolytic and kallikrein-like activities, cleaving β-chain of fibrinogen molecules specifically and releasing bradykinin from kininogen, respectively. Moreover, the purified enzymes indicated that they have specificities different from thrombin and thrombin-like proteases of snake venom reported previously by decreasing fibrinogen levels in plasma and prolonging bleeding without formation of fibrin clots. They also exhibit amidase activity against N-benzoyl-Pro-Phe-Arg-p-nitroanilide, which is a specific synthetic substrate for kallikrein-like proteases.

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 [35] (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 [36, 37], PA-BJ, a platelet aggregating enzyme isolated from the *B. jararaca* and *Trimeresurus mucrosquamatus* venoms [38], and RVV-V, a factor V-activating enzyme isolated from the *V. russelli* venom [39].

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 [40, 41]. Although a considerable amount of data

is now available, no standardised grouping of these venom serine proteases has yet been documented. However, in 2001 Wang et al. [27] 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. We therefore screened the *E. ocellatus* cDNA library in order to isolate and characterise different isoforms or variants of this enzyme superfamily.

2. Materials and Methods

2.1. Animals. Adult *E. ocellatus* (Nigeria) carpet viper used in this study was 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 *Echis ocellatus* snakes. The vipers were sacrificed 3 days after venom extraction when toxin gene transcription rates are at a peak. Glands were homogenized under liquid Nitrogen and total RNA extracted using guanidinium thiocyanate-phenol-chloroform as described previously [15]. Lambda phage cDNA libraries for *E. ocellatus* were constructed by RT-PCR using the SMART cDNA library construction kit (Clontech, California, USA). The lambda phage of the *E. ocellatus* 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 [42] was used to isolate sequences encoding serine proteinases from the cDNA libraries. A sense primer (5V-**GGA-TCC**-ATG-GTG-CTG-ATC-AGA-GTG-CTA-**ATC**-GCA-3V) and an antisense primer (5V-**CTC-GAG**-TGG-GGG-GCA-AGT-CGC-AGT-TGT-ATT-TCC-3V) complimentary to highly conserved amino-terminal signal peptide (M-V-L-I-R-V) and to the less conserved carboxy-terminal (T-T-A-T-C-P-P) domains of published serine proteinases DNA sequences of related viper species were synthesized commercially (Sigma-Genosys, UK). A TAG stop codon was inserted in the 3′ primer and BamH1 and Xho1 restriction endonuclease sites (bold) were included in the 5′ and 3′ primers, respectively, to facilitate future subcloning into mammalian expression plasmids. PCR was performed using an initial denaturation (95°C—6 minutes) and annealing (55°C—1 minute) step, followed by 35 cycles (1 minute each) of extension (74°C), denaturation (94°C), and annealing (55°C), and a terminal extension step (7 minutes)

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 BamH1 and Xho1 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 CEQk 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 Serine protienases of related vipers. The CLUSTALW program [43] with PAM 250 residue weight matrix was used to align deduced amino acid sequences representing each *E. ocellatus* Serineprotienases isoforms with analogues in venoms from related Viperidae species as illustrated in Table 1. Serine proteinase (CAB62591) from V. lebetina [44], Serine protease 1 (AAR24534) from B. gabonica [45], Thrombin-like enzyme pre. (AAK12273) from *D. acutus* [46], Venom serine protease 5 (AAN52350) from *T. stejnegeri* [47], Serine proteinase 3 pre. (O13063) from *gramineus* [48], Serine proteinase A Precursor (Q9PTU8) from *B. jararaca* [46, 49], Serine proteinase 2A pre. (O13060) from *T. gramineus* [45, 48], Serine protease (AAP42416) from *B. jararacussu* [50], KN-BJ2 (BAA20283) from *B. jararaca* [51], Serine proteinase 1 pre. (AAG10788) from *T. jerdonii* [52], Thrombin-like serine protease (AAL68708) from *G. ussuriensis* [53], and, finally, Serine protease catroxase I pre. (AAL77226) from *C. atrox* [54]. The phylogenetic trees constructed from the above alignments were generated by a neighbour-joining [55] algorithm in Lasergene software (DNASTAR, USA). The predicted antigenic profile [56] of the published and new *Echis ocellatus* serine protease (*EoSer*) isoforms analysed here was determined using Protean Software (DNASTAR).

3. Results

3.1. Isolation of cDNAs Encoding E. Ocellatus Serine Protease. PCR screening of the *Echis ocellatus* venom gland cDNA libraries resulted in a total of 14 *E. ocellatus* (*Eo*) cDNAs whose sequences matched (BLAST searches) those of published Serine proteases. The cDNAs consisted of 822 nucleotides (Figures 1 and 2(a)) and were predicted to encode an open reading frame proteins of 264 amino acids (28.5 kDa) (Figure 2(b)). Alignment of the predicted amino acid sequences of the 14 specific cDNAs encoding the *EoSP* proteins (Figure 2(b)) revealed sequence variations. 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. Where two or more identical sequences were obtained from any one of these libraries, a
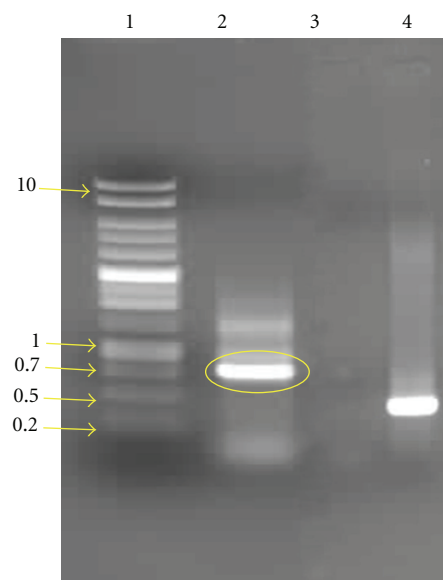

FIGURE 1: PCR product of the *E. ocellatus* serine proteases. Analysis of PCR amplification products by 0.7% agarose gel electrophoresis. Bands were visualised using the ultraviolet transillumination. Lane 2: represents the amplified PCR product (circled) of about 800 bp from *E. ocellatus* venom glands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

single representative cDNA was used for subsequent analysis. Structural properties analysis (Emin algorithm-DNASTAR, USA) (Figure 3) was used to categorise the 14 Serine protease sequences into four distinct groups, based solely on sequence alignment.

3.2. BLAST Search of the Predicted Amino Acid Sequence. Accession numbers assigned to the new *Echis ocellatus* Serine protease sequences are as follows: "group 1" *EoSer*-1 (GU562413), "group 2" EoSer-3 (GU592440), "group 3" *EoSer*-17 (GU592441), and "group 4" *EoSer*-7 (GU592439). 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 1) confirmed that the *EoSP* cDNAs encoded serine proteases.

3.3. Comparison of E. Ocellatus cDNAs with Analogous Serine Proteases from Other Viper Species. All the *EoSer*-variants contained the serine protease-consensus 24 amino acid signal peptide sequence (Figure 4, 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. The complete amino acid sequences of the *EoSP* variants were aligned with those of other venom serine proteases (Figure 4). Viper
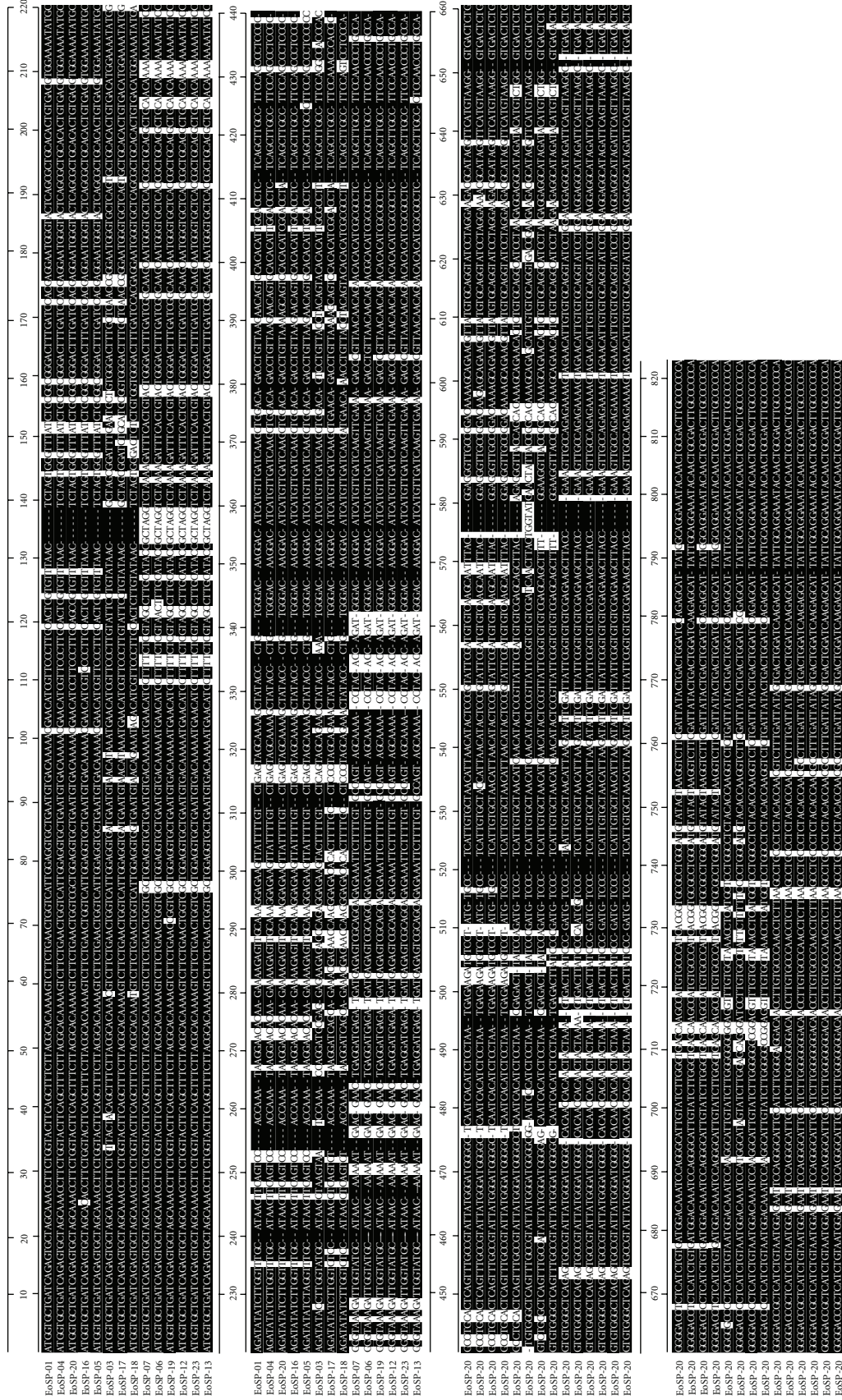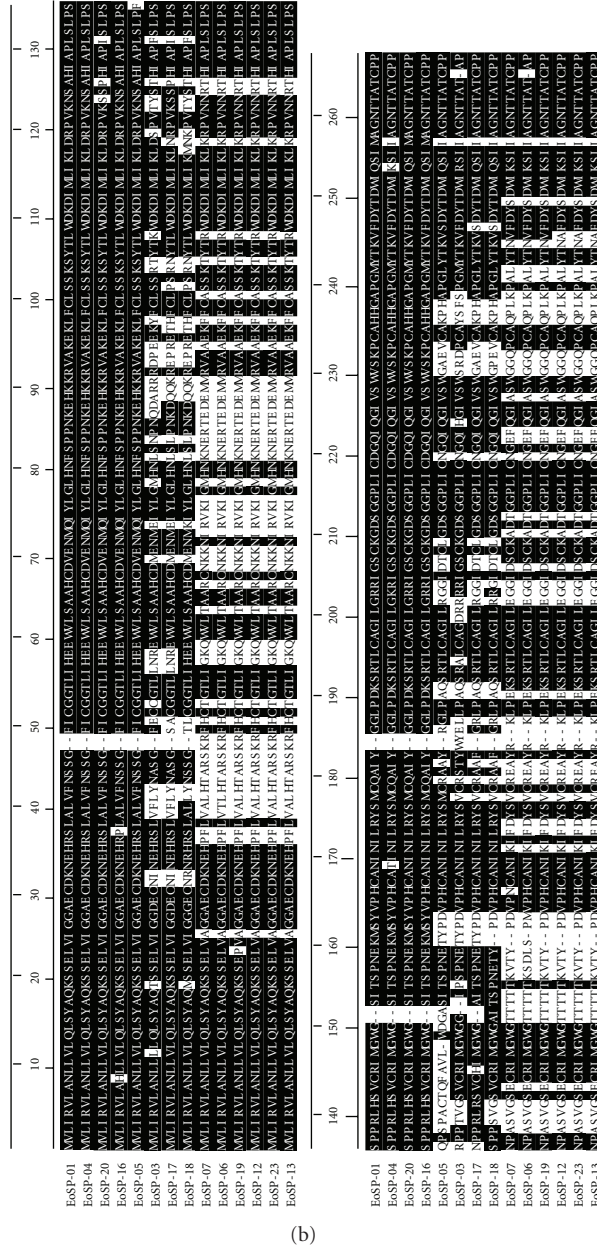
(a)

Figure 2: Continued.

(b)

FIGURE 2: (a) The nucleotide sequence of the fourteen *E. ocellatus* venom gland cDNAs resulting from PCR amplification. (b) Deduced amino acid sequences of *E. ocellatus* venom gland serine protease cDNAs.

venom SP sequences in the genetic databases were compared with the *E. o* groups (Table 2 and Figure 4) by BLAST. Groups 1–4 represent novel, highly similar, SP isomers with less than 65% sequence similarity to analogues in related viper species. Group 4 showed the greatest sequence similarity (80% and 82%) to the Serine protease of the African *V. lebetina* and *B. gabonica* vipers, respectively. Of all the *EoSP* clusters seemed to represent a SP sequence which showed the highest sequence similarity range between 62% to 70% to the SP of the vipers. None of the clusters showed more than 72% sequence similarity to the partial peptide sequences for the Thrombin-like serine protease isolated from the venom of

the *G. ussuriensis* viper [52]. Similarly, the Serine protease catroxase I pre. of *C. atrox* venom showed no greater than 65% sequence similarity to any of the *EoSP* sequences.

3.4. Predicted Antigenic Profile Analysis of E. ocellatus Serine Proteases with Analogous Molecules. 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 [15, 59, 60], we next compared the algorithm-predicted immunogenicity of the *E. ocellatus* serine protease cluster cDNA sequences with those of all the published SPs from vipers of African
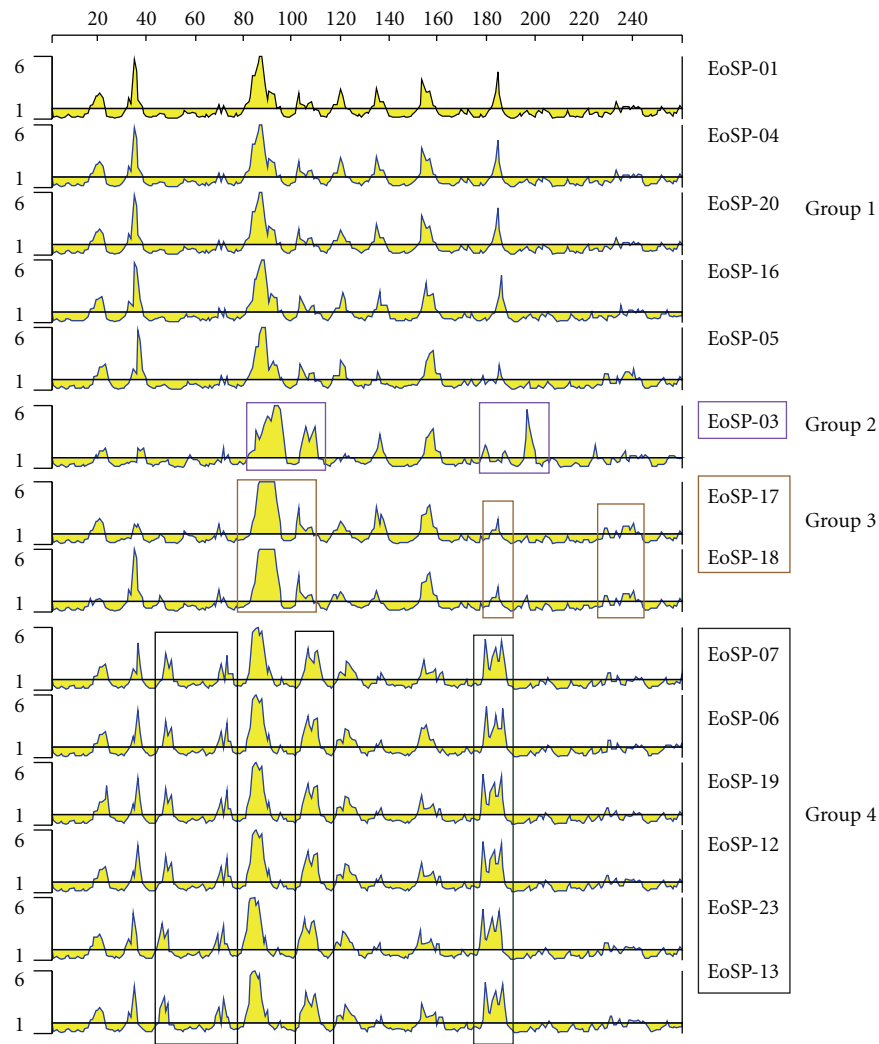
FIGURE 3: Differentiation of the fourteen cDNA-encoding *E. ocellatus* venom gland serine proteases. The predicted surface probabilities (Emin algorithm, DNASTAR, USA) of the 14 *E. ocellatus* serine protease cDNAs were aligned. The boxed areas indicate group specific structural motifs.

TABLE 1: Percent sequence similarity between *E. ocellatus* serine proteases and analogous molecules from related viper species.

| Species	Accession no.	References	Serine protease	*EoSP*-1	*EoSP*-3	*EoSP*-7	*EoSP*-17
V. lebetina	CAB62591	Siigur et al. [44]	Serine proteinase	65	66	80	64
B. gabonica	AAR24534	Francischetti et al. [45]	Serine protease 1	62	63	82	65
D. acutus	AAK12273	Liang et al. [46]	Thrombin-like enzyme pre.	67	67	71	70
T. stejnegeri	AAN52350	Lee and Zhang [47]	Venom serine protease 5	66	66	65	69
T. gramineus	O13063	Deshimaru et al. [48]	Serine proteinase 3 pre.	71	71	61	76
B. jararaca	Q9PTU8	Murayama, [49]	Serine proteinase A pre.	66	68	66	74
T. gramineus	O13060	Deshimaru et al. [48]	Serine proteinase 2A pre.	65	65	73	70
B. jararacussu	AAP42416	Kashima et al. [50]	Serine protease	63	63	72	68
B. jararaca	BAA20283	Serrano et al. [51]	KN-BJ2	69	68	62	69
T. jerdonii	AAG10788	Lu et al. [52]	Serine proteinase 1 pre.	65	65	71	70
G. ussuriensis	AAL68708	Zhao et al. [53]	Thrombin-like serine protease	67	67	72	71
C. atrox	AAL77226	Tsai et al. [54]	Serine protease catroxase I pre.	66	65	63	65
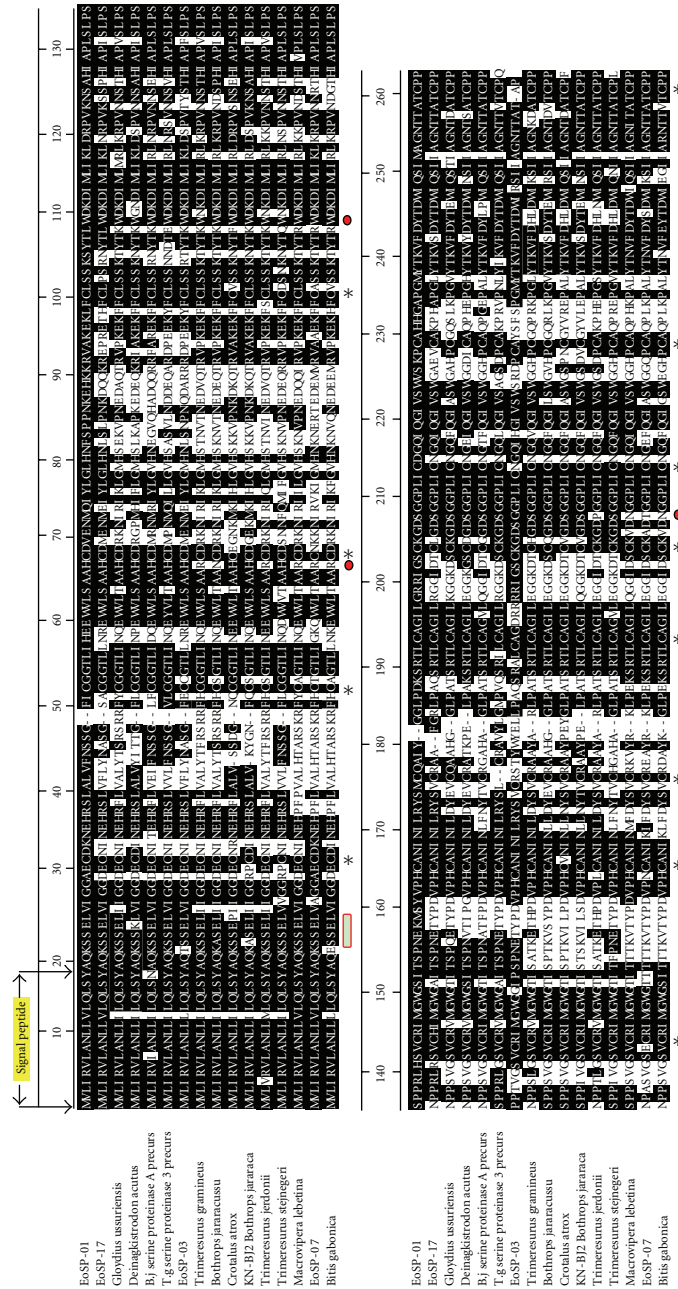
Figure 4: Amino acid sequence similarity between *EoSP* Variants and serine proteases from related vipers. The residues shaded in black correspond to residues that are identical to *EoSP-01*. The asteriks [*] represented the twelve conserved cysteine residues. The catalytic traid **His/Arg** (67), **Asp** (110) and **Ser** (208) are represented in red circles. Activated peptide where the mature proteine cleaved is represented by green rectangle.

origin (Figure 5). The predicted antigenic profiles of the published and new *E. ocellatus* serine proteases were analysed as shown in Figure 5 using Protean Software (DNASTAR, USA) [53]. 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 immunogenic

domains conservation common to all the new and published African viper venom SPs sequences as demonstrated in Figure 5.

4. Discussion

Serine proteases are a major component of viper venoms and are thought to disrupt several distinct elements of the blood coagulation system of envenomed victims.
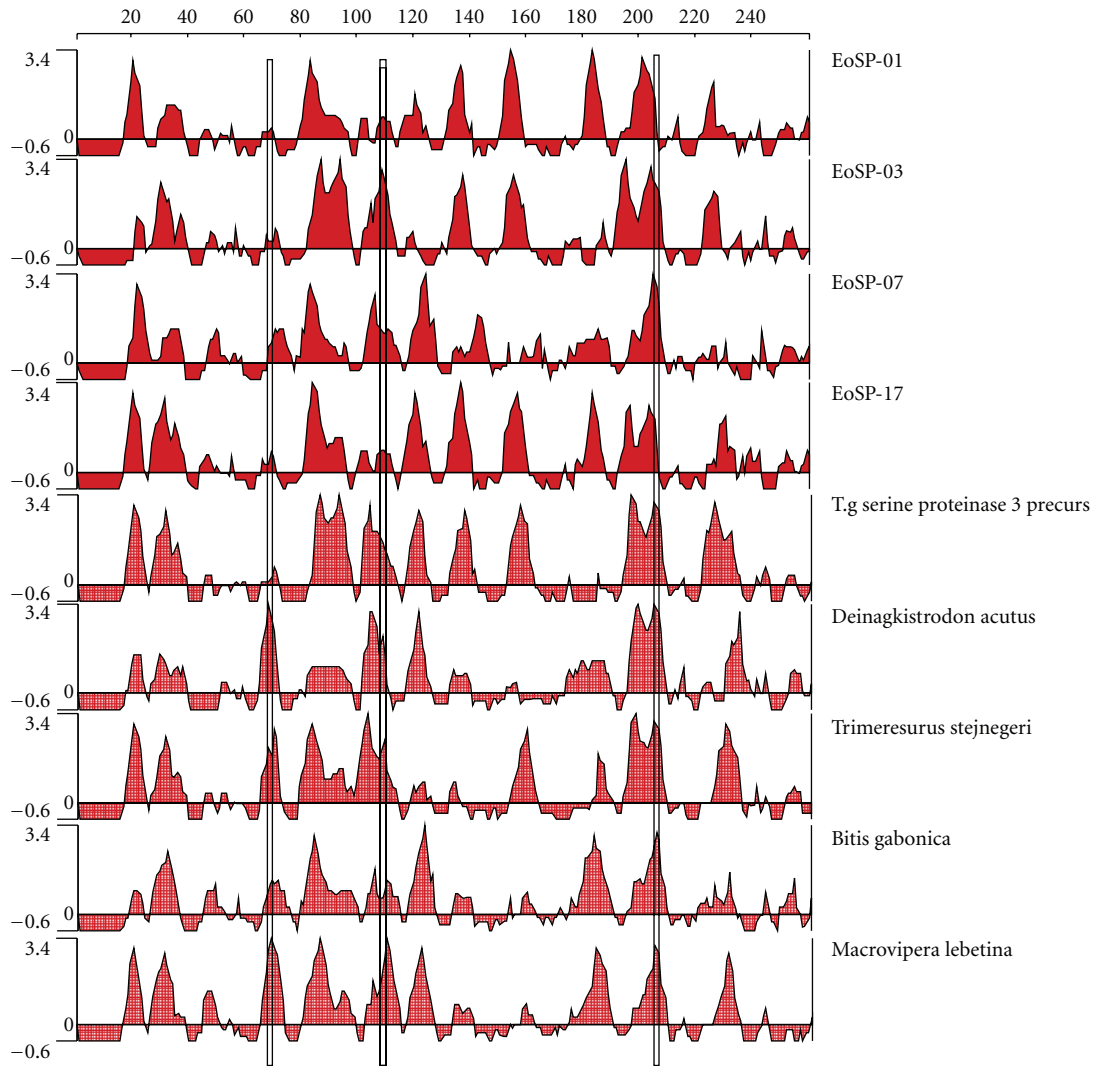
FIGURE 5: Comparison of antigenic profile of the *EoSer* variants with analogous serine proteases used in **Figure** 4. The top horizontal scale represents the number of amino acid residues. The conserved signal peptide is separated from the mature protein by a vertical dotted line. The three vertical boxes were drawn to indicate the conserved catalytic traid regions described in the text.

TABLE 2: 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}	
D	D^{102}	**D**^{102}	D^{102}	**D**^{112}	D^{112}	**D**^{112}	D^{112}	Braud et al. [57]
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. [58]
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. [57]
D	D^{96}	**N**^{96}	R^{96}	**Y**^{106}	Y^{106}	**Y**^{106}	Y^{106}	
D	D^{97}	**V**^{97}	T^{97}	**T**^{107}	T^{107}	**T**^{107}	T^{107}	Lee and Zhang [47]
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 kallikrein; DDE: substrate specificity to plasminogen.

A detailed understanding of the functions of these enzymes is important for both acquiring a full understanding of the pathology of envenoming and because these venom proteins have shown a vital role in treating blood coagulation disorders.

In general, serine proteinases including fibrinogenolytic enzymes are very abundant in *Viperidae* venoms in which they may account for 20% of their total protein content [61]. The unique specificity of snake venom proteinases makes them potentially useful in research of fibrinogen-depletion and limited proteolysis [62, 63]. This may be due to the existence of multiple forms of serine proteases in the venom of a single viper species which 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 work. The results obtained in this work 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, Figure 1) and encoded 260 amino acids (Figure 2(b)) 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 (Figure 3). A single representative clone from each group was chosen for further analyses as described earlier. 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 (Figure 4). The greatest sequence similarity was between *EoSer*-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 1. From the proteins with known biological activity, sequence similarities of the *EoSP* variants (i.e., *EoSer-01*, *EoSer-03*, *EoSer-07* and *EoSer-17*) were 62%–69% with the kinin-releasing and fibrinogen-clotting serine protease (KN-BJ) from venom of *B. jararaca* [51] (Table 1). 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 (Figure 4, 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** (Figure 4 in green) as proposed for batroxobin [64]; thus cleavage generates a hydrophilic zymogen peptide, based on the processing site of pre-peptides of mammalian serine proteinases [65–67]. 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 H67, D110 and S208 as shown in Figure 4. Such residues were highly conserved in groups 1–3, except proteins of group 4 (Figures 2(b) and 4) which contain R instead of H at the same position (Figure 4). Furthermore, comparison of the *EoSP* amino acid sequence alignment with analogous venom serine proteases (Figure 4) revealed a conserved consensus active site of L-T/S-A-A-H/R/N-C corresponding to position 63–68, as previously determined [68]. Most SVSPs are likely to be glycoproteins showing a variable number of N- or O-glycosylation sites in sequence positions that differ from one SVSP to the other [69]. Using the primary structure of *EoSP* variants (Figure 4) the putative N-linked glycosylation sites, Asn-X-Thr/Ser [45], were found and are located at two different positions. *EoSer-01, EoSer-03, and EoSer-17* [N^{44}-X^{45}-S^{46} and N^{257}-X^{258}-T^{259}] and *EoSer*-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 [30], confirmation of the roles of such motifs in venom proteases remain to be investigated. All serine proteases have a common pattern of 6-disulfide bridges [69, 70]. They contain twelve cysteine residues, ten of which form five disulfide bonds, based on the homology with trypsin [64]; the remaining two cysteines form a unique and conserved bridge among SVSPs, involving Cys245e (chymotrypsinogen numbering), found in the C-terminal extension [35].

From the results obtained this was found in all *EoSP* clones (Figure 2(b)) 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}** (Figure 4). 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 some cases certain amino acid sequences have been shown to be responsible for such effects as demonstrated in Table 2. 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 cannot be considered as a functional confirmation or even a categorization strategy to differentiate between the four *EoSP* cDNAs. However, from Figure 4 and Table 2 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 [56] 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 posttranslationally. The peaks shown by the *EoSP*s 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 *EoSP*s showed less similarity with that of the analogous venom SPs particularly those at residues 67 and 110, many antigenic residue similarities of *EoSP*s are shared with other SVSPs of related vipers. Therefore, it is likely that antibodies raised by *EoSP* 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.

Acknowledgments

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