

1 **Comparison of proteomic profiles of the venoms of two of the ‘Big Four’ snakes of India, the Indian**  
2 **cobra (*Naja naja*) and the common krait (*Bungarus caeruleus*), and analyses of their toxins.**

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11 **ABSTRACT:** Snake venoms are mixtures of biologically-active proteins and peptides, and several  
12 studies have described the characteristics of some of these toxins. However, complete proteomic profiling  
13 of the venoms of many snake species has not yet been done. The Indian cobra (*Naja naja*) and common  
14 krait (*Bungarus caeruleus*) are elapid snake species that are among the ‘Big Four’ responsible for the  
15 majority of human snake envenomation cases in India. As understanding the composition and complexity  
16 of venoms is necessary for successful treatment of envenomation in humans, we utilized three different  
17 proteomic profiling approaches to characterize these venoms: i) one-dimensional SDS-PAGE coupled  
18 with in-gel tryptic digestion and electrospray tandem mass spectrometry (ESI-LC-MS/MS) of individual  
19 protein bands; ii) in-solution tryptic digestion of crude venoms coupled with ESI-LC-MS/MS; and iii)  
20 separation by gel-filtration chromatography coupled with tryptic digestion and ESI-LC-MS/MS of  
21 separated fractions. From the generated data, 81 and 46 different proteins were identified from *N. naja*  
22 and *B. caeruleus* venoms, respectively, belonging to fifteen different protein families. Venoms from both  
23 species were found to contain a variety of phospholipases A<sub>2</sub> and three-finger toxins, whereas relatively  
24 higher numbers of snake venom metalloproteinases were found in *N. naja* compared to *B. caeruleus*  
25 venom. The analyses also identified less represented venom proteins including L-amino acid oxidases,

26 cysteine-rich secretory proteins, 5'-nucleotidases and venom nerve growth factors. Further, Kunitz-type  
27 serine protease inhibitors, cobra venom factors, phosphodiesterases, vespryns and aminopeptidases were  
28 identified in the *N. naja* venom, while acetylcholinesterases and hyaluronidases were found in the *B.*  
29 *caeruleus* venom. We further analysed protein coverage (Lys/Arg rich and poor regions as well as  
30 potential glycosylation sites) using in-house software. These studies expand our understanding of the  
31 proteomes of the venoms of these two medically-important species.

32 **Keywords:** toxins, venomics, venom variation, ESI-LC-MS/MS, tryptic digestion, toxin profile.

### 33 1. Introduction

34 Snake bite is a serious medical problem in tropical and sub-tropical regions, including India. The  
35 annual number of human deaths due to snake bite in India is conservatively estimated at 35,000 to 50,000,  
36 and the majority of bites are inflicted by the Indian or spectacled cobra (*Naja naja*), common krait  
37 (*Bungarus caeruleus*), saw-scaled viper (*Echis carinatus*) and Russell's viper (*Daboia russelii*) (Warrell,  
38 1999; Sharma *et al.*, 2015), the so-called "Big Four" venomous snakes of India (although there is debate  
39 as to whether a fifth species, the hump-nosed viper [*Hypnale hypnale*] should be included as a "big fifth"  
40 species; Joseph *et al.* 2007; Simpson and Norris 2007). Such a notably high number of snake bite related  
41 deaths are due to the close interaction between human and snake populations as well as the high toxicity  
42 of these snake venoms. Snake venoms are complex mixtures of proteins, peptides and other molecules  
43 that are produced by specialized venom glands and used for prey acquisition as well as defense against  
44 potential predators (Stocker, 1990). The diversity of bioactive molecules in venoms has been used to  
45 design pharmacologically active substances as potential drug leads and to develop precise research tools  
46 for better understanding of mammalian physiological pathways (Stocker, 1990; Koh and Kini, 2012).

47 In this study, we have chosen to evaluate the composition of the venoms of two elapid snakes  
48 belonging to the "Big Four": *N. naja* and *B. caeruleus*. *Naja naja* is native to the Indian subcontinent and  
49 found throughout Pakistan, Sri Lanka, Bangladesh and Southern Nepal. *Bungarus caeruleus* is found  
50 from the West Bengal plains of India to the Sindh province of Pakistan; occurs through South India and

51 Sri Lanka; and is recorded from Afghanistan, Bangladesh, and Nepal. Although neurotoxicity, which  
52 leads to acute neuromuscular paralysis (potentially causing morbidity and mortality), is a common effect  
53 of envenomation by these elapid snakes (Seneviratne and Dissanayake, 2002; Bawaskar and Bawaskar,  
54 2004; Ranawaka *et al.*, 2013), other toxins present in these venoms may also determine the overall effect.  
55 Analysis of venom composition along with characterization of individual new toxins is needed to  
56 elucidate mechanisms of venom toxicity (Yang, 1996) in an attempt to correlate the two (a field now  
57 termed “toxicovenomics” (Calvete 2015; Lauridsen *et al.* 2016,2017). Venom composition exhibits  
58 intraspecific (seasonal, geographical and ontogenetic) variations in numbers and types of toxins making  
59 species-level characterization an interesting challenge (e.g. Chippaux *et al.* 1991; Daltry *et al.* 1996A,B;  
60 Alape-Girón *et al.* 2008; Calvete *et al.* 2009). Previous studies have demonstrated regional differences in  
61 the compositional, biochemical and pharmacological properties of *N. naja* venoms within India, and these  
62 differences correlate with the severity of pathogenesis (Mukherjee and Maity, 1998; Shashidharamurthy  
63 *et al.*, 2002; Shashidharamurthy *et al.*, 2010; Saikumari *et al.*, 2015).

64 Relatively few proteomics studies of the venoms of either of these two dangerous elapids have  
65 been conducted. One previous study reported 26 proteins (with two or more associated peptide hits) from  
66 seven toxin families in the venom of a *N. naja* from Pakistan utilizing a combination of 2D gel  
67 electrophoresis and tryptic digest of crude venom followed by LC-MS/MS (Ali *et al.*, 2013).  
68 Sintiprungrat *et al.* (2016) compared the venomics of *N. naja* from western India (pool of venom from  
69 Rajasthan and Gujarat) and Sri Lanka, and six major venom protein families were reported in venoms  
70 from both areas. These were the three-finger toxin (3FTx), phospholipase A<sub>2</sub> (PLA<sub>2</sub>), cysteine-rich  
71 secretory protein (CRISP), snake venom metalloproteinase (SVMP), venom growth factor, and protease  
72 inhibitor protein families. Proteomic analysis of *N. naja* from eastern India revealed 43 proteins belonging  
73 to 15 venom protein families (Dutta *et al.*, 2017; minimum one peptide used to call protein). Further,  
74 earlier reports on the characterization of thermostable peptides from *N. naja* venom (Vietnam) revealed  
75 32 polypeptides and proteins belonging to the 3FTx, PLA<sub>2</sub>, cobra venom factor (CVF), L-amino acid

76 oxidase (LAAO), CRISP, and SVMP protein families (Binh *et al.*, 2010). A number of PLA<sub>2</sub>s (Bhat *et al.*,  
77 1991; Bhat and Gowda, 1991; Rudrammaji and Gowda, 1998; Sudarshan and Dhananjaya, 2016), SVMPs  
78 (Jagadeesha *et al.*, 2002), and other venom toxin components (Karlsson *et al.*, 1971; Müller-Eberhard and  
79 Fjellström, 1971; Shafqat *et al.*, 1990; Girish *et al.*, 2004) have also been isolated and characterized from  
80 *N. naja* venom. During the revision of this manuscript, venom analysis of *B. caeruleus* venom (pooled  
81 from Southeastern India and unspecified locales within India) was reported, with the observation that  
82 68.2% of total proteins were PLA<sub>2</sub>s, including  $\beta$ -bungarotoxins (Oh *et al.*, 2017). Moreover, only a few  
83 protein toxins have been purified and characterized from *B. caeruleus* venom (Bon and Changeux, 1975;  
84 Moody and Raftery, 1978; Sharma *et al.*, 1999; Singh *et al.*, 2001, 2005; Mirajkar *et al.*, 2005; More *et*  
85 *al.*, 2010).

86 Improvement of proteomic techniques, including the development of high resolution mass  
87 spectrometry and the deposition of protein sequences in accessible databases, has allowed for more  
88 thorough snake venom profiling in shorter time frames (Calvete *et al.*, 2007, 2009). Recent proteomic  
89 research has in turn spurred development of powerful methods for toxin characterization and  
90 immunological profiling of snake venoms (Pla *et al.*, 2012; Gutiérrez *et al.*, 2013). Hence, the information  
91 generated by such studies will potentially help in the production of more effective antivenoms and in the  
92 discovery of protein leads for the development of therapeutics (Vetter *et al.*, 2011). In the present study  
93 we utilized three different proteomic strategies to characterize the venoms of two medically-important  
94 elapid snake species. The first strategy was one dimensional SDS-PAGE, which was used to separate the  
95 constituent proteins after which tryptic digestion of bands and electrospray tandem mass spectrometry  
96 (ESI-LC-MS/MS) of resulting peptides was used to identify them. The second strategy was tryptic  
97 digestion of crude venom followed by ESI-LC-MS/MS. The third strategy was gel filtration (GF)  
98 followed by ESI-LC-MS/MS of the separated fractions. By combining these three complimentary  
99 techniques, we have generated more comprehensive proteomic profiles of the venoms of *N. naja* and *B.*  
100 *caeruleus* than have been previously reported.

## 101 **2. Materials and methods**

### 102 *2.1. Venoms*

103 Lyophilized crude *N. naja* and *B. caeruleus* venoms were procured from the Irula Snake  
104 Catcher's Society, Tamil Nadu, India. The snakes were from within Tamil Nadu state, and venoms were  
105 pooled from a number of individuals. The venoms were reconstituted in water prior to analysis unless  
106 stated otherwise.

### 107 *2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of venoms*

108 SDS-PAGE was performed in 12% polyacrylamide according to the method of Laemmli (1970).  
109 Crude venom samples (20 µg), each treated with non-reducing buffer were loaded in separate wells. The  
110 gels were stained with Coomassie blue and then destained by using 5% acetic acid in water. PageRuler™  
111 prestained protein marker (Thermo Scientific, Waltham, MA, USA) was used for estimating molecular  
112 weight.

### 113 *2.3. In-gel tryptic digestion*

114 Each band from SDS-PAGE gels was cut and destained using 200 µl MeOH: 50 mM NH<sub>4</sub>HCO<sub>3</sub>  
115 (1:1 v/v), and later dehydration was done using 200 µl ACN: 50 mM NH<sub>4</sub>HCO<sub>3</sub> (1:1 v/v). Next, 200 µl of  
116 100% ACN was added. The protein in the gel bands was reduced using 100 µl of 25 mM DTT in 50 mM  
117 NH<sub>4</sub>HCO<sub>3</sub> for 20 min followed by alkylation in the dark at room temperature with 100 µl of 55 mM  
118 iodoacetamide (Sigma, St. Louis, MO, USA) in 50 mM NH<sub>4</sub>HCO<sub>3</sub> for 15 min. Samples were then washed  
119 with 200 µl of 50 mM NH<sub>4</sub>HCO<sub>3</sub> in 50% ACN for 15 min and dehydrated with 200 µl of 100% ACN at  
120 room temperature for 15 min. The gels were digested using 20 µl of 12 ng/µl trypsin (Trypsin Gold,  
121 Promega, Madison, WI, USA). Next 30 µl of 0.01% ProteaseMAX in 50 mM NH<sub>4</sub>HCO<sub>3</sub> was added, and  
122 the sample was gently mixed and incubated at 37°C for 3 h. After incubation, 0.5 µl TFA was added to  
123 inactivate trypsin. The samples were centrifuged at 12,000 rpm and supernatants were loaded onto an  
124 HPLC-MS/MS system.

125 *2.4. Gel filtration chromatography of crude N. naja and B.caeruleus venom*

126 Crude venoms (100 mg) were dissolved in 2.5 ml of 50 mM Tris-HCl (pH 7.4) and filtered  
127 through 0.2 mm nylon syringe filters. The clear venom filtrates (1 ml) were loaded onto a HiLoad 16/60  
128 Superdex 200 pg gel filtration column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM  
129 Tris-HCl (pH 7.4). Proteins were eluted at a flow rate of 1 ml/min with the same buffer using an Äkta  
130 Purifier HPLC system (GE Healthcare), and the elution was monitored at 215 and 280 nm.

131 *2.5. In-solution tryptic digestion*

132 Individual freeze-dried crude venom samples and gel-filtration fractions were reconstituted in  
133 deionized water (4 µg/µl). Each sample (50 µl) was mixed with 41.5 µl of 50 mM NH<sub>4</sub>HCO<sub>3</sub> buffer, 1.0  
134 µl DTT (for reduction), and 2.0 µl of 1% ProteaseMAX detergent (in 50 mM NH<sub>4</sub>HCO<sub>3</sub>) and incubated at  
135 56°C for 20 min. Each sample was alkylated by adding 2.7 µl of 0.55 M iodoacetamide, and the mixture  
136 was incubated at room temperature (22-24°C) in the dark for 15 min. Trypsin (1.8 µg/µl in 50 mM acetic  
137 acid) was then added, and the samples were incubated at 37°C for 3 h. Next, 0.5 µl TFA was added and  
138 the mixture incubated at room temperature for 10 min. Samples were centrifuged at 10,000 rpm for 10  
139 min, and supernatants were analyzed by HPLC-MS/MS.

140 *2.6. Electrospray tandem mass spectrometry (ESI-LC-MS/MS)*

141 The tryptic digests of individual samples obtained by in-gel or in-solution digestion (described  
142 above) were loaded onto an Accela LCQ Fleet ion trap mass spectrometer. Each 80 µl sample was  
143 injected onto a Hypersil Gold C18 column (50 x 2.1 mm, Thermo Scientific, Waltham, MA, USA) pre-  
144 equilibrated with 0.1% formic acid (buffer A). Elution was carried out at a flow rate 200 µl/min with a  
145 linear gradient of 100% ACN in 0.1% formic acid (buffer B). The gradient was set from 0 to 40% buffer  
146 B over 38 min and then from 40 to 80% over 18 min. The eluent from the LC column was directly fed to  
147 the mass spectrometer, and the system was set to positive ionization mode. Spectra were obtained with a  
148 scan range of 500 to 2000 m/z.

## 149 2.7. Protein and peptide identification

150 MS/MS spectra were analyzed using SEQUEST and Proteome Discoverer 3.1 software with a  
151 false discovery rate of 0.01. The peptide fragments were assigned to protein families based on their  
152 similarity to sequences in the Lepidosauria subset of the National Centre for Biotechnology Information  
153 (NCBI) database. If at least two peptides were detected (with at least one being unique), protein species  
154 were considered to be present in the sample. Only proteins with a SEQUEST score of 2.0 or higher were  
155 utilized for analysis.

## 156 2.8. Visualization of SEQUEST output

157 The portions of the protein sequences covered by peptides detected via tandem mass spectrometry  
158 were visualized using an in-house Perl script. The program generates figures showing the peptide  
159 coverage, potential glycosylation sites and lysine (K) or arginine (R) rich and poor regions. As trypsin  
160 lyses proteins directly after K or R residues, the regions that are too high or too low in presence of these  
161 residues are either too small or too large (respectively) to be detected by MS based on the range  
162 parameters set. K/R-rich and K/R-poor regions were defined as protein segments containing more than  
163 two K/R within 3 to 5 residues or lacking K/R within 20 to 25 residues, respectively. The tryptic peptides  
164 with 5 to 20 residues were considered as potentially detectable with the standard experimental parameters.  
165 Although we did not specifically check for glycosylated peptides, their presence can cause failure in  
166 peptide detection due to uncertainty pertaining to the mass of sugar moieties, they may lead to peptides  
167 containing them being undetected. For this reason, we mapped potential glycosylation sites (predicted by  
168 the presence of NXT/NXS, where X is any residue except a proline).

## 169 3. Results and Discussion

### 170 3.1. Proteomics of SDS-PAGE gel bands

171 The abundance of high and low molecular weight proteins in both crude venoms was shown by  
172 SDS-PAGE (Fig.1). Visually, the banding patterns in the two venoms are distinct, with *N. naja* venom

173 appearing to have a greater number of large molecular weight proteins than *B. caeruleus* venom. Based  
174 on published molecular weights, the prominent bands indicate the potential proteins from various toxin  
175 families including 3FTx, PLA<sub>2</sub>, CRISP, LAAO, and SVMP, among others, in both the venoms. The  
176 individual gel bands were sliced, pooled into four fractions (Fig. 1) and further subjected to in-gel trypsin  
177 digestion and HPLC-MS/MS. From the SDS-PAGE bands of *N. naja* venom, 25 total proteins belonging  
178 to ten toxin families were identified (Table S1). Likewise, from the *B. caeruleus* SDS-PAGE bands, we  
179 identified twenty three proteins from six different toxin families (Table S2).

### 180 3.2. Proteomics of venom by direct tryptic digestion

181 Crude venoms of *N. naja* and *B. caeruleus* were subjected to tryptic digestion and further analysis  
182 using HPLC-MS/MS. Forty different proteins from 13 toxin families were identified from the *N. naja*  
183 venom (Table S1). Similarly, from the *B. caeruleus* crude venom, we identified 43 different proteins from  
184 eight toxin families (Table S2).

### 185 3.3. Proteomics of gel-filtration fractions

186 The *N. naja* crude venom subjected to gel filtration chromatography yielded nine distinct protein  
187 peaks (Fig. 2). Within these peaks, 75 total proteins belonging to 14 toxin families were detected. In the  
188 *B. caeruleus* crude venom, seven distinct peaks were seen (Fig. 3) and within these peaks 34 total proteins  
189 from eight toxin families were observed. Identities of individual proteins are listed in Tables S1 and S2.  
190 Interestingly, gel filtration of *N. naja* venom yielded 36 proteins not identified using other techniques, but  
191 gel filtration of *B. caeruleus* venom yielded no unique peptides (Fig. 4).

### 192 3.4. Analyses of toxin families and isoforms

193 The complete analysis of the peptides obtained using combined approaches provided a  
194 comprehensive overview of the different protein families and species present in both *N. naja* (Fig 5) and  
195 *B. caeruleus* venoms (Fig. 6). The most widely used proteomic profiling workflow (Lomonte and  
196 Calvete, 2017) has some difficulty in eluting larger proteins of low abundance from C<sub>18</sub> HPLC columns.



197 In this study, we have used complementary approaches so as to avoid such issues and to generate a more  
198 complete proteomic profile. The approaches using in-gel or crude venom tryptic digestion coupled with  
199 ESI-LC-MS/MS detected a similar number of proteins in both *N. naja* and *B. caeruleus* venoms. This  
200 might be due to the abundance of low to medium sized (in terms of molecular weight) proteins in both of  
201 the venoms. The presence of a larger number of high molecular weight proteins in *N. naja* crude venom  
202 compared to *B. caeruleus*, as observed in SDS-PAGE profiles, has resulted in 36 proteins being identified  
203 in *N. naja* venom using the third approach (in-solution tryptic digestion of gel-filtration fractions and ESI-  
204 LC-MS/MS) compared to none in *B. caeruleus* venom. The differences and similarities in protein  
205 profiles between the two species on examination of the proteomic results are discussed in detail.

#### 206 3.4.1. PLA<sub>2</sub> enzymes

207 PLA<sub>2</sub> enzymes were found to be present in relatively high abundance and were represented  
208 overall by 17 and 19 proteins in *N. naja* and *B. caeruleus* venoms, respectively (Figs. 5 and 6). The  
209 members of this family induce a wide range of pharmacological effects including myotoxicity,  
210 neurotoxicity, hypotension, haemolysis, cardiotoxicity, antibacterial, coagulopathy, hemorrhage, edema,  
211 tissue damage and convulsion (Kini, 2003). Neurotoxic PLA<sub>2</sub> enzymes are among the most lethal toxic  
212 components in cobra and krait venoms (Tan *et al.*, 1989; Yanoshita *et al.*, 2006). Peptides belonging to a  
213 previously reported acidic PLA<sub>2</sub> and a group I PLA<sub>2</sub> were detected in both *N. naja* and *B. caeruleus*  
214 venoms (Tables S1 and S2). In the *N. naja* venom, peptides with sequences similar to several PLA<sub>2</sub>  
215 enzymes from the elapid species *N. sagittifera*, *N. atra*, *N. kaouthia*, *N. melanoleuca* and *Micropechis*  
216 *ikaheka* as well as some viperid species were detected (Table S1). In the *B. caeruleus* venom, peptides  
217 similar to several PLA<sub>2</sub> isoforms originally described from the elapid snakes *B. multicinctus*, *B. fasciatus*,  
218 *B. candidus*, *N. naja*, *N. sagittifera*, *Brachyuropsis roperi*, *Laticauda colubrina*, *Pseudechis australis*, and  
219 *Cacophis squamulosus* were detected (Table S2).

#### 220 3.4.2. 3FTxs

221 We identified 24 proteins in the *N. naja* venom belonging to the 3FTx family and 14 in the *B.*  
222 *caeruleus* venom (Figs. 5 and 6). The 3FTxs have a distinctive structural arrangement composed of three  
223  $\beta$ -stranded loops that protrude from a small hydrophobic globular core that makes them appear like a  
224 hand with three extending fingers. This protein scaffold is stabilized by four conserved disulfide bonds  
225 located in the globular core (Kini and Doley, 2010). Despite having similarities in structure, they are  
226 known to exert diverse activities such as neurotoxicity, cardiotoxicity, cytotoxicity, and anti-platelet  
227 effects (Kini and Doley, 2010). The 3FTxs detected in the *N. naja* and *B. caeruleus* venoms belong to the  
228 short-chain neurotoxin, long-chain neurotoxin,  $\kappa$ -neurotoxin, muscarinic toxin and orphan subfamilies  
229 (Tables S1 and S2). In both venoms, peptides identical to those from *N. naja* cytotoxin 7, long neurotoxin  
230 1, and long neurotoxin 4; *N. oxiana* short neurotoxin 1; and *N. nivea* or *N. kaouthia* cytotoxin 2 were  
231 detected. (Tables S1 and S2). In *N. naja* venom, 3FTx peptides were obtained similar to sequences from  
232 the elapids *N. naja*, *N. kaouthia*, *N. mossambica*, *N. atra*, *N. nivea*, *N. annulifera*, *N. oxiana* and *N.*  
233 *annulata annulata* (Table S1). In the *B. caeruleus* venom, peptides similar to 3FTxs from the elapids *B.*  
234 *candidus*, *B. multicinctus*, *N. kaouthia*, *N. naja*, *N. melanoleuca*, and *N. atra* were identified (Table S2).

### 235 3.4.3. SVMPs

236 Analysis of the peptides obtained in our study revealed 13 SVMPs in the *N. naja* venom and five  
237 in the *B. caeruleus* venom (Figs. 5 and 6). SVMPs are organized into three broad classes- PI, PII (with  
238 subclasses a through e), and PIII (with subclasses a through d) based on differences in structure (Fox and  
239 Serrano, 2005, 2009, 2010). In addition to hemorrhagic activity, members of the SVMP family may also  
240 have fibrin(ogen)olytic activity, inhibit platelet aggregation, or be pro-inflammatory (Markland and  
241 Swenson, 2013). There is generally very little metalloproteinase content in elapid venoms (Li *et al.*, 2004;  
242 Markland and Swenson, 2013) compared to viperids (Francischetti *et al.*, 2004; Serrano *et al.*, 2005;  
243 Cidade *et al.*, 2006; Sanz *et al.*, 2006). The SVMPs identified mostly belonged to the PIIIa subfamily, and  
244 peptides from zinc metalloproteinase disintegrins and other domains of SVMPs were found in both  
245 venoms (Tables S1 and S2). In *N. naja* venom, SVMP peptides showed similarity to sequences originally

246 from the elapids *N. kaouthia*, *N. atra*, *B. multicinctus*, *Hoplocephalus bungaroides*, *Micrurus fulvius* and  
247 *Drysdalia coronoides* as well as the viperid *Echis carinatus sochureki* (Table S1). SVMP peptides from  
248 *B. caeruleus* venom had similar sequences to those from the elapids *B. multicinctus*, *N. kaouthia*, and  
249 *Micrurus fulvius* (Table S2).

#### 250 3.4.4. CRISPs/helveprins

251 A total of six CRISPs were identified in *N. naja* venom as compared to two in *B. caeruleus*  
252 venom, although they were the second largest group of non-enzymatic proteins found in both venoms  
253 (Figs. 5 and 6). The members of the CRISP family inhibit cyclic nucleotide-gated ion channels in  
254 photoreceptor and olfactory cells (Brown *et al.*, 1999) and potassium activated smooth muscle contraction  
255 (Osipov *et al.*, 2005; Ito *et al.*, 2007). CRISP peptide sequences in the *N. naja* venom were similar to  
256 sequences from the elapids *N. atra* and *N. kaouthia* and from the viperids *Trimesurus gracilis* and *D.*  
257 *russelii* (Table S1). In the *B. caeruleus* venom, peptides with similar sequences to CRISPs originally  
258 identified from elapid *B. candidus* and *Ophiophagus hannah* were detected (Table S2).

#### 259 3.4.5. LAAOs

260 A comparison of the number of LAAOs detected in both the venoms revealed six in the *N. naja*  
261 venom and two in the *B. caeruleus* venom (Figs. 5 and 6). LAAOs are homodimeric, high molecular  
262 weight enzymatic proteins (Tan and Fung, 2010) responsible for oxidizing hydrophobic L-amino acids  
263 thereby releasing  $\alpha$ -keto acids, ammonia and  $H_2O_2$  (Du and Clemetson, 2002; Dineshkumar and  
264 Muthuvelan, 2011). The product of this reaction,  $H_2O_2$ , causes edema formation; ADP or collagen-  
265 induced platelet aggregation inhibition or activation; apoptosis; and antibacterial, antiparasitic,  
266 anticoagulant, hemolytic and hemorrhagic effects (Suhr and Kim, 1996). LAAO peptides in the *N. naja*  
267 venom were similar to peptide sequences of proteins from the elapids *N. atra*, *Notechis scutatus*, and  
268 *Oxyuranus scutellatus* as well as the viper *D. russelii* (Table S1). In the *B. caeruleus* venom, LAAO  
269 peptides were similar to those from the elapid *B. multicinctus* (Table S2).

#### 270 3.4.6. 5'-Nucleotidases (5'-NTDs)

271 There were two 5'-NTDs detected in the *N. naja* venom compared to one in the *B. caeruleus*  
272 venom (Figs. 5 and 6). Ubiquitous enzymes, 5'-NTDs are primarily responsible for causing disruption of  
273 physiological homeostasis in humans by inducing anticoagulant effects and by inhibiting platelet  
274 aggregation (Dhananjaya *et al.*, 2006). They also act synergistically with other toxins to exert a more  
275 pronounced anti-coagulant effect during envenomation (Dhananjaya and D'Souza, 2010). Peptides of 5'-  
276 NTDs from *N. naja* and *B. caeruleus* venoms showed sequence similarity to 5'-NTD sequences from  
277 *Micrurus fulvius* (Table S1) and *Ovophis okinavensis* (Table S2), respectively.

#### 278 3.4.7. Toxins specific to *N. naja* venom

279 There were four proteins belonging to the CVF family detected in the *N. naja* venom (Fig. 5).  
280 CVF is a complement-activating protein present in cobra venom. It is a three-chain protein that  
281 functionally resembles C3b, the activated form of complement component C3 (Vogel *et al.*, 2004).  
282 Peptides from this protein family showed similarity to CVF sequences from *N. kaouthia* (Table S1).

283 Peptides belonging to two proteins of the poorly characterized toxin family of aminopeptidases  
284 were also found only in *N. naja* venom (Fig. 5). Aminopeptidases are exo-metalloproteases, and in  
285 mammals they are involved in normal physiological functions such as maintenance of blood pressure  
286 (Vajyapuri *et al.*, 2010). Aminopeptidase peptides identified from *N. naja* venom showed similar  
287 sequence to those from the elapid *Micrurus fulvius* and the viperid *Crotalus horridus* (Table S1).

288 Two proteins belonging to the PDE family were identified in *N. naja* venom (Fig. 5). The  
289 findings correlate with the earlier reports that claim low levels of PDEs in elapid venoms and none in  
290 *Bungarus* sp. (Aird, 2002). The members of the PDE family are responsible for the successive release of  
291 5'-mononucleotides from the 3' end of polynucleotides (Iwanaga and Suzuki, 1979) and for supplying  
292 nucleotide substrates for the activity of other toxins, such as 5'-NTDs (Aird, 2002; Valério *et al.*, 2003).  
293 PDEs are also reported to inhibit platelet aggregation and lower mean arterial pressure (Russell *et al.*,

294 1963). The detected PDE peptides showed similarity to sequences from the elapid *Micrurus fulvius* and  
295 the viperid *Macrovipera lebetina* (Table S1).

296 Two ohanin/vespryn proteins were identified in *N. naja* venom (Fig. 5). Ohanin identified from  
297 *O. hannah* venom was the first snake venom protein shown to induce hypolocomotion and hyperalgesia  
298 in experimental mice (Pung *et al.*, 2005). The peptides identified from *N. naja* venom showed similarity  
299 to the isoforms from *N. kaouthia* and *O. hannah* (Table S1).

300 Only one SVSP was identified from the *N. naja* venom (Fig. 5). These proteins are widely  
301 distributed in viperid venoms but are found quite rarely in elapid venoms (Jin *et al.*, 2007). SVSPs show  
302 fibrin(ogen)olytic activities and are not susceptible to hirudin, heparin, or other serine protease inhibitors,  
303 so they result in abnormal fibrin clots (Matsui *et al.*, 2000). The identified sequences showed similarity to  
304 SVSPs from *N. atra* (Table S1).

305 The analysis also revealed peptides belonging to one previously reported VNGF protein from *N.*  
306 *naja* venom (Table S1). VNGFs isolated from the elapids have properties similar to those of nerve growth  
307 factor isolated from mouse submaxillary glands and are responsible for the survival and differentiation of  
308 neurons (Angeletti, 1970; Barbacid, 1995).

309 One previously reported isoform of Kunitz-type serine protease inhibitor (KPI) was identified in  
310 *N. naja* venom (Table S1). Several KPIs have been purified and characterized from elapid and viperid  
311 snake venoms (Shafqat *et al.*, 1990). A wide variety of biological functions such as inhibition of serine  
312 proteases, blocking of ion channels, interference in blood coagulation, inflammation and fibrinolysis are  
313 exhibited by KPIs (Mukherjee *et al.*, 2014).

#### 314 3.4.8. Toxins specific to *B. caeruleus* venom

315 In *B. caeruleus* venom, peptides were detected for two hyaluronidase isoforms (Fig. 6). The  
316 members of this protein family cause degradation of hyaluronic acid in the extracellular matrix which is  
317 important in the diffusion of toxins from the site of a bite into the circulation. Hence, they are also called

318 “spreading factors” (Kemperaju and Girish, 2006). Hyaluronidases generate a wide range of metabolic  
319 products through hyaluronic acid degradation (Kemperaju and Girish, 2006). Hyaluronidase peptides in  
320 the *B. caeruleus* venom showed similarity to those from the viper *Bitis arietans* (Table S2).

321 One AChE was detected in the *B. caeruleus* venom (Fig. 6). These proteins play a crucial role in  
322 cholinergic transmission and have been reported to occur at high levels in the venoms of elapid snakes,  
323 particularly in *Bungarus* (Frobert *et al.*, 1997). Identified peptides for this protein showed sequence  
324 similarity to AChEs from *B. fasciatus* (Table S2).

325 In-depth analyses of identified proteins in *N. naja* and *B. caeruleus* venoms indicated that both  
326 are rich in PLA<sub>2</sub>s and 3FTxs. This correlates well with the severe neurotoxicity and cytotoxicity observed  
327 in *N. naja* and *B. caeruleus* envenomations (Law *et al.*, 2014). The other protein families, with relatively  
328 low abundance, seem to have minor physiological effects on envenomated humans.

### 329 3.5. Peptide coverage analysis

330 The prediction of K/R rich or poor regions and potential glycosylation sites in proteins is critical for  
331 a complete proteomic profiling, as it helps predict coverage to be expected based on any given technique.  
332 Our in-house MS visualization program generated figures that show the overlapping of individual tryptic  
333 peptides and the overall coverage in the identified proteins. Here, we have shown coverage data for toxins  
334 from the PLA<sub>2</sub>, 3FTx, CRISP, and SVMP families in both *N. naja* and *B. caeruleus* venom (Figures 7 and  
335 8). Similarly, figures were generated for other toxin families (data not shown). The coverage and amount of  
336 overlap of tryptic peptides were higher for PLA<sub>2</sub>s and 3FTxs than for CRISPs and SVMPs in both *N. naja*  
337 and *B. caeruleus* possibly due to a number of factors such as smaller sizes of 3FTxs and PLA<sub>2</sub>s, tryptic  
338 cleavage patterns (numbers and locations of K/R residues), and relative concentrations of each toxin in the  
339 venom. In the *N. naja* venom potential glycosylation sites were observed in proteins belonging to the 3FTx,  
340 CRISP, PLA<sub>2</sub> and SVMP families, although only 3FTx and SVMP proteins in *B. caeruleus* venom showed  
341 the same possibilities.

#### 342 **4. Conclusion**

343 Analysis of the proteomes of *N. naja* and *B. caeruleus* venoms using the complementary  
344 approaches of gel electrophoresis, liquid chromatography and mass spectrometry has generated an in-  
345 depth characterization of these venoms. We have identified 81 protein species from *N. naja* venom (a  
346 much greater number than previously reported in proteomic studies) and 46 from *B. caeruleus* venom.  
347 The detection of such a large number of venom proteins, including PLA<sub>2</sub>s, 3FTxs and SVMPS, correlate  
348 well with clinical reactions reported in the literature. Hence, it can be concluded that using the three  
349 complementary strategies, rather than one can yield comparatively more information and give a better  
350 overall characterization of venom proteomes.

351 In this study we noted the presence of AChEs, PDEs, 5'-NTDs, and SVSPs specifically in *N. naja*  
352 venom as well as hyaluronidases and AChEs solely in *B. caeruleus* venom. This analysis clearly shows  
353 the differences in the toxin composition between the venoms and contributes further to the current  
354 knowledge of venom proteins in these species. To determine such variations in venom composition,  
355 analysis of samples from specific localities needs to be done, and that will help in designing more  
356 efficacious, region-specific anti-venoms.

357 The range of molecular sizes of proteins and peptides is a major obstacle in proteomic analysis of  
358 snake venoms. With the extensive developments in proteomic techniques, it has become possible to better  
359 elucidate the complete toxin profiles of snake venoms. As such, we have utilized complementary  
360 techniques to create an expanded understanding of the proteomes of the venoms of these two medically-  
361 important species.

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- 592

593 **Fig.1.** SDS-PAGE profiles of the crude venoms of *Naja naja* and *Bungarus caeruleus*. Lanes: 1, standard protein ladder;  
594 2, *N. naja* crude venom; and 3, *B. caeruleus* crude venom. Abbreviations: 3FTxs, three finger toxins; CRISPs, cysteine-  
595 rich secretory proteins (helveprins); CVFs, cobra venom factors; KPIs, Kunitz-type serine protease inhibitors; LAAOs, L-  
596 amino acid oxidases; PLA<sub>2</sub>s, phospholipases A<sub>2</sub>; PDEs, phosphodiesterases; SVMPs, snake venom metalloproteinases;  
597 and VNGFs, venom nerve growth factors

598

599 **Fig.2.** Gel filtration chromatography profile of crude *Naja naja* venom. Crude venom (100 mg in 2.5 ml of 50 mM  
600 Tris-HCl, pH 7.4) was loaded onto a Superdex 200 column pre-equilibrated with the same buffer. Proteins were  
601 eluted with the same buffer at a flow rate of 1 ml/min and monitored at 215 nm. Insets indicate the snake venom  
602 protein families identified by tandem mass spectrometry in individual peaks. Numbers in parenthesis are the number  
603 of protein species identified for each family. Abbreviations: 3FTxs, three finger toxins; 5'-NTDs, 5'-nucleotidases;  
604 CRISPs, cysteine-rich secretory proteins (helveprins); CVFs, cobra venom factors; KPIs, Kunitz-type serine  
605 protease inhibitors; LAAOs, L-amino acid oxidases; PLA<sub>2</sub>s, phospholipases A<sub>2</sub>; PDEs, phosphodiesterases; SVMPs,  
606 snake venom metalloproteinases; and VNGFs, venom nerve growth factors.

607

608 **Fig.3.** Gel filtration chromatography profile of crude *Bungarus caeruleus* venom. Crude venom (100 mg in 2.5 ml of  
609 50 mM Tris-HCl, pH 7.4) was loaded onto a Superdex 200 column pre-equilibrated with 50 mM Tris-HCl (pH 7.4).  
610 Proteins were eluted with the same buffer at a flow rate of 1 ml/min and monitored at 215 nm. Insets indicate the  
611 snake venom protein families identified by tandem mass spectrometry in individual peaks. Numbers in parenthesis  
612 are the number of protein species identified for each family. Abbreviations: AChEs, acetylcholinesterases; 3FTxs,  
613 three finger toxins; 5'-NTDs, 5'-nucleotidases; CRISPs, cysteine rich secretory proteins (helveprins); LAAOs, L-  
614 amino acid oxidases; PLA<sub>2</sub>s, phospholipases A<sub>2</sub>; and SVMPs, snake venom metalloproteinases.

615

616 **Fig.4.** Venn diagrams of the number of protein species obtained from A) *Naja naja* and B) *Bungarus caeruleus*  
617 venoms using three proteomic approaches. Differently shaded areas indicate overlap among the various techniques.

618

619 **Fig.5.** Relative abundance of venom protein families identified by ESI-LCMS/MS of crude *Naja naja* venom. Each  
620 section represents the number of protein species found within that specific toxin protein family, with relative  
621 percentage in parentheses. Abbreviations: 3FTxs, three finger toxins; 5'-NTDs, 5'-nucleotidases; CRISPs, cysteine-  
622 rich secretory proteins (helveprins); CVFs, cobra venom factors; KPIs, Kunitz-type serine protease inhibitors;  
623 LAAOs, L-amino acid oxidases; PLA<sub>2</sub>s, phospholipases A<sub>2</sub>; PDEs, phosphodiesterases; SVMPS, snake venom  
624 metalloproteinases; and VNGFs, venom nerve growth factors.

625

626 **Fig.6.** Relative abundance of venom protein families identified by ESI-LCMS/MS of *Bungarus caeruleus* crude  
627 venom. Each section represents the number of protein species found within that specific toxin protein family, with  
628 relative percentage in parentheses. Abbreviations: AChEs, acetylcholinesterases; 3FTxs, three finger toxins; 5'-NTDs,  
629 5'-nucleotidases; CRISPs, cysteine rich secretory proteins (helveprins); LAAOs, L-amino acid oxidases; PLA<sub>2</sub>s,  
630 phospholipases A<sub>2</sub>; and SVMPS, snake venom metalloproteinases.

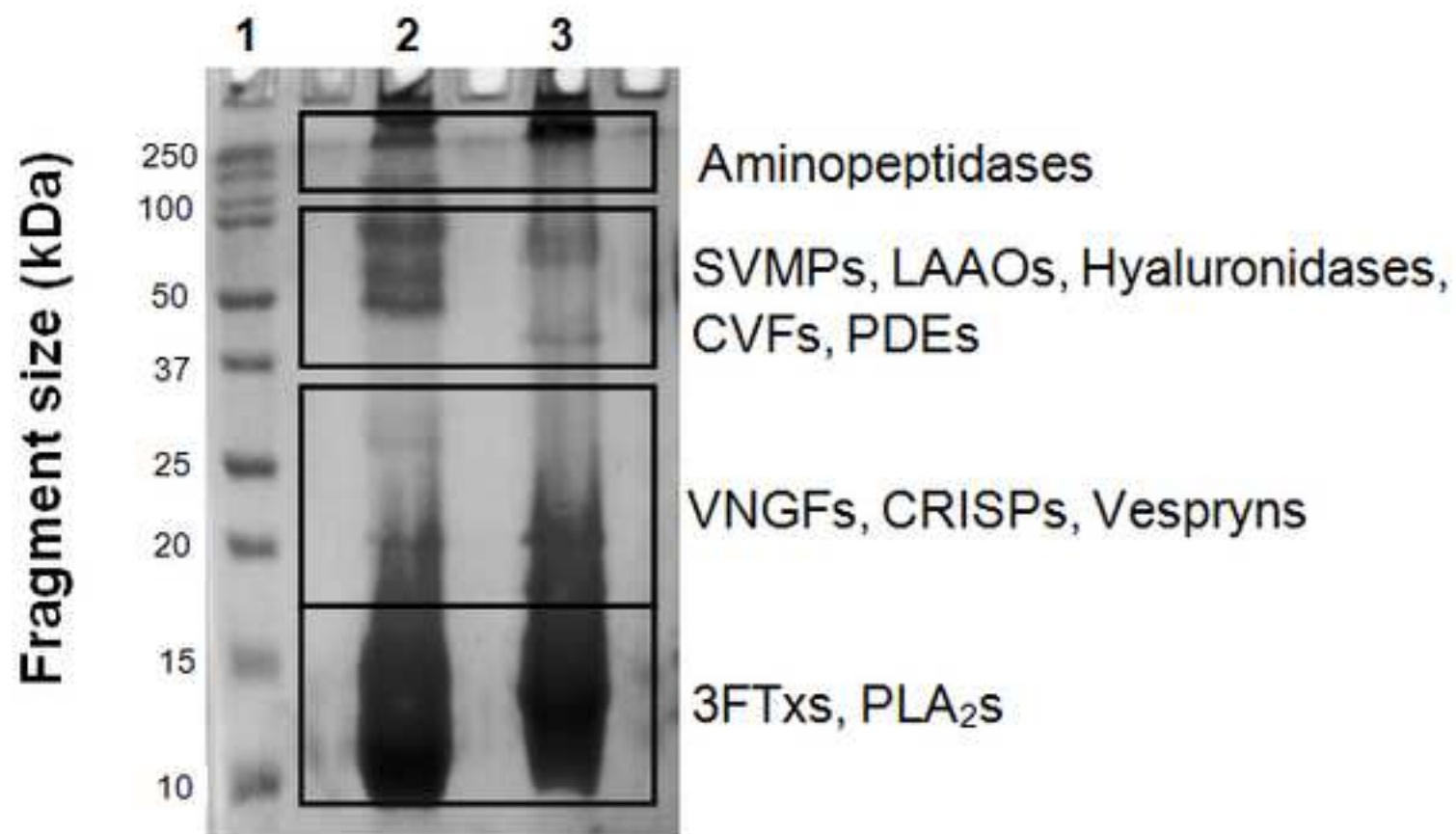
631 **Fig.7.** Sequence coverage of the major protein families in *Naja naja* venom. Alphanumeric designations on the left of  
632 each panel represent NCBI accession numbers. Panels: (A) 3FTxs, three-finger toxins; (B) PLA<sub>2</sub>s, phospholipases A<sub>2</sub>;  
633 (C) CRISPs, cysteine-rich secretory proteins (helveprins); and (D) SVMPS, snake venom metalloproteinases. Blue,  
634 black and brown regions show the lysine and arginine poor, medium and rich regions, respectively. Red, purple and  
635 green bars indicate tryptic peptides of low, medium and high score, respectively, based on SEQUEST analysis of the  
636 corresponding proteins. In addition, cyan regions in the protein sequence represent potential glycosylation sites.

637

638 **Fig.8.** Sequence coverage of the major protein families in *Bungarus caeruleus* venom. Alphanumeric designations  
639 on the left of each panel represent NCBI accession numbers. Panels: (A) 3FTxs, three-finger toxins; (B) PLA<sub>2</sub>s,  
640 phospholipases A<sub>2</sub>; (C) CRISPs, cysteine-rich secretory proteins (helveprins); and (D) SVMPS, snake venom  
641 metalloproteinases. Blue, black and brown regions show the lysine and arginine poor, medium and rich regions,  
642 respectively. Red, purple and green bars indicate tryptic peptides of low, medium and high score, respectively, based  
643 on SEQUEST analysis of the corresponding proteins. In addition, cyan regions in the protein sequence represent  
644 possible glycosylation sites.

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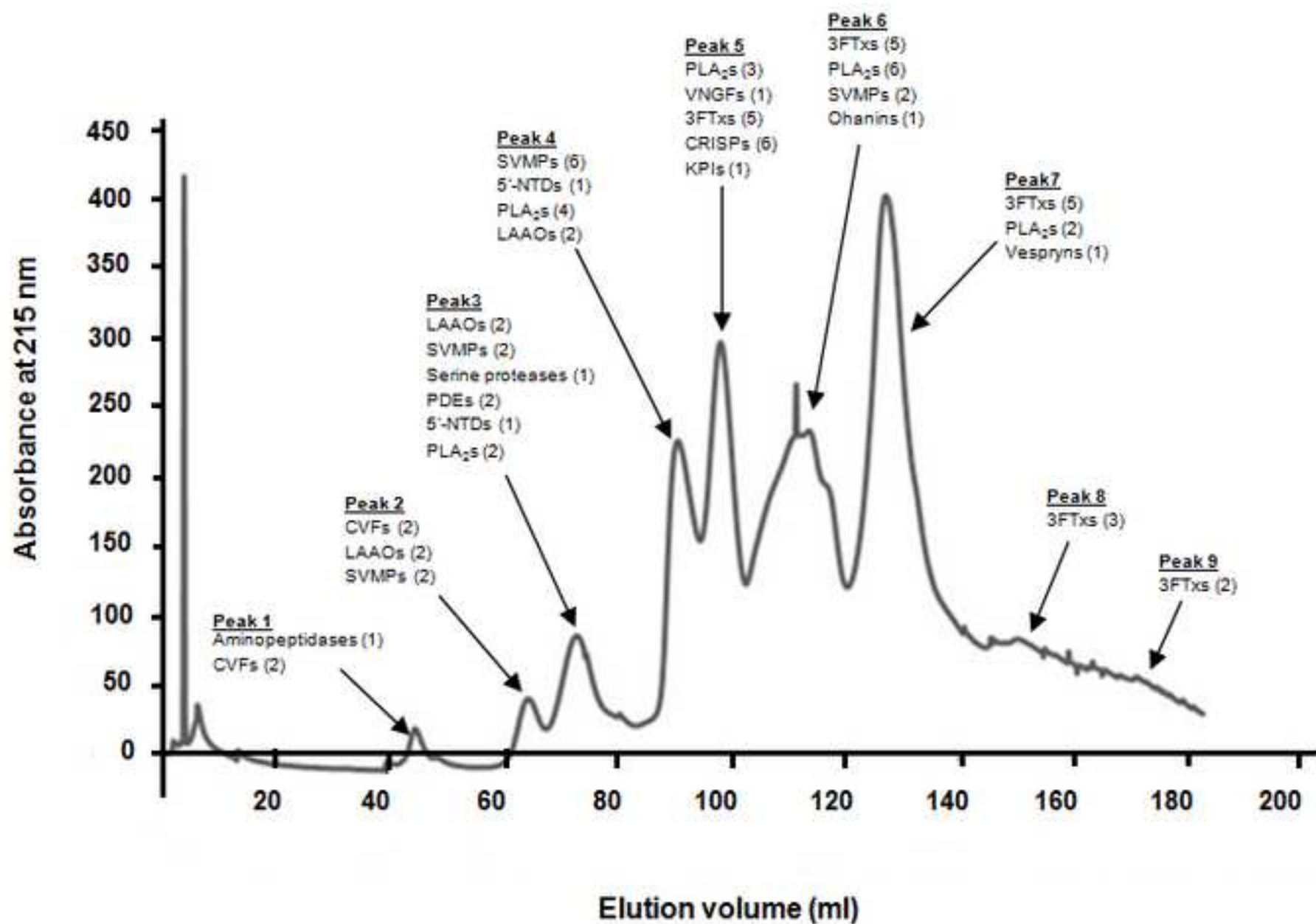
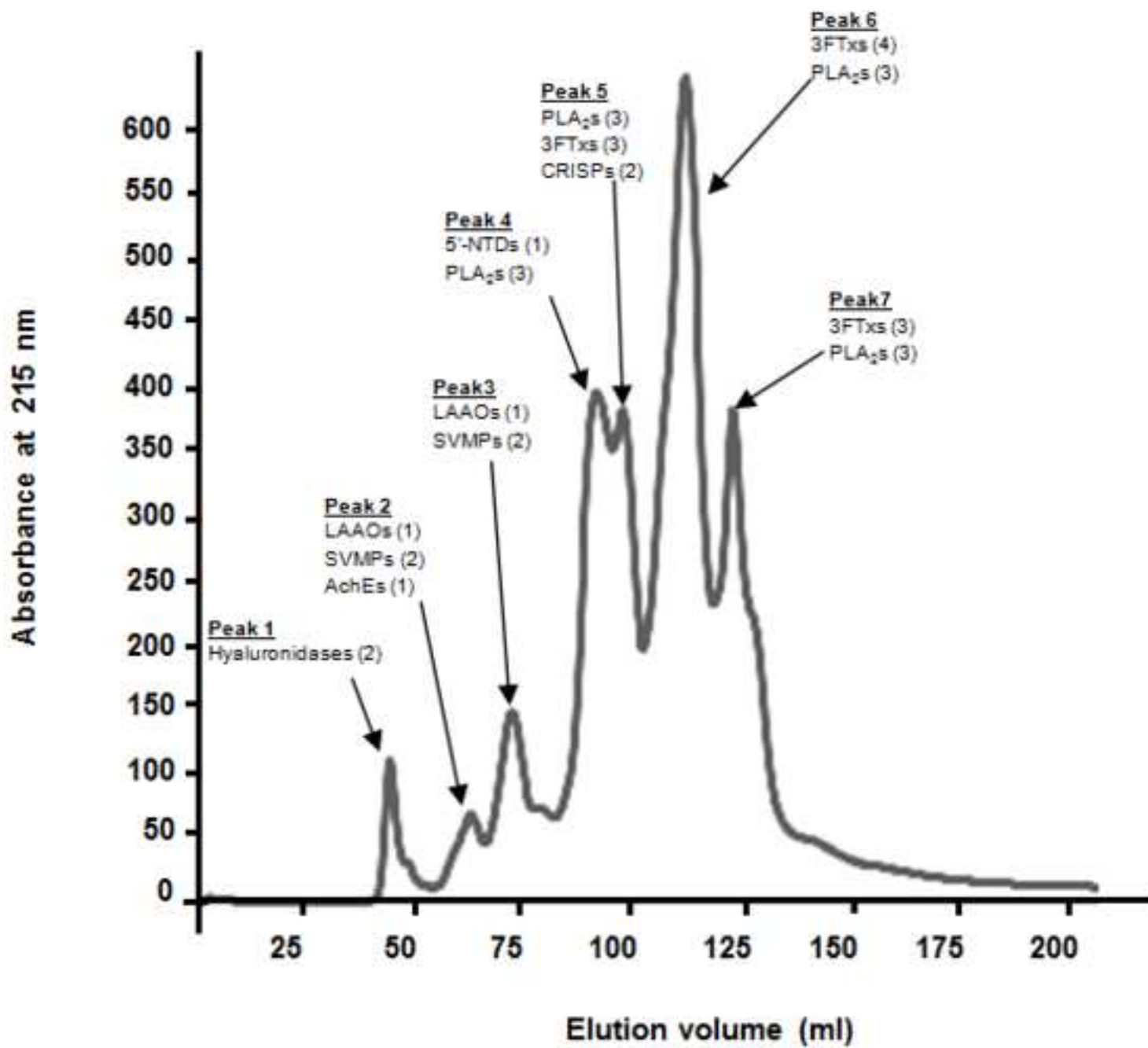
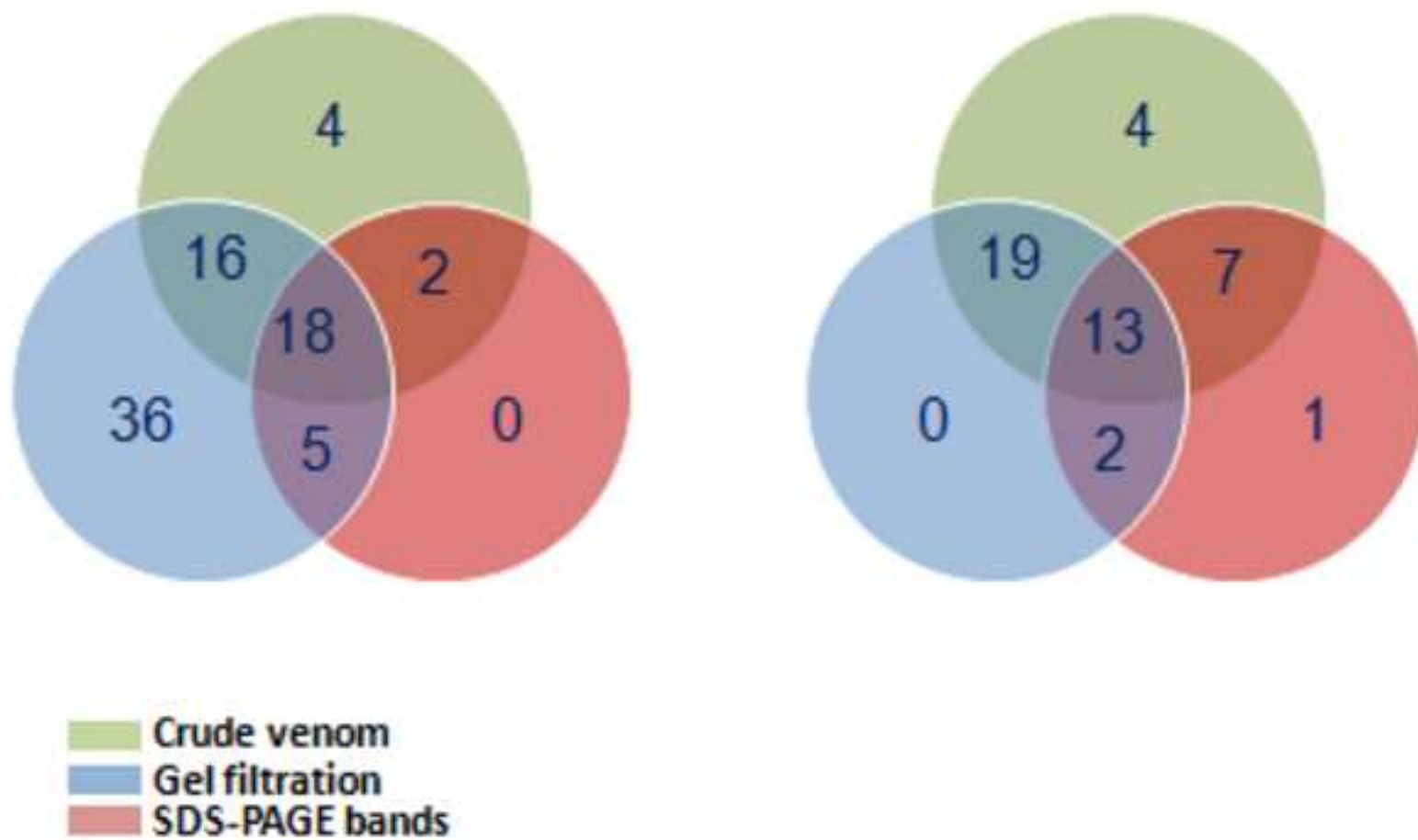


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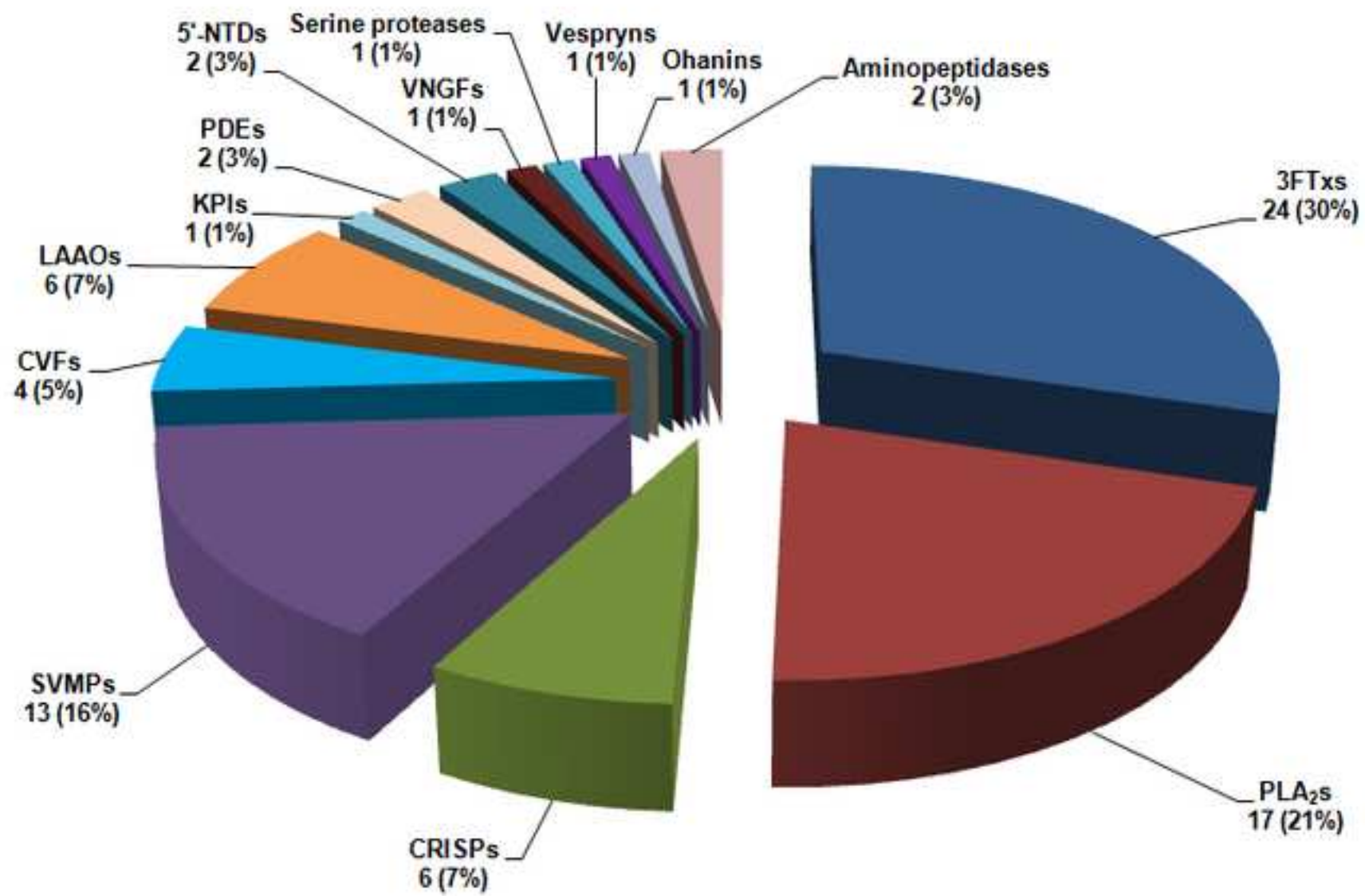
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