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Identification of < 10 KD peptides in water extraction of Venenum Bufonis from Bufo gargarizans using Nano-LC-MS/MS and De novo sequencing

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1 **Identification of <10KD peptides in the water**
2 **extraction of *Venenum Bufonis* from *Bufo gargarizans***
3 **using Nano LC-MS/MS and *De novo* sequencing**

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14
15 **Abstract**

16 Skins of anurans (frogs and toads) are rich sources of bioactive peptides. However,
17 the peptides secreted by the skin glands of *Bufo gargarizans*, the most common toad
18 in China, remained unexplored to date. Here, a strategy combines LC-MS/MS, RNA
19 sequencing and bioinformation analysis was applied to unravel the peptides in the
20 *Bufo gargarizans* secretions. Data-dependent LC-MS/MS acquisitions of intact
21 peptides followed by automated chromatographic alignment, *De novo* analysis,
22 database and homology searches with manual validations showed that the venom is
23 composed by 939 features, with masses ranging from 0.7–4 kDa. These peptides
24 derived from 85 proteins were identified using the PEAKS software with acquired MS
25 and MS/MS spectra of *Venenum Bufonis* against the house-built protein database
26 using *De novo* RNA sequencing, while only 23 peptides from 8 proteins were found

27 when searching known amphibian database. Moreover, it was found that many
28 peptides with high abundance in *Venenum Bufonis* derived from proteolytic
29 processing of a larger precursor protein, named as CL4590. Molecular cloning was
30 applied to validate a short domain of CL4590 to evident the accuracy of these
31 obtained sequences. Although the bioactivities of peptides identified by MS/MS are
32 unknown, the next function annotation showed that they may involve in the cell
33 killing, immune and metabolic process, antioxidant activity and antimicrobial actions.
34 Therefore, the peptidomics analysis on *Bufo gargarizans* discover abundant novel
35 toad peptides which broaden our horizons on the secretion multiplicity and supplied
36 an assortment of pharmacological candidates.

37 **key words** *Bufo gargarizans*; Peptidomics; Mass Spectrometry; *De novo* sequencing;
38 Molecular Cloning

39

40 **1. Introduction**

41 Amphibians have evolved various defense strategies for survival. Their bioactive
42 peptides, especially the antimicrobial and anticancer peptides from the secretions,
43 have attracted much attention during the past decades. To date, more than a thousand
44 bioactive peptides from amphibian have been deposited into the Antimicrobial
45 Peptides Database (<http://aps.unmc.edu/AP/main.php>) and Anticancer Peptides
46 Database (<http://crdd.osdd.net/raghava/cancerppd/index.php>). In addition, many
47 antimicrobial peptides have been found to have antitumor effects [1]. Nowadays,
48 secretion of several species (snake, sorption, toad, frog) and their active components
49 (protein, peptides, enzymes) have revealed various therapeutic effect. PGLa, an
50 antimicrobial peptide from the magainin family, has been found in the skin of
51 *Xenopus laevis*. It demonstrates a wide spectrum activity against Gram-positive and
52 Gram-negative bacteria [2]. Melittin, a 28-mer α -helical peptide from bee venom,
53 inhibited several drug resistant bacteria in vitro and degraded the biofilm layer [3]. A
54 peptide from the venom of *Buthus Martensii Karsch* inhibited the propagation of
55 fibrosarcoma cells [4]. Magainin 2 could lyse hematopoietic tumor rapidly, without
56 damaging normal cells [5]. Bengalin, a protein separated from Indian black scorpion

57 (*Heterometrus bengalensis*) venom, showed antiproliferative activity on leukemic cell
58 lines [6]. A neuropeptide, isolated from the venom of Stony Creek Frog *Litoria*
59 *lesueuri*, showed anticancer activity on 60 different human cancer cells [7].
60 Antibacterial and anticancer studies on extraction from animal secretions are explored
61 all over the world. However, research on proteins and peptides from the secretion of
62 Chinese toad (*Bufo gargarizans*) is relatively insufficient. This important natural
63 property of antimicrobial or antitumor peptides to be potential drugs represents an
64 excellent value for therapeutic research.

65 *Bufo gargarizans*, one of the biggest species of *Bufo* in China, whose secretion
66 contains various small molecules with significant anticancer activity, has drawn much
67 attention so far [8]. The parotid secretion of *Bufo gargarizans*, *Venenum Bufonis*, has
68 been used in clinic as a traditional Chinese medicine for nearly 2000 years [9], and it
69 is well-known because of its anticancer activity as well as toxicity. It has been widely
70 acknowledged that its efficient and poisonous components were some small
71 molecules such as bufalin and cinobufagin [10,11]. However, the anticancer
72 concentration of small molecules *in vivo* resulted in serious cardiotoxicity [12]. While
73 two peptides from *Bufo gargarizans* and their derivations showed remarkable
74 antimicrobial and antitumor action. One of which is Buforin, which was discovered in
75 the stomach mucosa of the Asian toad *Bufo gargarizans* [13] and the other is a novel
76 Cathelicidin termed BG-CATH, which was identified by analysis of the toad skin
77 transcriptome [14].

78 Nowadays, identifying small peptides is a great challenge because mass spectrometry
79 tools are not permanently appropriate for the detection of molecules within small
80 mass range. Usually, fragmentation of small peptides produces low peaks with poor
81 responsibility which lead to vague identified sequences [15]. The enormous
82 complication and considerable number of peptides result in the extreme challenge to
83 investigation on animal secretions. Nevertheless, studying the comprehensive
84 multiplicity of secretion and toxicities in venom is considerable because of the massive
85 potential substances in animal venoms [16]. As earlier studies in articles, there are
86 many useful methods for identification of peptide at transcriptomic, peptidomics or
87 genomic level [17]. Despite the increase in the number of reports on venom
88 peptidomics, the comprehensive knowledge of the intricacy and dynamic range of

89 proteins present within animal venom remains a great challenge [18]. Furthermore,
90 there are lots of limitations of present technique, the combination strategy is necessary
91 for identify peptides with high-efficiency [19,20]. Currently, the methods are
92 improved by the combination of peptidomics and transcriptomic studies [21,22], which
93 could successfully unmask the composition of animal secretion. Here, we explored the
94 combination technique to reveal the peptidomic constitution of one of the most
95 important animal-venom-derived Chinese medicine, *Bufo gargarizans*, which could
96 improve the understanding of the molecular biological mechanisms of *Bufo*
97 *gargarizans* in the treatment of human disease.

98 **2. Materials and Methods**

99 *2.1 Reagents and materials*

100 DL-Dithiothreitol (DTT), Iodoracetamide (IAA), penicillin-streptomycin solution,
101 fetal bovine serum (FBS) and MTT powder were purchased from Sigma (St. Louis,
102 MO, USA). RPMI-1640 culture medium and DMSO were supplied by Invitrogen
103 (Paisley, UK). Water was purified using a Mil-lipore Milli-Q system (Bedford, MA,
104 USA). Acetonitrile and Formic acid were bought from TEDIA (Fairfield, USA) The
105 SMMC-7721 cell lines were obtained from Shanghai Cell Institute, China Academy of
106 Sciences.

107 *2.2 Animals*

108 Specimens of Chinese toad, *Bufo gargarizans* (n=3) were obtained in Jiangsu Province
109 in China. The parotid secretion was obtained via mild squeezing and massaging of the
110 glands. The viscous white parotid secretion was washed using deionized water,
111 snap-frozen in liquid nitrogen, lyophilized and stored at -20 °C prior to analysis.

112 *2.3 Venom extraction*

113 200 mg parotid secretion extracted from *Bufo gargarizans* were dissolved in 1.5 ml
114 PCR water and then ultrasonicated by a ultrasonication for 6 min and extracted by
115 ultrasonic for another 30 min by ice-bathing. After that the tubes were incubated in an
116 incubator at 4 °C for 5 h. Next, the supernatant (about 1.5 ml) were divided in two parts
117 and transferred drop by drop onto the center of 10 KD molecular weight cut-off

118 separately. Afterwards, these two filter cartridges were centrifuged at a speed of 8000
119 r/min for 20 min. Subsequently, combined the products by completely pipetting. 200µl
120 filtered products was allocated into two well-prepared PCR tubes. And 12.5 µl DTT
121 was added in one PCR tube at room temperature. Then, 7.5 µl IAA was also added in
122 the tube in a dark environment for 45 min. Finally, all the samples were desalted by
123 oasis HLB columns (Waters, USA) and dried by a concentrator (Eppendorf, Hamburg,
124 Germany) at 45 °C.

125 *2.4 Anticancer activity of total peptides on human hepatocarcinma cell* 126 *lines*

127 The human hepatocarcinma cell lines (SMMC-7721) were maintained in RPMI-1640
128 culture medium. RPMI-1640 were supplemented with 1% penicillin-streptomycin
129 solution and 10% fetal bovine serum. The cells were placed in an incubator (Stuart,
130 UK) with a 90% humidified environment containing 5% CO₂ at 37 °C. The cell line
131 was prepared at the density of 5×10³ cells per well onto 96 well plates. Following this,
132 cell lines were loaded with peptides at final concentrations ranged between
133 0.01mg/ml~500µg/ml and incubated over 24 h. After this, 10 µL of 5 mg/mL MTT
134 solution were added into each well and incubated for 4 h. The supernatants were
135 removed by a syringe, followed by addition 100 µL of 1% DMSO. The absorbance of
136 each well was recorded by PowerWaveX340 (BioTek instruments, USA) at 490 nm.

137 *2.5 Mass spectrometry*

138 Each sample was resuspended in 0.1%FA and centrifuged at 14000 g for 5 min. The
139 supernatant (4 µl) was loaded onto a 2 cm Thermo column (PepMap, 75 µm*2 cm C18,
140 3 µm, 100 Å) (Dionex, USA) with a Nano-LC system (Dionex UltiMate 3000) (Thermo,
141 USA) and the peptides were eluted onto a resolving 15 cm analytical C18 column
142 (Acclaim PepMap RSLC, 50 µm*15 cm C18, 2 µm, 100Å) (Dionex, USA). A gradient
143 elution of solvent A (formic acid: acetonitrile: water, 0.1/4/95.9) and solvent B (formic
144 acid: water: acetonitrile, 0.1/4/95.9) was applied as follows: 0~10 min, 1% B; 10~12
145 min, 1%~3% B; 12~102 min, 3% ~ 20% B; 102 ~116 min, 20%~30% B; 116~120 min,
146 30%~90% B; 120~128 min, 90%B; 128~129 min, 90%~1% B; 129~150 min, 1% B.
147 The peptides were performed to a Nano-electrospray ionization followed by tandem

148 mass spectrometry (MS/MS) in an LTQ Orbitrap Velos Pro (Thermo, USA) coupled
149 online to the HPLC. The mass spectrometry was operated in data-dependent mode,
150 automatically switching between MS and MS2 acquisition. Survey full-scan MS
151 spectra (m/z 350 –1800) were acquired in the Orbitrap with a resolution of 60,000.
152 The 15 most intense ions were sequentially isolated and fragmented by collision
153 induced dissociation (CID). Peptides with unassigned charge states, as well as less
154 than +2, or more than +6, were excluded from fragmentation. Fragment spectra were
155 recorded in the Orbitrap mass analyzer with resolution of 17,500. The dynamic
156 exclusion was enabled with repeat count two, and an exclusion duration 20 s.

157 *2.6 RNA sequencing*

158 A 5 mg sample of the lyophilized bufo parotid secretion powder was dissolved in 1ml
159 of cell lysis buffer (DynaL Biotech, UK). Polyadenylated mRNA was extracted from it
160 by Dynabeads® mRNA DIRECT™ Kit (Ambion, Life Tech, Oslo, Norway), then
161 subjected to cDNA library construction procedure by using NEBNext® Ultra™
162 Directional RNA Library Prep Kit for Illumina® (New England BioLabs Inc, UK).
163 According to the instructions, the cDNA library construction consisted of these main
164 steps. First of all was the mRNA fragmentation by incubating at 94 °C with random
165 primers. Then the RNA fragments were subjected to first strand cDNA and second
166 strand cDNA synthesis. After purification with 1.8×Agencourt AMPure XP beads, and
167 repair of the cDNA library was performed by incubating with the end repair reaction
168 buffer, the nucleotide A was added to the 3' end of the DNA fragments to avoid ligated
169 to each other. Therefore, the adapters with the nucleotide T at the 3' end were ligated to
170 the DNA fragments. At last, the DNA fragments with adapters were enriched by PCR
171 reaction and purified by AMPure XP beads. The quality of the cDNA library was
172 verified by using Agilent 2100 Bioanalyzer (Agilent Technologies, USA) with Agilent
173 DNA 1000 kit (Agilent Technologies, USA). The quantity of the cDNA library was
174 validated by qPCR with KAPA SYBR® FAST qPCR kit (KAPA Biosystems, USA).

175 The cDNA library was then loaded into a flow cell with oligos complementary to the
176 adapters to generate clusters through bridge amplification. Finally, the transcriptome
177 was obtained by doing RNA-Sequencing on the Illumina HiSeq 2000 platform. The

178 raw data obtained from Hiseq platform were analyzed as bellows. Firstly, the index
179 primers used to identify different samples were removed. Secondly, the data was
180 transferred to the program FastQC 0.1.0.1 to filter the reads with low quality and less
181 than 25 nucleotides. The filtered data was saved as fastq files. At last, the clean reads
182 were *De novo* assembled using the software Trinity to obtain the transcriptome.
183 RNA-seq generated 52,028,702 sequence reads that were assembled into 90,986
184 transcripts. The complete transcriptomic analyses of these samples are under
185 development and will be published elsewhere.

186 For the identification of the mRNA corresponding to the *Venenum Bufonis* proteins
187 investigated in this work, Trinity contigs were subjected to a BlastX search against a
188 compiled set of spider toxins downloaded from GenBank and Uniprot KB databases,
189 and those showing some degree of similarity were separated in a subset. The translated
190 amino acid sequences of this subset were used as house-built database for the MS/MS
191 analysis.

192 *2.7 Molecular cloning*

193 The isolation of pure mRNA from thick parotid secretion was achieved by utilizing a
194 magnetic oligo-dT bead kit (DynaL Biotech, Merseyside, UK) which could bind
195 polyadenylated mRNA in the cell lysis buffer supplied with the kit. Reverse
196 transcription and synthesis of the first-strand cDNA were followed by a 3'-RACE
197 reaction to isolate target antimicrobial peptide precursor nucleic acid sequence data
198 with a SMART-RACE kit (Clontech, Palo Alto, CA, USA). 3'-RACE was facilitated by
199 a sense primer (B.G. S1: 5'-CCATTCAGACGTTTCCTTCTCAGTA-3') and an
200 antisense primer (B.G.AS1: 5'-TAGCCCTGTACTTCTCCATGTATC-3'), which
201 were then designed for the polymerase chain reaction according to the discovered
202 RNA-Seq data of CL4590. The PCR cycling procedure included an initial denaturation
203 step at 94 °C maintained for 60 s, then 40 thermal cycles which involved 20 s at 94 °C
204 for denaturation, primer annealing for 10 s at 62 °C, and 240 s for extension at 72°C.
205 The PCR products were purified by gel electrophoresis and cloned using a pGEM-T
206 vector system (Promega Corporation, Southampton, UK). The DNA sequences of
207 clones were obtained by use of an ABI 3100 automated capillary sequencer (Applied
208 Biosystems, Foster City, CA, USA).

209 2.8 Bioinformatic analysis

210 Mass spectrometry raw data of peptide fractions were submitted to database searches
211 in PEAKS Studio using tolerance of precursor and fragment mass of 10 ppm and
212 0.025 Da. *De novo* peptides, whose average local confidence (ALC) scores $\geq 80\%$
213 were selected for database searches against the house-built transcriptome-derived
214 database and amphibian database downloaded from NCBI with the same conditions.
215 The peptide false discovery rate (FDR) was predictable by the decoy fusion method
216 and was selected at a maximum of 1%. Post-translational modifications and homology
217 searches were performed by PEAKS PTM and SPIDER, respectively. Only features
218 detected above the intensity threshold of 150 and in at least 2 out of 3 replicates were
219 considered for further analysis.

220 3. Results and discussion

221 Studies on animal venoms are widely conducted from all over the world [23]. However,
222 research on proteins and peptides from *Venenum Bufonis*, secretion of *Bufo*
223 *gargarizans*, is relatively insufficient. And the sequences and structures of these
224 peptides are worth to be valued. Bioactive peptides are fragment of the innate immune
225 system of amphibian, insects and mammals and are generally small (<10 kDa) [24].

226 3.1 LC-MS analysis of peptides in *Venenum Bufonis*

227 In this study, to focus on small peptides, peptides were extracted by water and filtered
228 by 10 kDa ultrafilters. The ion map after full-scan of peptides was exhibited in Figure
229 1. *De novo* sequencing performed by PEAKS software generate large numbers of
230 peptides focused on the 700–4000 Da mass range. The analysis gave out sequence
231 data and peptide identifications in *B. gargarizans* venom peptidome at this moment. It
232 functioned as a complementary approach to analysis generate main information of
233 intact masses of native peptides. *De novo* analysis of peptide fragments leads to
234 substantial sequence assignments. The number of peptides were detected with
235 molecular weight from 0.7 to 1 kDa, 1 to 2 kDa, 2 to 3 kDa, 3 to 4 kDa were 135, 822,
236 77 and 2, respectively (Figure 2). The 10 highest intense ions presented main clusters
237 of masses around 1.2 kDa, with precursor ion charges ranging from 2 to 3. Most ions

238 eluted between retention times of 60–116 min (Table 1), which were equal to
239 gradients around 11 – 30% of acetonitrile.

240 In total, the native peptide fraction of *B. gargarizans* venom LC-MS/MS analysis
241 followed by database, PTM and SPIDER searches resulted in a total of 939 unique
242 peptides identified from 85 proteins against the house-built protein database using *De*
243 *novo* RNA sequencing. Part of them were list in Supplemental Table 1 and Table 2.
244 However, only 23 peptides from 8 proteins were found when searching downloaded
245 amphibian database in Uniprot (Supplemental Table 2). The proteins identified with
246 the highest scores and the maximum number of peptides named CL4590 from
247 *Venenum Bufonis*. To the best of our knowledge, this is a functional unknown protein.
248 CL4590 precursor is composed of 897 amino acids and is cleaved to produce 296
249 peptides detected by LC-MS/MS (Supplemental Figure 1). In addition, these intact
250 unique peptides derived from CL4590, covering 54% of the whole protein sequence
251 (Supplemental Figure 2). Usually there are three kinds of poly-peptide skeleton on the
252 general fracture mode, the a/b/c are behalf of N-terminal of peptide and x/y/z are
253 representing the C-terminal of peptide. For example, the best peptide sequence
254 matches to the protein is M(+15.99)LANEDYASLTKGIQNL, the charge of the
255 sequence is 2, m/z 948.9697. This can be verified by the mass of each amino acid,
256 such as the amino acid between ion y-14 and y-13 by the presence between the ion
257 b-4 and b-3, which corresponds to an amidated ASN. Comparing to another peptide
258 LANEDYASLTKGIQNL, N-Terminal absence of Met and none oxidized
259 modification rendered the observation of 147 mass decrease. The mass spectra for
260 both peptides are displayed in Figure 3. The peptides derived from CL4590 mainly
261 focus on four parts of the protein, Figure 4 showed one part of the protein. BLAST
262 searches and sequence alignments exhibited the protein homology to protein
263 NP_0010881 and all the four main parts are possibly a TRPM8 channel-associated
264 factor (Supplemental Figure 3). TRPM8 is a member of the transient receptor
265 potential (TRP) superfamily of cation channels, which is a calcium-channel protein.
266 The expression of TRPM8 is strongly positive-regulated in cancer cells.

267

268

Table 1. The 10 highest intense ions detected in the *Bufo gargarizans* venom peptidome by LC-MS/MS analysis.

Protein Accession	Peptide	Unique	-10lgP	Mass	ppm	m/z	z	RT	Intensity
Unigene2127_All	K.TKPTDDELKEL.Y	Y	69.37	1287.656	2.7	644.8369	2	68.9	6.02E+07
Unigene2127_All	Q.AEFDKAAEDVKKLLK.T	Y	98.21	1590.862	0.6	796.4386	2	68.52	5.67E+07
CL4590.Contig1_All	L.SEWEENPM(+15.99)NKYVS	Y	88.84	1627.682	0.7	814.8491	2	72.25	4.31E+07
CL4590.Contig1_All	E.WEENPMNKYVS	Y	72.48	1395.613	1.1	698.8145	2	91.03	3.29E+07
CL4590.Contig1_All	L.ANEDYASLTKGIQNL.N	Y	108.77	1635.81	-0.3	818.9122	2	114.87	2.43E+07
CL127.Contig3_All	A.TPVDWKPGDRVM.V	N	67.98	1399.692	1.6	467.572	3	86.36	2.37E+07
CL4590.Contig1_All	F.FDFPGNKIT.S	Y	79.6	1037.518	0.3	519.7665	2	96.87	1.91E+07
CL4590.Contig1_All	F.FDFPGNKITSVAGVY.F	Y	75.42	1613.809	2.1	807.9135	2	118.22	1.89E+07
CL4590.Contig1_All	G.WDPFKQVF.S	Y	70.7	1065.528	0.3	533.7716	2	123.67	1.72E+07
CL4590.Contig1_All	W.GWPIDDATTEKL.S	Y	97.75	1344.656	0.3	673.3355	2	110.63	1.54E+07

Table2. Proteins (partial) identified in the secretion of *Bufo gargarizans* by LC-MS/MS through searching transcriptome-derived database.

Accession	-10lgP	Coverage (%)	Intensity	#Peptides	#Unique	#Spec	Description
CL4590.Contig1_All	610.2	54	7.26E+08	296	296	635	minus strand unknown [<i>Bufo gargarizans</i>]
Unigene2773_All	491.62	60	2.75E+08	110	110	297	LOC733366 protein [<i>Xenopus laevis</i>]
Unigene2127_All	423.53	93	3.13E+08	74	74	187	minus strand diazepam binding inhibitor [<i>Bufo gargarizans</i>]
Unigene13919_All	405.09	42	9.87E+07	76	76	154	catalase [<i>Glandirana rugosa</i>]
CL6550.Contig2_All	364.42	21	1.16E+07	36	36	57	inner-ear cytokeratin [<i>Rana catesbeiana</i>]
Unigene18322_All	355.25	56	1.72E+07	36	36	52	brain abundant membrane attached signal protein 1 [<i>Xenopus (Silurana) tropicalis</i>]
CL7798.Contig1_All	311.05	49	7.59E+07	15	15	40	diazepam binding inhibitor [<i>Xenopus laevis</i>]
Unigene19123_All	266.99	89	1.59E+07	20	20	25	minus strand Predicted: protein diaphanous homolog 1 [<i>Xenopus (Silurana) tropicalis</i>]
Unigene12309_All	229.43	23	1.53E+06	10	10	11	Predicted: calpain small subunit 1 [<i>Xenopus (Silurana) tropicalis</i>]
CL2275.Contig5_All	229.09	5	2.48E+06	8	8	9	Predicted: filamin-A isoform 3 [<i>Ornithorhynchus anatinus</i>]

270 3.2 Validation of CL4590 by molecular cloning

271 The peptide with the largest number of unique peptides derived from CL4590 was
272 identified, which derived from the parotid secretion of *Bufo gargarizans* and it consists of
273 2970 nucleotides (Supplemental Figure 2). The translated open reading frame of CL4590
274 contains 897 amino acids. We focused on this protein due to its substantial quantity in the
275 secretions. Therefore, a further identification of a short domain in CL4590 at the genetic
276 level was performed using specific primers. The band around 500 bp of PCR product is
277 consistent with the size of target domain (Figure 5). The validated fragment, including 502
278 nucleotide acids, shows only 5 nucleotide acids are inconsistent with CL4590 and result in
279 two amino acids different from the corresponding sites of CL4590 on the aspect of amino
280 acid sequences. The CL4590 sequence established by *De novo* RNA-Sequencing had more
281 than 99% overlap with the validated fragment using molecular cloning (Figure 6A, 6B).
282 This illustrated that our peptide sequences identified in *Venenum Bufonis* were accurate.
283 The full-length nucleotide sequence of CL4590 and the validated partial sequence were
284 deposited into Genbank database under the accession numbers Banklt 2053205 Seq1
285 MG189609 and Banklt 2053207 Seq1 MG189610, respectively.

286 The transcriptome method based on RNA-seq reveals a powerful implement to improve the
287 number of the peptides identification. RNA-Seq reveals the presence of RNA in a
288 biological sample at a given moment in time. RNA-Seq eliminated several challenges
289 raised by microarray technologies due to the limitation of dynamic range of detection.
290 However, during the procedure of reverse transcription, the emerging cDNA was synthesised
291 which can sometimes separate from the template RNA and re-anneal to another stretch of
292 RNA with a sequence similar to the original model, generating an artefactual chimeric cDNA
293 [25]. Furthermore, the RNA-Seq method used here relies on fragments assembling, which
294 might place false positive on the final sequencing results [26]. This strategy might not be
295 helpful when encountering the needs for *De novo* sequencing without any reference data.
296 Thus, a short sequence of the protein was verified by molecular cloning to prove the
297 accuracy of RNA-Seq. We focused on protein CL4590 due to its high contents, and up to 296
298 peptides derived from the protein. This specific sequence comparison between the
299 nucleotide sequence of cloned cDNA and the corresponding part in RNA-Seq data showed
300 an extraordinary sequence similarity, which illustrates the result of direct RNA sequencing
301 is reliable.

302 3.3 Anticancer activity

303 The total peptides in *Bufo gargarizans* secretion were extracted by water and filtered with
304 10 KD ultrafiltration. We performed anticancer activity assays on the total peptides and the
305 result of MTT showed obvious inhibition on SMMC-7721 cancer cells in the concentration
306 of 0.1981 µg/ml. Thus, the total peptide extraction obtained from *Venenum Bufonis* are
307 possibly contain peptides that can inhibit the growth of cancer cells (Figure 7).

308 3.4 Bioinformation analysis

309 A peptide named Buforin II isolated from the stomach of *Bufo gargarizans* is well known
310 owing to its strong antibacterial and anticancer activity. The cDNA was found by RNA-Seq,
311 but the mature peptide was not found by LC mass spectrometry. It could be speculated that
312 the peptide is not expressed after translation or it only existed in toad stomach [13]. Buforin
313 II shows a complete sequence identity with the N-terminal region of histone 2A. Four
314 peptides identified by LC/MS were found homology to histone H2B (*Gallus gallus*) and
315 one was identified from histone H3 (*Chaperina fusca*) (Supplemental Table 2). Histones
316 belong to alkaline proteins usually existed in eukaryotic cell nucleus who can package and
317 instruct DNA into nucleosomes [27]. Histone H2A and histone H3 mentioned here belong
318 to core histone, which are the principal proteins of chromatin. And all histones have a
319 highly positively charged N-terminus with many lysine and arginine residues [28].
320 Regularly, antimicrobial peptides are positively charged while the cell membranes have
321 negative charges, they would bind to each other by electrostatic attraction. Thus, five
322 peptides derived from histone proteins are possibly kinds of antimicrobial or anticancer
323 peptides. To sum up, some further analysis need to be done in the future to explore the
324 bioactivity of these peptides.

325 Apart from these peptides searched in amphibian database with known functions, a large
326 number of peptides obtained by *De novo* analysis without annotation are considered to
327 treasure-houses, expected to be explored in the future. Gene annotation is an ideal method
328 to connect the transcriptome to the peptidome. More importantly, the expression analysis in
329 the transcript and peptide level could describe the network of the overall gene. In the
330 functional analysis of a set of peptides, it is necessary to have a comprehensive analytical
331 tool. Gene ontology is an international standardized gene functional classification system.

332 The recognized proteins are automatically annotated [29]. The whole transcripts obtained
333 by RNA-Seq was categorized into three main categories: biological process, cellular
334 component and molecular function (Figure 8). Functional annotation and classification
335 provide predicted information that these peptides and proteins most involved in the cell
336 killing, immune process, steroid biosynthesis, antioxidant and antimicrobial activity.
337 Fourteen proteins get from this study were found in the list, four of them were predicted to
338 take part in immune system process, six might participate in the process of metabolic and
339 the rest of them are regarded to have antioxidant activity (Supplemental Table 3), which
340 indicate the directions for following research.

341 **4. Conclusion**

342 We offered extensive information on the parotid secretion of *Bufo gargarizans*
343 transcriptome and peptidome by mass spectrometry and bioinformation analytical methods
344 in this study. Interpretation of the transcriptomic and peptidomics data uncovered a number
345 of candidate peptides with their precursor proteins which involved in the secretion. The
346 peptides also presented promising results of anti-proliferated activity on SMMC-7721.
347 However, the functions of most peptides are unknown, much more work is required to
348 elucidate these proteins and peptides functions, our findings will aid further research aiming
349 to identify key proteins and peptides in the secretion.

350 **Conflicts of Interest**

351 The authors declare no conflict of interest.

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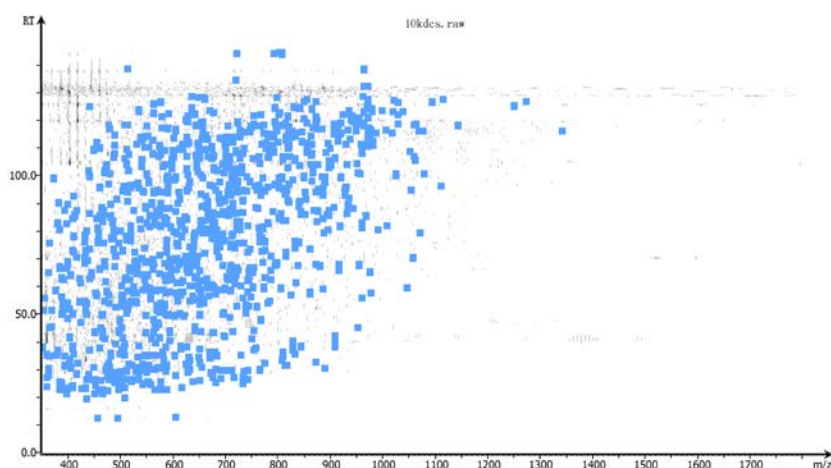
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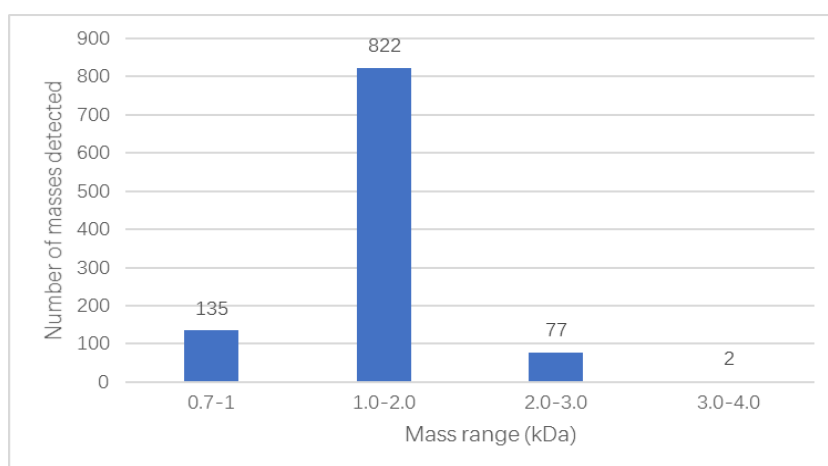
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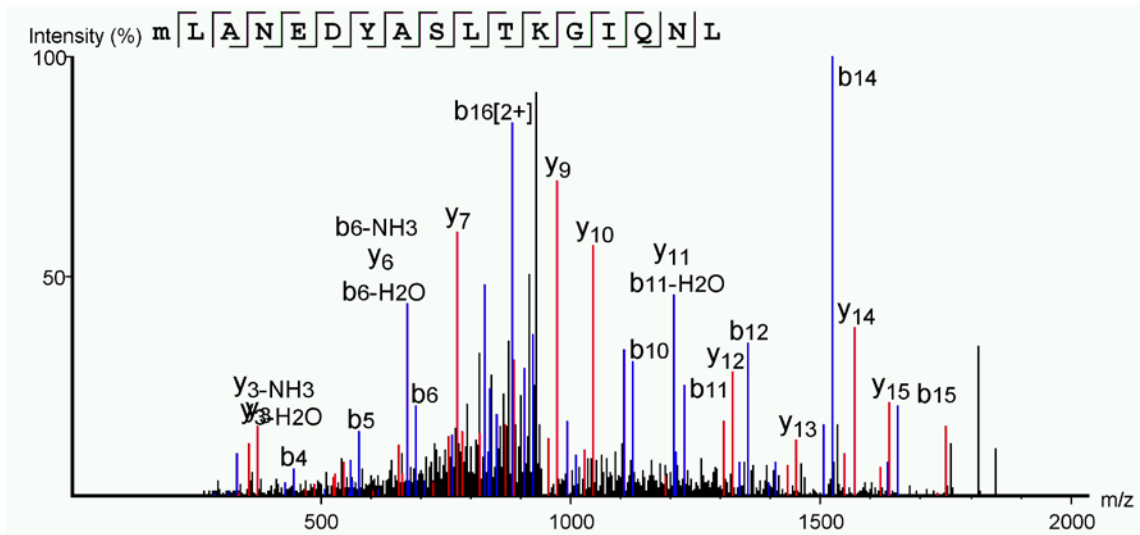
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452 **Figure 1.** Mass map of *Venenum bufonis* peptides ion data displayed by PEAKS after
453 processing of LTQ-Orbitrap velos Pro.



454

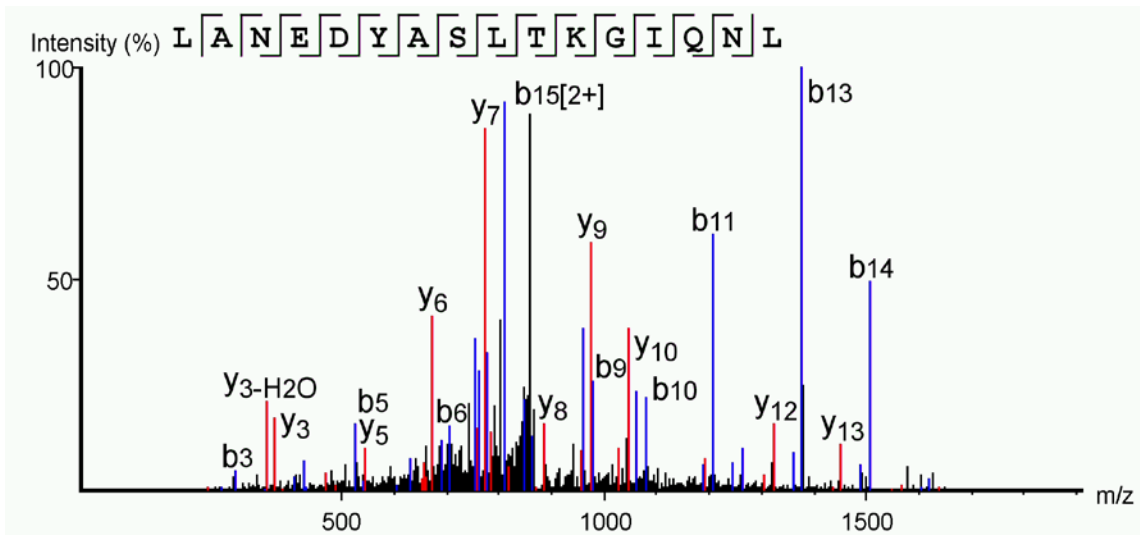
455 **Figure 2.** The molecular weight of peptides from *Venenum Bufonis*. *x-axis* represents the
456 mass ranges of these peptides and *y-axis* represents the number of masses that were
457 detected.



458

459

A



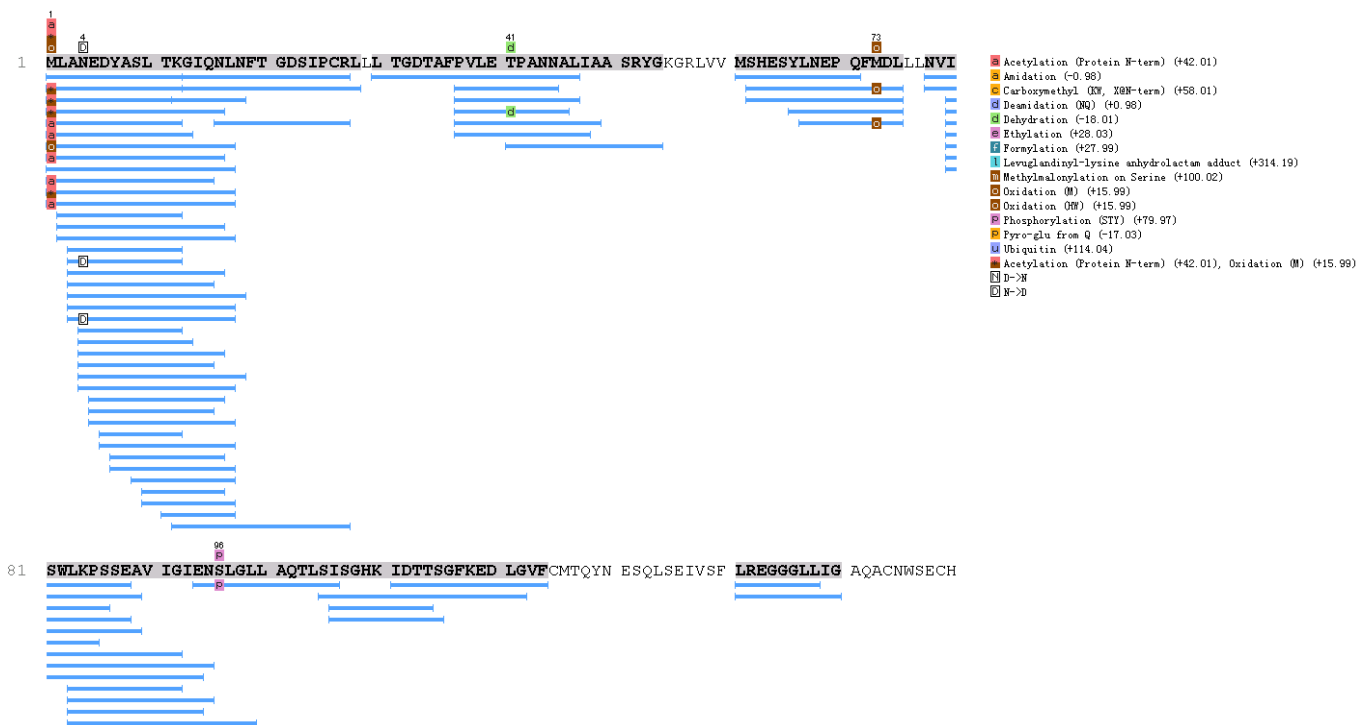
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B

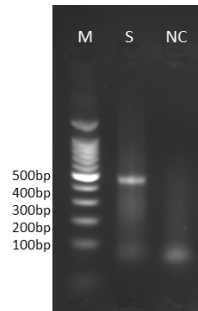
462 **Figure 3.** The MS/MS spectrum of the best unique peptide
 463 M(+15.99)LANEDYASLTQNL (m/z 948.9697) (A) and LANEDYASLTQNL
 464 (m/z 875.4536) (B) identified in CL4590.

465



466

467 **Figure 4.** Peptide spectrum matches indicated by blue lines below the sequences of the precursor protein CL4590 (partial).



468

469 **Figure 5.** Gel electropherogram of PCR products generated from *Bufo gargarizans*
470 secretion-derived cDNA library. Lane M contains a standard DNA ladder as a
471 marker, and each band represents 100bp increments. Lane S accounts for the PCR
472 product from cDNA library of *Bufo gargarizans* secretion at 62°C. Lane NC
473 represents the PCR product using deionized water at 62°C as a negative control.

474

475

CL4590	(365)	HSDVPSQYPGNKILNRFGISILERTI	INGNYKVPESTTNAYHFLRSVCQFLRDLKNGVEI
Validated	(1)	HSDVPSQYPGNKILNRFGISILERTI	INGNYKVPESTTNAYHFLRSVCQFLRDLKNGVEI

CL4590	(425)	KPPLISWLSQLNQDVSSYLKLPATPLRVSLWQELSYLVQC�LPEVSKENPVKNQSKAEFL
Validated	(61)	KPPLISWLSQLNQDVSSYLKLPATPLRVSLWQELSYLVQC�LPEVSKENPVKNQSKAEFL

476

CL4590	(485)	ICLAQEVNCLYDS	QEDTGELDVQGEPTRIDIDGTTLDGDTWRSTGL
Validated	(121)	ICLAQEVNCLYDS	QEDTGELDVQGEPTRIDIDGTTLDGDTWRSTGL

477

A

CL4590	(1092)	CCATTGACAGCGTTCCTTCTCAGTATCCAGGAAACAAATCCTCAATAGATTTGGGATAAG
Validated	(1)	CCATTGACAGCGTTCCTTCTCAGTATCCAGGAAACAAATCCTCAATAGATTTGGGATAAG

CL4590	(1152)	CATTCTGGAA	AGAAC	CATA	ACAAATGGCAATTATAAAGTTCCCGAGTCTACTACTAACGC
Validated	(61)	CATTCTGGAGAGAACTATAGAAAATGGCAATTATAAAGTTCCCGAGTCTACTACTAACGC			

CL4590	(1212)	ATATCATTTTCTCAGATCAGTGTGTGCTAGTTTCTGAGAGACTTGAAAAATGGAGTGGAGAT
Validated	(121)	ATATCATTTTCTCAGATCAGTGTGTGCTAGTTTCTGAGAGACTTGAAAAATGGAGTGGAGAT

CL4590	(1272)	TAAACCCCTCTAATCTCCTGGTTATCACAGCTCAATCAAGATGTCTCTAGCTATCTGAA
Validated	(181)	TAAACCCCTCTAATCTCCTGGTTATCACAGCTCAATCAAGATGTCTCTAGCTATCTGAA

CL4590	(1332)	GTTGCCTGCCACACCTCTTAGAGTGTCCCTATGGCAAGAACTTTCATATCTGGTACAATG
Validated	(241)	GTTGCCTGCCACACCTCTTAGAGTGTCCCTATGGCAAGAACTTTCATATCTGGTACAATG

CL4590	(1392)	TAATTTACCCGAAGTTAGTAAAGAGAATCCAGTGAAAAACCAATCCAAGAGGCTTTTCT
Validated	(301)	TAATTTACCCGAAGTTAGTAAAGAGAATCCAGTGAAAAACCAATCCAAGAGGCTTTTCT

CL4590	(1452)	AATATGTTTGGCTCAAGAAAGTCAACTGCCTATATGACTCAG	CTCAAGAGGACACAGGAGA
Validated	(361)	AATATGTTTGGCTCAAGAAAGTCAACTGCCTATATGACTCAG	CTCAAGAGGACACAGGAGA

CL4590	(1512)	GCTTGATGTTCAAGGAGAACCCACACGTATAGATATTGATGGCACAACTCTTGATGGTGA
Validated	(421)	GCTTGATGTTCAAGGAGAACCCACACGTATAGATATTGATGGCACAACTCTTGATGGTGA

CL4590	(1572)	TACATGGAGAAGTACAGGGCTA
Validated	(481)	TACATGGAGAAGTACAGGGCTA

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B

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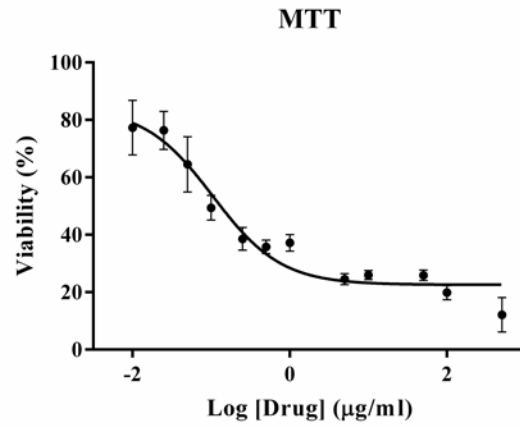
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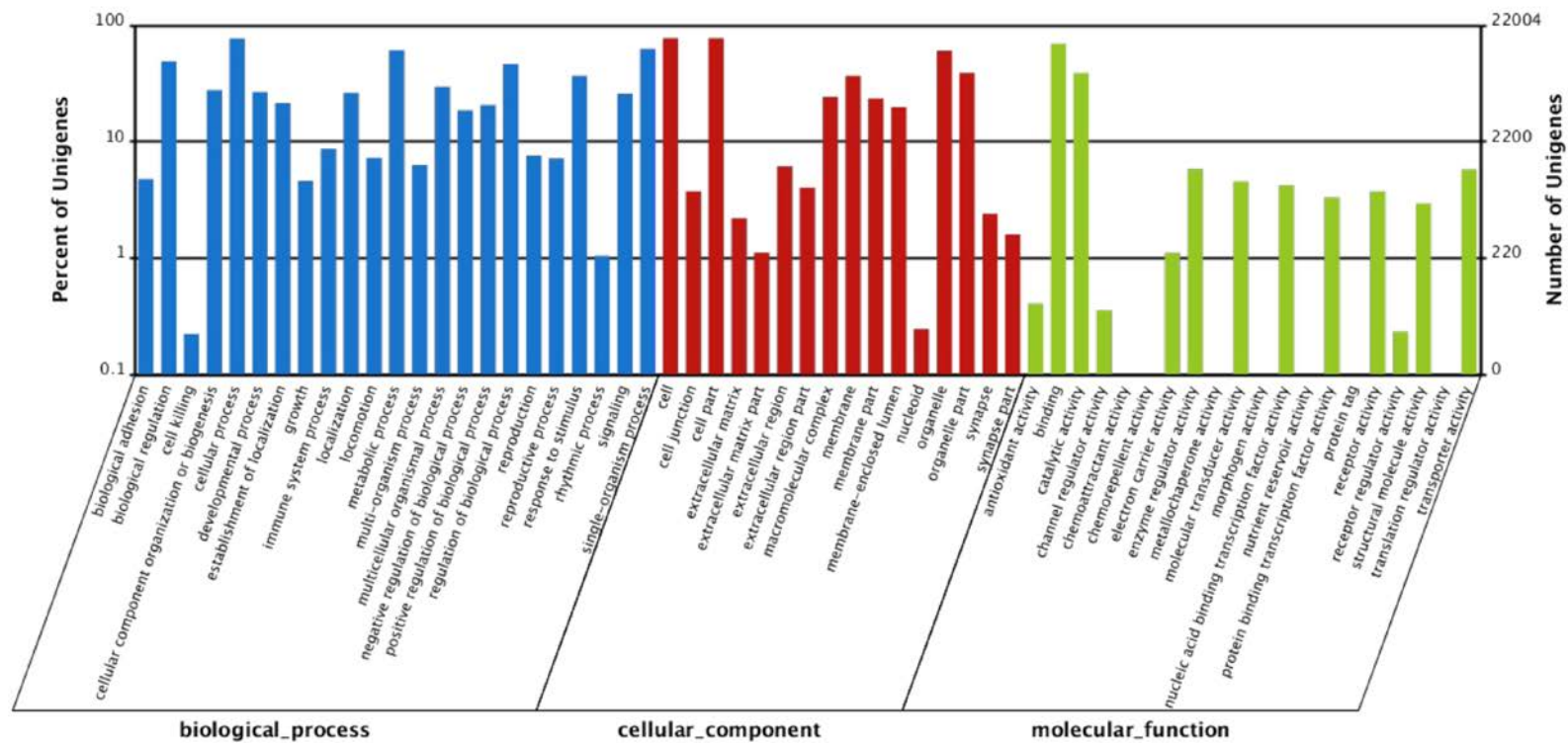
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Figure 6. (A) Alignment of translated open-reading frame amino acid sequence of the corresponding fragment of CL4590 and cloned fragment(Validated). (B) Alignment of the corresponding fragment of CL4590 and cloned nucleotide sequence (Validated). The discrepant nucleotides or amino acid residues are indicated by blue.



485

486 **Figure 7.** The viability of SMMC-7721 treated by peptides extracted from secretion of
487 *Bufo gargarizans*.



488

489 **Figure 8.** GO classification analysis of Unigenes in All-Unigene. GO functions is showed in X-axis. The right Y-axis shows the number of genes
 490 which have the GO function, and the left Y-axis shows the percentage.

**Identification of <10KD peptides in the water
extraction of *Venenum Bufonis* from *Bufo gargarizans*
using Nano-LC-MS/MS and *De novo* Sequencing**

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M L A N E D Y A S L T K G I Q N L N F T
1 ATGTTGGCTA ATGAAGATTA CGCCTCACTT ACCAAAGGCA TCCAGAATCT AAATTTTACA
TACAACCGAT TACTTCTAAT GCGGAGTGAA TGGTTTCCGT AGGTCTTAGA TTTAAAATGT
G D S I P C R L L L T G D T A F P V L E
61 GGAGACAGTA TTCCTTGTAG GCTTCTTCTT ACTGGTGACA CAGCATTCCC AGTGTGGAG
CCTCTGT CAT AAGGAACATC CGAAGAAGAA TGACCACTGT GTCGTAAGGG TCACGACCTC
T P A N N A L I A A S R Y G K G R L V V
121 ACTCTGCAA ATAATGCTCT CATTGCTGCA TCCAGATACG GCAAGGGCCG CTTGTTGTG
TGAGGACGTT TATTACGAGA GTAACGACGT AGGTCTATGC CGTCCCAGGC GAACCAACAG
M S H E S Y L N E P Q F M D L L L N V I
181 ATGAGTCATG AATCATATCT GAATGAGCCA CAATTATATG ATTTACTGCT GAATGTGATA
TACTCAGTAC TTAGTATAGA CTTACTCGGT GTTAAATACC TAAATGACGA CTTACACTAT
S W L K P S S E A V I G I E N S L G L L
241 TCCTGGTTGA AACCATCTTC GGAAGCAGTA ATTGGCATTG AAAACAGCCT AGGTCTCCTG
AGGACCAACT TTGGTAGAAG CCTTCGTCAT TAACCGTAAC TTTTGTGCGA TCCAGAGGAC
A Q T L S I S G H K I D T T S G F K E D
301 GCACAACTC TTTCTATCTC TGGCCATAAA ATTGACACGA CCTCTGGCTT TAAGGAAGAT
CGTGTTTGAG AAAGATAGAG ACCGGTATTT TAACTGTGCT GGAGACCGAA ATTCCTTCTA
L G V F C M T Q Y N E S Q L S E I V S F
361 TTGGGAGTGT TCTGCATGAC ACAATATAAT GAAAGCCAGT TATCAGAAAT TGTGTCATTT
AACCCCTACA AGACGTA CTGTTATATTA CTTTCGGTCA ATAGTCTTTA ACACAGTAAA
L R E G G L L I G A Q A C N W S E C H
421 TTGAGGGAAG GTGGCGGTCT TCTTATTGGA GCTCAAGCCT GCAATTGGTC TGAATGCCAC
AACTCCCTTC CACCGCCAGA AGAATAACCT CGAGTTCGGA CGTTAACCCAG ACTTACGGTG
P E L N V F F D F P G N K I T S V A G V
481 CCAGATTAA ACGTTTCTT CGATTTTCCCT GGAACAAAA TAACATCTGT AGCTGGAGTC
GGTCTTAATT TGCAAAAGAA GCTAAAAGGA CCTTTGTTTT ATTTAGTACA TCGACCTCAG
Y F T N K T G E N G I F P V S E K P P P
541 TATTTACTA ATAAGACTGG AGAAAATGGG ATTTTCCCAG TGAGTGAGAA ACCACCACA
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F P S F D D V D F S M D L K Q I F N G V
601 TTTCCGCTT TTGATGATGT GGACTTCTCC ATGGACCTAA AACAAATCTT CAATGGAGTA
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S S L D I T G S S V V S K L L L H G P L
661 TCCAGTCTGG ATATTACTGG GAGTCTGTGT GTTTCTAAAC TCCTGCTACA CGGACCGTTG
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T F P L G L T S S H Q C F F A A A Y Y G
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781 AGAGGACGTG TTGTAGTGGG GTCTCATGAA GGCTATATTA CAAGACTAGA GCTGAAGTCT
TCTCCTGCAC AACATCACCC CAGAGTACTT CCGATATAAT GTTCTGATCT CGACTTCAGA
F I L N A I S W L D A G R S G R I G V N
841 TTTATCCTAA ATGCCATTT ATGGCTGGAT GCAGGAAGAA GTGGGAGGAT TGGTGTAAAT
AAATAGGATT TACGGTAAAG TACCGACCTA CGTCTTCTT CACCCTCCTA ACCACAATTA
K A M R G L I P I L Q A E G I S C A M S
901 AAGGCAATGC GAGGCCTGAT TCCCATACTG CAGGCTGAAG GTATCTCTTG TGCAATGTCC
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N V S S E F S I Y C C N S Y S D A E V D
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K I H Q F V A E G G S L L I A G H A W Y
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W S Y S H S D V P S Q Y P G N K I L N R
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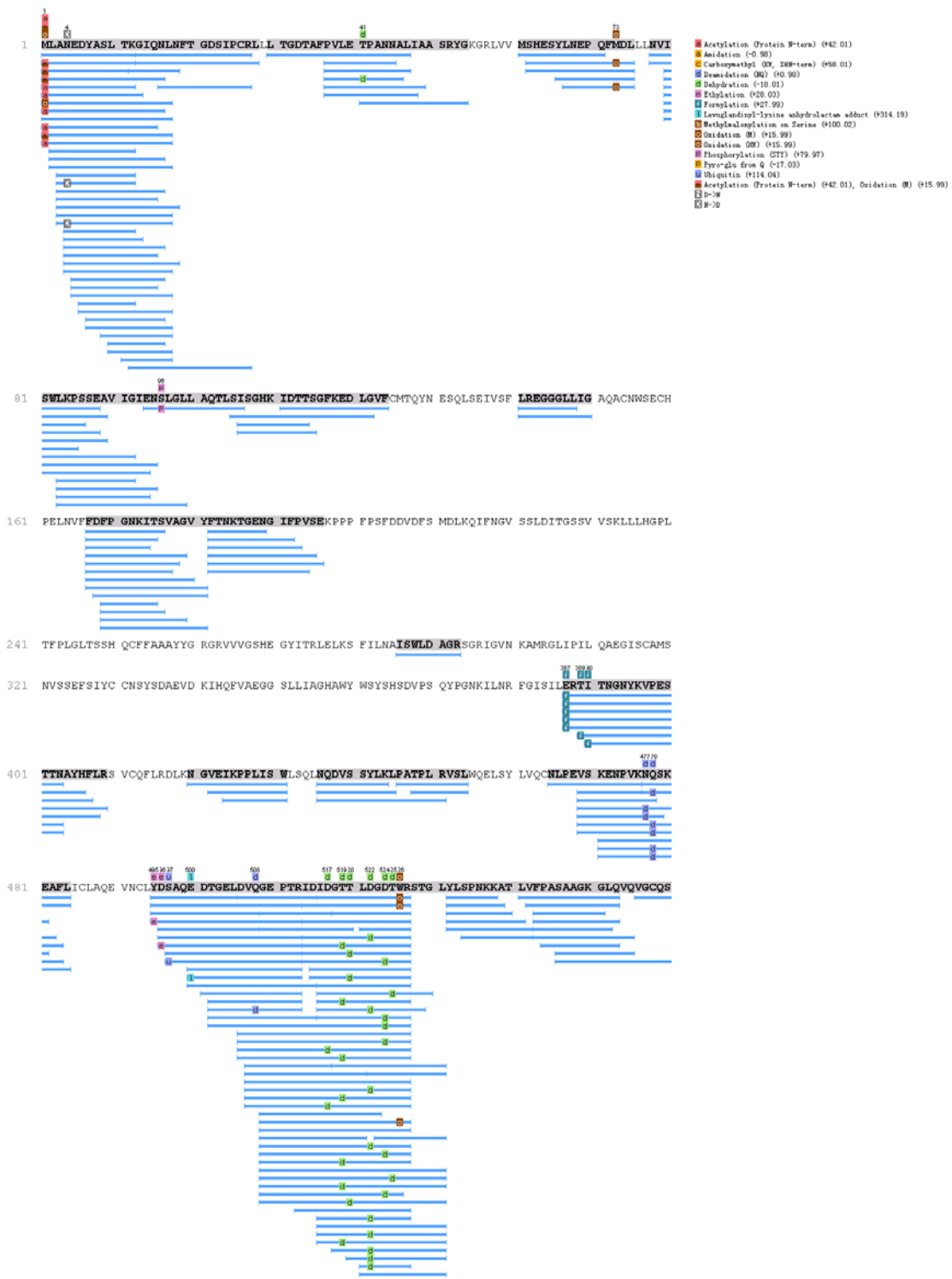
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 E A F L I C L A Q E V N C L Y D S A Q E
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 L D G D T W R S T G L Y L S P N K K A T
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 L V F P A S A A G K G L Q V Q V G C Q S
 1621 CTTGTATTTC CAGCCTCAGC TGCAGGGAAG GGTCTCCAGG TACAAGTTGG CTGTCAATCC
 GAACATAAAG GTCGGAGTCG ACGTCCCTTC CCAGAGGTC ATGTTCAACC GACAGTTAGG
 D D L S G A A E L C R A P V V I H R K N
 1681 GATGACCTGA GCGGAGCAGC AGAGTTGTGT CGTGCTCCAG TGGTTATACA TAGAAAGAAC
 CTACTGGACT CGCCTCGTCG TCTCAACACA GCACGAGGTC ACCAATATGT ATCTTTCTTG
 V V D E K V V I S C I W G G L L Y V I V
 1741 GTTGTGATG AGAAGGTTGT GATATCCTGT ATCTGGGGAG GACTTCTTTA CGTTATTGTA
 CAACAACTAC TCTTCCAACA CTATAGGACA TAGACCCTC CTGAAGAAAT GCAATAACAT
 K G K S Q L G N V P V T I Y G A E P A P
 1801 AAGGGGAAGA GTCAACTAGG AAATGTTCCA GTTACAATCT ATGGGGCAGA ACCAGCTCCA
 TTCCCTTCT CAGTTGATCC TTTACAAGGT CAATGTTAGA TACCCCGTCT TGGTCGAGGT
 T F I N G Q T K V S S W L D T L R T S T
 1861 ACATTTATAA ATGGACAAC TAAAGTTTCT TCGTGGTTGG ATACATTAGC CACTCAACA
 TGTAATATT TACCTGTTG ATTTCAAAGA AGCACCAACC TATGTAATGC GTGAAGTTGT
 S P W A E L S A E N I T L T V P T D K V
 1921 TCTCCTTGGG CTGAGCTTAG CGCAGAGAAT ATTACCCTGA CTGTCCCTAC TGATAAAGTC
 AGAGGAACCC GACTCGAATC GCGTCTCTTA TAATGGGACT GACAGGGATG ACTATTTGAG
 R S L E D P E S L M S L W D N I M S T V
 1981 CGCTCACTAG AGGATCCTGA ATCGCTTATG TCTTTATGGG ATAACATCAT GTCAACAGTG
 GCGAGTGATC TCCTAGGACT TAGCGAATAC AGAAATACCC TATGTAGTA CAGTTGTCAC
 T D L A S I P K K L P R P E R I V A D V
 2041 ACTGATCTGG CATCTATCCC AAAGAACTG CCTCGTCCAG AAAGAATTGT GGCCGATGTT
 TGACTAGACC GTAGATAGGG TTTCTTTGAC GGAGCAGGTC TTTCTTAACA CCGGTACAA
 Q I S A G F M H A G Y P I M C H V P T A
 2101 CAGATCTCAG CAGGTTTTAT GCATGCTGGA TACCCAATAA TGTGCCATGT GCCAACAGCA
 GTCTAGAGTC GTCCAAAATA CGTACGACCT ATGGGTTATT ACACGGTACA CGGTTGTCGT
 S S L V S V T S L K K G M W G A A H E L
 2161 TCTTCTTTAG TAAGTGTTAC ATCATTGAAG AAAGGCATGT GGGGGGCTGC ACATGAGCTT
 AGAAGAAATC ATCACAATG TAGTAACTTC TTTCCGTACA CCCCCGACG TGTACTCGAA
 G H N Q Q R G V W E F P P H T T E A T C
 2221 GGTCAACAAC AGCAGAGAGG AGTTTGGGAA TTCCCTCCTC AACTACAGA AGCCACCTGT
 CCAGTGTGG TCGTCTCTCC TCAAACCTT AAGGGAGGAG TGTGATGTCT TCGGTGGACA
 N L W S V Y V H E I L L G I P R D N A H
 2281 AACCTGTGGT CAGTATATGT ACATGAAATC TTGCTGGGGA TCCAAGAGA TAACGCTCAT
 TTGGACACCA GTCATATACA TGTACTTTAG AACGACCCCT AGGGTTCTCT ATTGCGATA
 P A L K P G D R E K R I K Q Y L K N G A
 2341 CCTGTCTCA AGCCAGGAGA CAGAGAAAAA AGAATCAAGC AGTATCTGAA GAATGGAGCC
 GGACGAGAGT TCGGTCCTCT GTCTCTTTTT TCTTAGTTCG TCATAGACTT CTTACCTCGG
 K L D E W S V W T A L E T Y L Q L Q E C
 2401 AAACCTGATG AGTGGAGCGT GTGGACAGCG CTTGAGACTT ATCTTCAGCT ACAAGAGTGC
 TTTGAACTAC TCACCTCGCA CACCTGTCGC GAACTCTGAA TAGAAGTCGA TGTTCTCAGC
 F G W D P F K Q V F S E Y Q T M S N V S
 2461 TTTGGCTGGG ATCCCTTCAA GCAAGTTTTT TCAGAGTATC AGACCATGTC CAATGTCACT
 AAACCGACCC TAGGGAAGTT CGTCAAAAAG AGTCTCATAG TCTGGTACAG GTTACAGTCA

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      N V K D V K M N L W A E K F S H A V N K
2521 AATGTCAAGG ATGTTAAGAT GAATCTCTGG GCAGAGAAGT TTTCTCACGC AGTCAATAAG
      TTACAGTTCC TACAATTCTA CTTAGAGACC CGTCTCTTCA AAAGAGTGCG TCAGTTATTC
      N L A S F F K A W G W P I D D A T T E K
2581 AACCTAGCTT CATTCTTCAA GGCTTGGGGA TGGCCCATTG ATGATGCTAC CACCGAAAAA
      TTGGATCGAA GTAAGAAGTT CCGAACCCCT ACCGGGTAAC TACTACGATG GTGGCTTTT
      L S I L S E W E E N P M N K Y V S *
2641 TTATCCATAT TAAGTGAATG GGAAGAGAAT CCAATGAATA AATATGTCAG CTAAATTTCA
      AATAGGTATA ATTCACTTAC CCTTCTCTTA GGTTACTTAT TTATACAGTC GATTTAAAGT
2701 TAAAAGAAAT ATATAAAATG ATCCTTCTAT AGTAATATGC TTTTGGTGGC TTAAGAGGAA
      ATTTTCTTTA TATATTTTAC TAGGAAGATA TCATTATACG AAAACCACCG AATTCTCCTT
2761 TGCATTATAG GCTCAAAAGT GTTAAAGGAA AATATAAAAA TCACATCATA ATAATATTGC
      ACGTAATATC CGAGTTTTCA CAATTCCTT TTATATTTTT AGTGTAGTAT TATTATAACG
2821 AACAGAGCCT GTTAGATTAT CATCTGAATA TGGAAAATA ATAGAATATA ATCATATGCA
      TTGTCTCGGA CAATCTAATA GTAGACTTAT ACCTTTTGAT TATCTTATAT TAGTATACGT
2881 ATGCATGCAA TGTTCAATAA AGAATTCAGA TTCCTGCTCC AACTTAGTTT TGTTTTTTTG
      TACGTACGTT ACAAGTTATT TCTTAAGTCT AAGGACGAGG TTGAATCAAA ACAAAAAAAC
2941 TTTTTTT
      AAAAAAA

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Figure S1. The transcript obtained from the de novo assembled clean reads and the translated amino acid sequence is shown above the nucleotides. The stop codon is indicated by an asterisk.



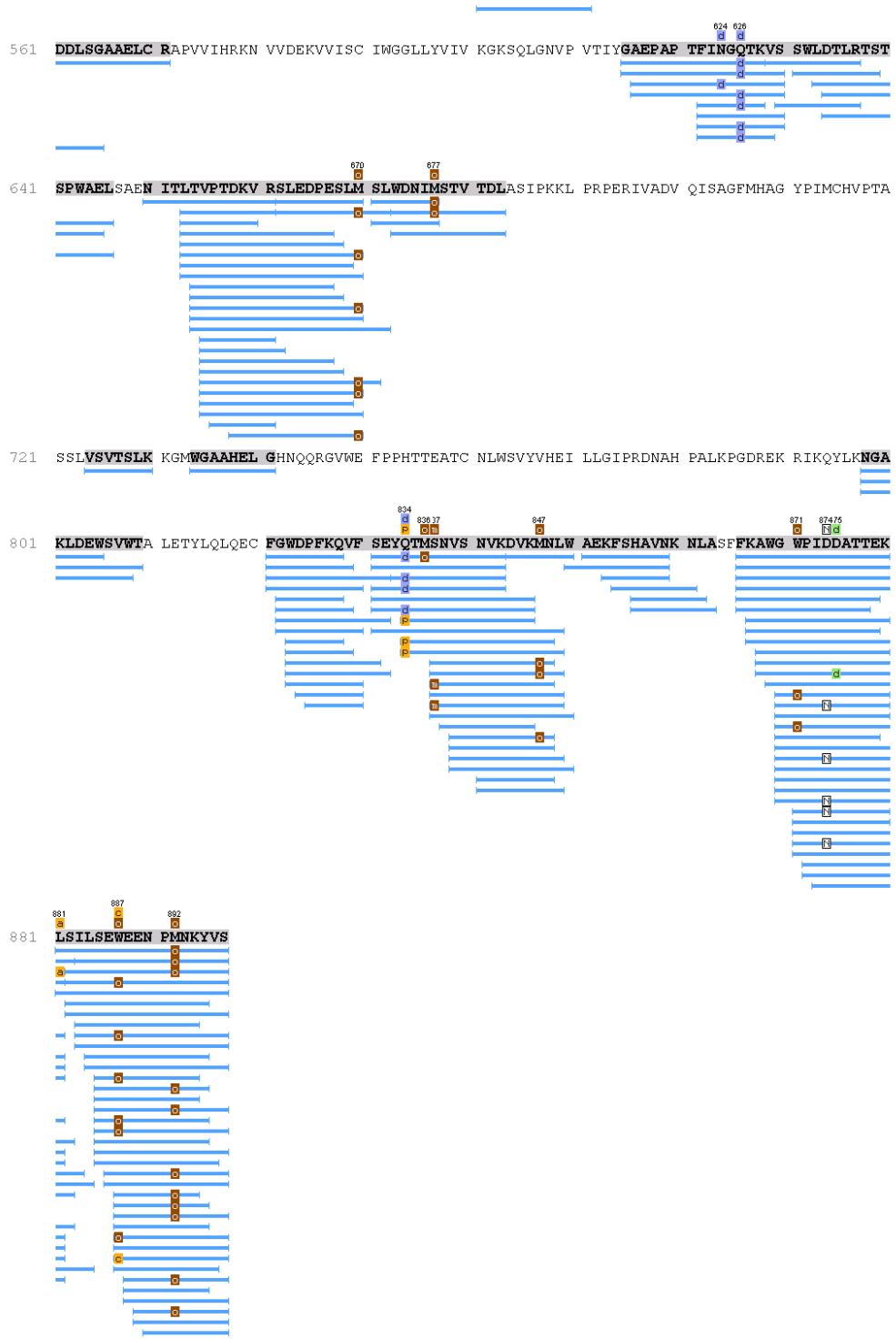


Figure S2. Peptide spectrum matches indicated by blue lines below the sequences of the protein identified.

CL4590.Con : MLANEDYASLTKGIQNLNFTGDSIPCRLLLTGDTAFPVLETPANNALIAASRYGKGRLLVVMESHESYLNPEQFMDLLLNVTISWLKESSEAVIGIENSICLLAQTLISISCHKIDTTS : 115
 NP_0010881 : MKVLEDYRSLVHGIGSLDFSGDAVPCKLLLTGDTAFPVLVTPRKDVLIAASRYGKGVVVMASHESYLNNTNAFMDFLKNAVSWLSENSEANIGVHKGLNLLTDNLSANGSKVQNTS : 115

CL4590.Con : GFKEDLGVFCMTQYNEQLSEIVSFLREGGGLLIGAOACNWSSECHPELNVFDFPFGNKIISVAGVYFTNKTCENGIFPVSEKPPFPFSEDDVDFSMDLKQIFNGVSSLDITGSSV : 230
 NP_0010881 : TLIEGLGVFCTIGYDPSQDKQIISFVREGGGLLIGAOAWHWSYSHKQENVLHHFPGNKIISVSGVHFTSDYGEKGNICVMENIPQAPIYTSFDFSLDQNYLLKGMSSQLDISGSSI : 230

CL4590.Con : VSKLLHLHGPLTFPPLGLTSSHCQFFAAAYYGRGRVVVGSHEGYITRLELKSFTLNNAISWLDAGRSGRIGVNAKMRGLIPIILQAEGISCAMSNVSSEFSIYCCNSYSDAEVDKTHQF : 345
 NP_0010881 : PSDLLHLHGLTSFPVGLSENKQCFLGATYYKGRVVVAHEGYLSKSELKTIIMLNNAISWLDINQNRRIQVHKGLRQFAELLQKENIPCNISLDPDLSVYCCTSYSDAEAKATHF : 345

CL4590.Con : VAEGGSLLIAGHAWYWS--YSHSDVPSQYPGNKILNRFGISILERTITNG--NYKVEESTINAYHELRVSVQFLRDLKNCVEIKPPLISWLSQLNODVSSYLKLPATPIRVSLWQ : 456
 NP_0010881 : VAEGGGLLIAGHAWYSSQNSDLVLIQYPGNKILNRFGISILDRITPGCNVNAINEDESSQYVHRRGLCNLQAELRSCAEVKPPLSIWMNKLRQDVTAFMRLPANPIISISIQS : 460

CL4590.Con : ELSYLVQ-CNLPEVSKENPVKNQSKAEFLICLAEVNCIYDSAQEDTGEELDVQGEPTRIDTGTTLDGDITWRSTGLYLSENKKATLVFPASAAGKGLQVQVGCQSDDLLSCAAELC : 570
 NP_0010881 : QFVEMMQICEIPNVTKQCPVSSCSKEAFILCLAEVNCIYDSAQEDTGEELDVQGEPTRIDTGTTLDGDITWRSTGLYLAERKTAVLEFPASAVHQGLQVQVGCQSDDLLSSADKYC : 570

CL4590.Con : RAPVVLRKKNVDEKVVISCIWGLLYIVKKGKSQLGNVPVITYGAEPAPIFINGQIKVSSWLDITLRTSTSPWAEISAENITLTVPTDKVRSLEDPEILMSLWDNIMSTVTDLAS : 685
 NP_0010881 : RAPVVVRRFHWDSQRVSVSCFWGGLVYIVKANSNLGITPVKVVYEAEPAPIYTKCKTSLDTWIQSIRNLPAPWAEELITENIILTVPSDAIRSLSDPEALLSLWDKIMVAITELAA : 685

CL4590.Con : IPKKLPRPERIVADVQISAGFMHAGYPIIMCHVPTASSLVSVTSIKKG-MWGAHELGHNQQRGVWEFPPHTTEATCNLWSVYVHEILLGIPRDNAPALKPGDREKRKQYLNKNG : 799
 NP_0010881 : IPKKLPRPERFVADVQISAGWMHAGYPIIMCHLESAKELTDLNIQTGGIWGPIHELGHNQQTINWELPPHTTEATCNLWSVYVHETVVLGIPRSDAHCCLQAEFRANHIQEYLRNG : 800

CL4590.Con : AKLDEWSVWTALETYLQLECFGWDPFKQVFSEYQTMNSVSNVKDVKMNLWAEKFSHAVNKNLASFFKAWGWPIDDATTEKLSIISEWEEENPMNKYVS----- : 897
 NP_0010881 : SNLEQMNWVWTALETYLQLECFGWDPFKQLFKDYQSMSGLRNENKSKMNLWAEKFSHAVQTNLVPFFKAWGWPIEEATHSKLSVLPVWEKDPMKSYLSARKSNSN : 905

Figure S3. The amino acids sequence alignment of protein CL4590 and NP_0010881.

Supplement Table 2

proteins identified by homology search with PEAKS of peptide sequences obtained by de novo analysis of <i>B. gargarizans</i> secretion. Database: Uniprot (amphibian).							
Peptide	-10lgP	Mass	m/z	RT	Intensity	Accession	Protein Description
HAVSEGTKAVT	73.53	1098.5669	550.291	26	2.67E+05	POC1H3H2B1_CHICK	Histone H2B 1/2/3/4/6 (<i>Gallus gallus</i>)
HAVSEGTKAVTK	68.14	1226.6619	409.8929	22.72	3.22E+05	POC1H3H2B1_CHICK	
IFERLAGEASRLA	59.55	1431.7833	716.8997	106.22	1.84E+05	POC1H3H2B1_CHICK	
PEPAKSAPAPK	49.85	1091.5974	546.8065	22.99	6.16E+04	POC1H3H2B1_CHICK	
DSYVGDEAE(sub Q)SKR	48.54	1354.6001	678.3071	36.73	2.00E+04	P53506 ACT8_XENLA	Actin cytoplasmic type 8 (<i>Xenopus laevis</i>)
EAPLNPKAN	51.69	952.4977	477.256	32.37	1.87E+05	P53506 ACT8_XENLA	
GQKDSYVGDEAQ	68.71	1295.563	648.7886	34.87	3.11E+04	P53506 ACT8_XENLA	
ISKQYDESGPS	78.92	1338.5939	670.3048	37.4	4.43E+05	P53506 ACT8_XENLA	
LVV(sub I)DNGSGMCK	48.2	1121.5209	561.7679	58.35	3.97E+05	P53506 ACT8_XENLA	
MGQKDSYVGDEAQ	60.93	1426.6034	714.3116	43.9	1.10E+05	P53506 ACT8_XENLA	
MGQKDSYVGDEAQSKRGILT	73.42	2182.0688	728.3621	68	8.96E+05	P53506 ACT8_XENLA	
RVAPEEHPV	59.39	1032.5352	517.2799	33	6.47E+06	P53506 ACT8_XENLA	
VAPEEHPVLL	50.61	1102.6022	552.3084	90.5	2.01E+05	P53506 ACT8_XENLA	
VV(sub I)DNGSGMCK	61.91	1008.4368	505.2258	31.08	3.70E+06	P53506 ACT8_XENLA	
YVGDEAE(sub Q)SKRG	60.05	1209.5625	605.7894	26.35	1.16E+05	P53506 ACT8_XENLA	
YVGDEAQSKRG	73.34	1208.5785	605.2971	24.47	1.47E+05	P53506 ACT8_XENLA	
R(+42.01)PQYDASELK	66.01	1247.6146	624.8091	61.6	1.48E+06	tr D5KU33 D5KU33_BOM MX	Annexin (<i>Bombina maxima</i>)
AQGPP(+31.99)GPAGPAGER	48.2	1292.6108	647.3131	32.56	1.09E+05	tr O93251 O93251_LITCT	Alpha 1 type I collagen (<i>Lithobates catesbeiana</i>)
GPP(+31.99)GPAGPAGERGEQ	49.93	1407.6378	704.826	30.44	2.64E+05	tr O93251 O93251_LITCT	
GPP(+31.99)GPAGPAGERGEQGPAG	60.41	1689.7706	845.8885	39.69	1.10E+05	tr O93251 O93251_LITCT	
AK(+14.02)YGAVPGGAGV	50.49	1059.5713	530.7991	65.24	1.28E+06	tr Q0Q5Z1 Q0Q5Z1_DAN RE	Tropoelastin 1 (<i>Danio rerio</i>)
K(+42.01)PGYAGAGLIPG	48.2	1198.6345	600.3192	53.6	5.94E+06	tr Q0Q5Z2 Q0Q5Z2_XEN TR	Tropoelastin 1 (<i>Xenopus tropicalis</i>)
EIAQDFKTDL	51.26	1178.5819	590.298	95.32	8.33E+05	tr Q1WH09 Q1WH09_9NE OB	Histone H3 (<i>Chaperina fusca</i>)

Supplement Table 3

Classification of proteins identified from *Venenum Bufonis* by GO annotation

Class	Protein Name
immune system process	Unigene8539_All
	CL4669.Contig1_All
	Unigene35022_All
	Unigene20657
metabolic process	CL2148.Contig1_All
	CL4920.Contig2_All
	CL4920.Contig3_All
	Unigene20657_All
	Unigene21587
	Unigene30732_All
antioxidant activity	CL127.Contig1_All
	CL127.Contig2_All
	CL127.Contig3_All
	Unigene13919_All