

## Natterins, a new class of proteins with kininogenase activity characterized from *Thalassophryne nattereri* fish venom

G.S. Magalhães<sup>a</sup>, M. Lopes-Ferreira<sup>a</sup>, I.L.M. Junqueira-de-Azevedo<sup>a</sup>, P.J. Spencer<sup>b</sup>,  
M.S. Araújo<sup>c</sup>, F.C.V. Portaro<sup>a</sup>, L. Ma<sup>d</sup>, R.H. Valente<sup>d,e</sup>, L. Juliano<sup>c</sup>, J.W. Fox<sup>d</sup>, P.L. Ho<sup>a</sup>,  
A.M. Moura-da-Silva<sup>a,\*</sup>

<sup>a</sup> Laboratório de Imunopatologia, Instituto Butantan, Av. Vital Brasil, 1500, 05503-900 São Paulo, SP, Brazil

<sup>b</sup> IPEN/CNEN-SP, São Paulo, Brazil

<sup>c</sup> Universidade Federal de São Paulo, São Paulo, Brazil

<sup>d</sup> University of Virginia, Charlottesville, USA

<sup>e</sup> FIOCRUZ, Rio De Janeiro, Brazil

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### Abstract

A novel family of proteins with kininogenase activity and unique primary structure was characterized using combined pharmacological, proteomic and transcriptomic approaches of *Thalassophryne nattereri* fish venom. The major venom components were isolated and submitted to bioassays corresponding to its main effects: nociception and edema. These activities were mostly located in one fraction (MS3), which was further fractionated. The isolated protein, named natterin, was able to induce edema, nociception and cleave human kininogen and kininogen-derived synthetic peptides, releasing kallidin (Lys-bradykinin). The enzymatic digestion was inhibited by kallikrein inhibitors as Trasylol and TKI. Natterin N-terminal peptide showed no similarity with already known proteins present in databanks. Primary structure of natterin was obtained by a transcriptomic approach using a representative cDNA library constructed from *T. nattereri* venom glands. Several expressed sequence tags (ESTs) were obtained and processed by bioinformatics revealing a major group (18%) of related sequences unknown to gene or protein sequence databases. This group included sequences showing the N-terminus of isolated natterin and was named Natterin family. Analysis of this family allowed us to identify five related sequences, which we called natterin 1–4 and P. Natterin 1 and 2 sequences include the N-terminus of the isolated natterin. Furthermore, internal peptides of natterin 1–3 were found in major spots of whole venom submitted to mass spectrometry/2DGE. Similarly to the ESTs, the complete sequences of natterins did not show any significant similarity with already described tissue kallikreins, kininogenases or any proteinase, all being entirely new. These data present a new task for the knowledge of the action of kininogenases and may help in understanding the mechanisms of *T. nattereri* fish envenoming, which is an important medical problem in North and Northeast of Brazil.

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### 1. Introduction

In venomous animals, evolution has done an extraordinary job of making toxins with a wide variety of sequence

motifs, which are essential for targeting important physiological components with affinity high enough to kill or immobilize preys and predators. Therefore, venoms are extensively studied revealing tools to understand physiological pathways and eventually discover new drugs. Moreover, the knowledge of toxin function helps in the understanding and treating the human victims inflicted by venomous animals. Amongst venomous animals, several species of fishes can produce severe human envenoming and interesting toxins have already been described from their venoms [1–3]. In the North and Northeast of Brazil, *Thalassophryne nattereri* fishes,

**Abbreviations:** EDDnp, *N*-[2,4-dinitrophenyl]-etilenediamine; EDTA, ethylenediaminetetraacetic acid; ESTs, expressed sequence tags; hK1, human kallikrein 1; PMSF, phenyl methyl sulfonyl fluoride; 2DGE, two-dimensional gel electrophoresis; TKI, phenylacetyl-FSR-EDDnp; TLCK, tosyl Lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone.

\* Corresponding author. Tel.: +55 11 3726 7222; fax: +55 11 3726 1505.

E-mail address: [anamoura@butantan.gov.br](mailto:anamoura@butantan.gov.br) (A.M. Moura-da-Silva).

belonging to Batrachoididae family, are responsible for several accidents amongst fishermen [4]. Human accidents occur by contact with the fish spines located on their dorsum and both sides of the head, which are connected to the venom glands. Envenoming symptoms include severe edema and pain followed by a fast settling necrosis, both in human victims and experimental animals [5]. It is estimated that hundreds of accidents occur every year and the incidence is underestimated because patients seldom look for medical care due to its lack of efficacy.

*T. nattereri* venom is composed of proteins endowed with proteolytic and myotoxic properties, but devoid of phospholipase A<sub>2</sub> activity [6]. Analysis of its local effects showed myotoxicity with difficult muscle regeneration [7]. The blood flow at microvessels was also impaired with stasis and presence of thrombi in venules, focal transient constrictions in arterioles and increased vascular permeability. Venom lacked a direct pro-coagulant activity, but exerted a strong cytolytic action on platelets and endothelial cells in vitro [8]. Recently, we also have demonstrated a kininogenase activity in the whole venom, which was correlated, by the use of specific antagonists, with the major symptoms of envenoming including local edema and nociception [9].

With the purpose of understanding the nature of *T. nattereri* venom toxicity, we carried out a structural characterization of the major venom toxins through proteomic and transcriptomic techniques. Under these approaches we isolated the major venom toxin with a kininogenase activity as well as its and other related cDNA sequences from the venom gland cDNA library. These are novel sequences in databanks, with minor resemblance to other proteinases, characterizing a new family of proteins named natterins, presenting a kininogenase activity.

## 2. Material and methods

### 2.1. Characterization of venom toxins

*T. nattereri* venom was collected from 40 specimens collected at Mundaú lagoon, State of Alagoas, Brazil. Venom was extracted from the openings at the tip of the spines by applying pressure at their bases. Venom was pooled and conserved at  $-80^{\circ}\text{C}$  before use. For toxin purification, 3 mg venom was diluted in 500 ml 50 mM Tris–HCl pH 8.0 and subjected to fractionation in a Mono-S FPLC-column equilibrated with the same buffer, and eluted with a linear gradient from 0 to 2 M NaCl in the same buffer at a flow rate of  $1\text{ ml min}^{-1}$ , monitored at 280 nm. Fractions showing nociceptive and edematogenic activities were pooled, dialyzed against 50 mM Tris–HCl pH 8.0 and applied to a TSK 3000-Toso Hass-75  $\times$  7.5 mm gel filtration exclusion HPLC-column. Chromatographies were run at a flow rate of  $1\text{ ml min}^{-1}$  and monitored at 214 nm, using the same buffer. Fractions were analyzed for purity on a 12% SDS-PAGE gel and stained with Coomassie Blue. For amino-terminal

sequencing, the isolated toxin was subjected to electrophoresis in a 12% SDS/PAGE and transferred to PVDF membrane as described previously [10]. The membrane was stained with 0.025% Coomassie Blue in the absence of acetic acid. Stained bands were cut from the PVDF membrane and subjected to Edman degradation in a Procise sequencer (Perkin–Elmer Corporation) according to manufacturer's instructions.

Pooled crude *T. nattereri* venom was also subjected to two-dimensional gel electrophoresis (2DGE) with a Protean IEF cell System (Bio-rad). Isoelectric-focusing was performed on an 11-cm Immobiline DryStrip (Amersham Biociences) in a pH range of 6.0–11.0 using the manufacturer's specifications. Before SDS-PAGE the strips were subjected to equilibration buffer with reducing agent (dithiothreitol) followed by cysteine carbamidomethylation with iodoacetamide. The electrophoresis was performed using precast gels PROTEAN II Ready Gel 10–20% acrylamide concentrations (Bio-rad) and the proteins bands were stained with Coomassie Blue.

Stained spots of crude venom 2DGE or SDS-PAGE bands of isolated toxins were excised from the gels and incubated with trypsin followed by extraction of tryptic peptides by chromatography on capillary column (Agilent 1100 Series with an 8 cm  $\times$  75 mm) with the effluent directly entering the electrospray source of a Finnigan LCQ ion trap mass spectrometer. Collisionally activated dissociation (CAD) spectra were generated for each peptide found in the digestion mixture and analyzed by the Sequest program [11] for identification of the source protein. Alternatively, peptide masses were compared to a homemade databank constructed with translated expressed sequence tag (EST) sequences from *T. nattereri* venom glands. From the digestion mixture of each spot, some peptides were partially de novo sequenced [12].

### 2.2. Biological and enzymatic activities

Nociception and edema activities were estimated by injection of the isolated fractions or crude venom (1 or 3  $\mu\text{g}/30\text{ }\mu\text{l}$ ) in the intraplantar region of the hind foot paw of Swiss mice ( $n = 6$ ). Nociception was assessed as the time in seconds spent by the animals licking the envenomed paw during a 30 min observation time [13]. Edema-forming activity was evaluated by measuring the thickness of injected paws with paquimeter (Mytutoyo) 2 h after injection. The results were expressed by the difference between experimental and control footpad thickness [14]. For both tests, as control group, mice were injected with 30  $\mu\text{l}$  of sterile phosphate buffered saline, and results are expressed as mean  $\pm$  S.D. of three independent experiments.

Kininogenase activity of venom fractions was assayed by hydrolysis of internally quenched fluorogenic substrates including the region flanking the N-terminal residues of bradykinin derived from human kininogen and also by radioimmunoassay of released kinins after incubation of high or low molecular weight kininogen with venom toxins. The hydrolysis of the Abz-MISLMKRPQ-EDDnp substrate was accomplished at  $37^{\circ}\text{C}$  in 50 mM Tris–HCl buffer pH 7.4 containing

100 mM NaCl. Fluorescence was measured at  $\lambda_{em} = 420$  nm and  $\lambda_{ex} = 320$  nm in a Hitachi F-2000 spectrofluorometer using 1 cm path-length cuvette. Substrate and toxins were incubated in a thermostatically controlled cell and the increase in fluorescence with time was continuously recorded for 5–10 min [15]. The fraction concentrations for initial rate determinations were chosen at a value intended to hydrolyze less than 5% of the substrate present in the reaction. The inner-filter effect was corrected using an empirical equation as previously described [16]. The cleaved bonds were identified by HPLC comparing the retention times of the produced fragments with synthetic peptides encompassing the expected hydrolysis products. For inhibition studies, the stock solutions and the work concentration of the synthetic inhibitors used in the characterization of the natterin proteolytic activity were made as described elsewhere [17]. The set of inhibitors included E-64, pepstatin, *o*-phenantroline, EDTA, PMSF, TLCK (purchased from Sigma Co.). The trasylol was purchased from Bayer Pharmaceutical Co. and the TKI and PKSI<sub>527</sub> were synthesized as described [18]. The results were recorded as the percentage of residual activity relative to control reactions run simultaneously in the absence of the inhibitor.

For radioimmuno assay, fractions (1 µg) were incubated with human high or low molecular mass (MM) kininogen (200 nM) in 50 mM Tris buffer, pH 7.4, 100 mM NaCl in a final volume of 100 µl for 10 min at 37 °C. Kinins were extracted in ethanol (four times the reaction's final volume) for 10 min at 70 °C. Solutions were freeze-dried and dissolved in 200 µl of egg albumin buffer (0.1% egg albumin in 0.01 M phosphate buffer, pH 7.0, 0.14 M NaCl, 0.1% NaN<sub>3</sub>, 30 mM EDTA, 3 mM *ortho*-phenanthroline). Aliquots (50 µl) were incubated with 100 µl of antibody anti-bradykinin (1:80,000) as described [19] and 100 µl of [<sup>125</sup>I]-labeled Tyr-bradykinin for 20 h at 4 °C. Four hundred microliters of 0.1% bovine γ-globulin in 0.01 M phosphate buffer, pH 7.0, 0.14 M NaCl, 0.15 0.1% NaN<sub>3</sub> and 800 µl of 25% polyethyleneglycol 6000 solution were added to the samples, which were incubated for 10 min at 4 °C. Finally, the samples were centrifuged at 2000 × *g* for 20 min at 4 °C; the supernatants were removed and the pellets submitted to radiation counting [20].

### 2.3. cDNA library construction

The cDNA library was constructed from mRNA prepared from nine glands extracted from three specimens of *T. nattereri* fish collected in Mundaú lagoon, State of Alagoas, Brazil. To stimulate the production of messenger RNAs (mRNAs) venom was extracted 4 days before gland extraction and the fishes were further kept in the natural reservoir. The total RNA was extracted using Trizol reagent (Invitrogen) and the mRNA purification was performed in an oligo-dT cellulose column (Amersham-Pharmacia Biotech). The cDNA was prepared with Superscript Plasmid System for cDNA Synthesis and Cloning (Life Technologies, Inc.), according to the supplier's method, linked to Eco RI adapters and selected by size

(400–800 pb and up 800 pb) in agarose gel electrophoresis [21]. All cDNAs were ligated directionally into pGEM11Zf+ (Promega) at Eco RI/Not sites and used to transform calcium competent *Escherichia coli* DH5α cells which were subsequently plated on a 2YT agarose plates containing 100 µg ml<sup>-1</sup> of ampicillin [22].

### 2.4. DNA sequencing and bioinformatics analysis

Single-pass sequencing of the 5'-termini of 775 randomly selected cDNA clones was conducted with standard M13 forward primers on an ABI 3100 automatic DNA sequencer using ABI prism Big Dye Terminanator kit (PE Applied Biosystems) following manufacturer's instructions. All ESTs were subjected to the Phred program [23] to remove poor quality sequences with the general parameters of trimming sequences using a window length of 75 bases with 75% of standard quality (Phred = 20). The CrossMatch program was used to remove adapters, vector and poly (A/T) sequences as well as to eliminate sequences less than 150 pb in length. CAP3 program [24] were used to assemble all ESTs in contiguous sequences, using parameters > 95% of identity in a high quality region, in such a way that, only sequences close related were kept in the same cluster. For the identification of the cDNA sequences, the FASTA file containing all clusters was blasted against the GenBank NCBI database using the BLAST client program, found at <ftp://ftp.ncbi.nlm.nih.gov/blast/network/netblast/> [25]. Signal peptide and glycosylation prediction was accomplished using the programs SignalP 3.0, NetOglyc and NetNglyc servers available at CBS Prediction Servers (<http://www.cbs.dtu.dk/services/>).

## 3. Results

### 3.1. Analysis of the main venom toxins

*T. nattereri* venom was fractionated by current chromatographic methods and the main fractions characterized for primary structure and screened for the most important venom activities: induction of edema, nociception and kininogenase activity. Following a cation-exchange chromatography (FPLC, Mono-S column, pH 8.0), five fractions were isolated: two acidic/neutral (MS1, MS2) collected before the initiation of the NaCl gradient and three basic (MS3, MS4 and MS5) fractions collected with NaCl concentrations of approximately 1, 1.15 and 1.67 M, respectively (Fig. 1A). Nociceptive activity was distributed in all fractions, in levels significantly higher than control; however, comparable or lower than the levels induced by the same doses of the whole venom (Table 1). The highest activity was observed in fractions MS3/MS4, which was similar to nociception induced by the whole venom. Edema-inducing activity was also predominant in fractions MS3/MS4 presenting similar levels to those of whole venom. Fraction MS2 also induced edema in levels significantly higher than control but much lower than fraction MS3

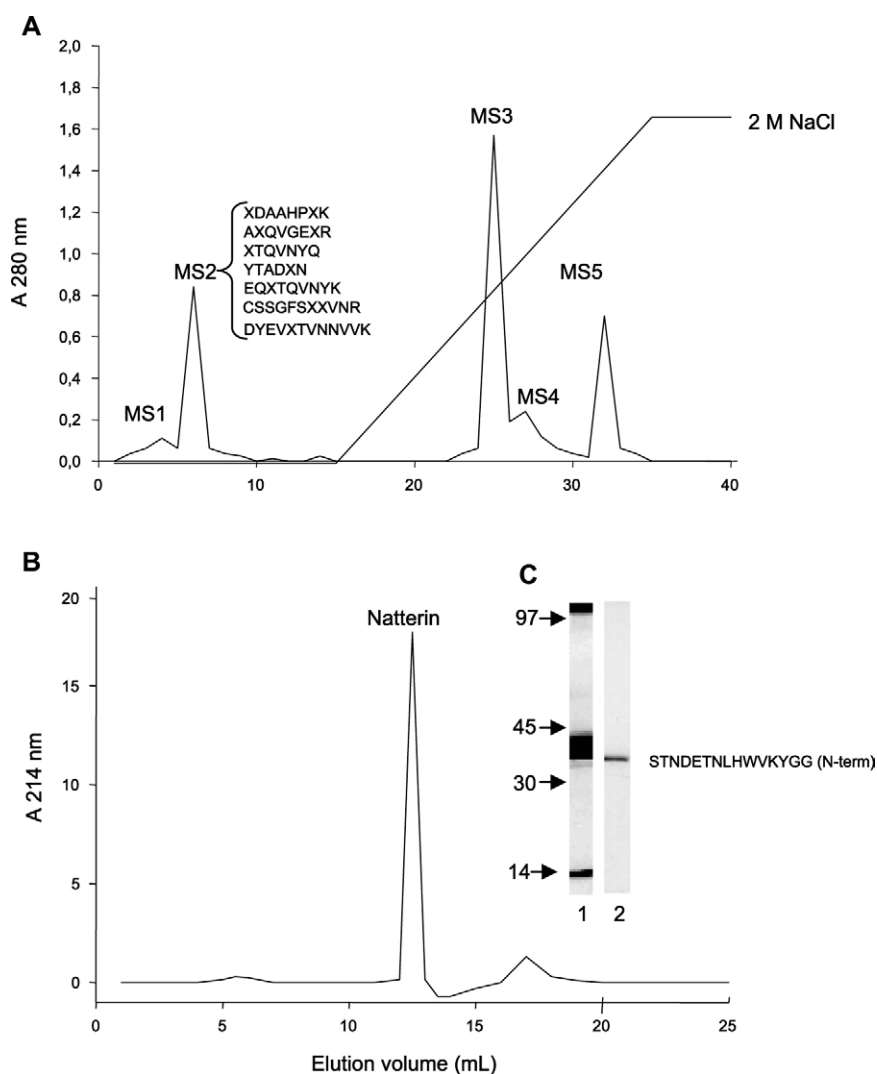


Fig. 1. Characterization of major toxins from *T. nattereri* venom. (A) Cation-exchange chromatography (Mono S, FPLC-column, pH 8.0) of crude *T. nattereri* venom with a linear gradient from 0 to 2 M NaCl, displaying the internal peptides of fraction MS2. (B) Gel filtration chromatography of fraction MS3 on HPLC-column (TSK 3000-Toso Hass-75 × 7.5 mm). (C) SDS/PAGE analysis of crude venom (1) and natterin (2) under reducing conditions, exhibiting its N-terminal sequence.

(Table 1). It is important to point out that all fractions presented nociceptive and edema-inducing activities, thus suggesting that all contribute to the whole venom toxicity. This

may explain why the isolated fractions presented similar activity compared to the whole venom and not higher, as expected for isolated proteins. Fraction MS2 was subjected to partial

Table 1  
Biological activities of fractions isolated from *T. nattereri* venom

	Nociception (s)	Edema (mm)	HK cleavage (pg released kinins)	LK cleavage (pg released kinins)
Control	14 ± 1	0.4 ± 0.1	200	530
Venom (3 µg)	201 ± 12	2.8 ± 0.5	NT	NT
MS1 (3 µg)	58 ± 11	0.6 ± 0.2	NT	NT
MS2 (3 µg)	87 ± 10	1.0 ± 0.3	NT	NT
MS 3 (3 µg)	189 ± 16	2.7 ± 0.4	NT	NT
MS4 (3 µg)	113 ± 18	1.8 ± 0.4	NT	NT
MS5 (3 µg)	31 ± 9	0.4 ± 0.2	NT	NT
Venom (1 µg)	108 ± 23	1.2 ± 0.4	240	6300
Natterin (1 µg)	110 ± 21	1.3 ± 0.6	250	6700
Kallikrein (1 µg)	NT	NT	16,000	13,500

The whole venom or fractions isolated in FPLC Mono-S column (MS1–5) or size exclusion HPLC-column (natterin) were tested for induction of nociception and edema or release of kinins after incubation with high molecular weight (HK) or low molecular weight (LK) kininogens as described in Section 2; NT = not tested.



primary structure analysis (MS/MS spectrometry) revealing the internal peptides shown in Fig. 1A. Those sequences were not found in databanks. Since induction of edema and nociception was predominant in MS3 fraction, it was further subjected to a size exclusion chromatography using HPLC system, which resulted in a major peak corresponding to the isolated toxin that we named natterin (Fig. 1B). This fraction yielded a single protein band with a MM of approximately 40 kDa by SDS-PAGE under reducing conditions. Its N-terminal sequence (Fig. 1C) did not show any hit in databanks. Natterin toxin presented edema and nociception-inducing activities values comparable to those obtained by injection of the same doses of venom (Table 1).

### 3.2. Enzymatic properties of natterin

Natterin kininogenase activity was assayed both by cleavage of synthetic peptides and release of kinins by radioimmune assay. As shown in Table 1, when incubated with low molecular weight kininogen, isolated natterin released similar amounts of kinins as released by equal concentrations of the whole venom. Neither natterin nor the whole venom was able to release kinins from high molecular weight kininogen. It is important to note that cleavage of the peptide containing bradykinin region of human kininogen (Abz-MISLMKRPEDDnp) occurred between methionine and lysine as evaluated in HPLC by comparison of retention times of the reaction products and the authentic synthesized fragments correspondent to peptides cleaved at the M–K bonds. This pattern of hydrolytic activity was comparable with the one from human tissue kallikrein. In order to further investigate this activity, several inhibitors for the major groups of proteolytic enzymes were assayed. The inhibition of synthetic peptides cleavage was relevant only when using the specific tissue-kallikrein inhibitor TKI (1 mM) and trasylol (200 U ml<sup>-1</sup>) which rendered an inhibition of 40% and 65%, respectively. On the other hand, plasma kallikrein inhibitor PKSI<sub>527</sub> (1 mM) and classical inhibitors of serine-, metallo-, thiol- or aspartate-peptidases evoked a minor inhibition of the peptide digestion (below 20%). Those results suggest that natterin toxin exerts a kininogenase activity similar to those found in the tissue kallikrein.

### 3.3. Identification and sequencing natterin cDNAs

The mRNA extracted from *T. nattereri* venom glands was used to construct a unidirectional cDNA library. Seven hundred and seventy-five clones were sequenced by single-pass DNA sequencing from 5' ends, with an average readable sequence length of 390 pb (200–580). Resulting ESTs were assembled using stringency parameters of > 95% identity, in such a way that the generated clusters could be considered unigenes.

After searching the databases, 51.3% sequences showed similarity with genes coding for cell functions, cell defense, retroelements and lectins (data not shown). However, a high

numbers of clusters (48.6% of total transcripts) showed to be unknown to the databases, which prompted us to carry out a further sequence analysis. For this purpose, all unknown clusters were additionally assembled in families under lower stringency parameters of identity using CAP3 program (>65%, overlap percent identity cutoff) and a tBlastx (translated query × translated database) in this case using all clusters as database and also as a query (e-value 10<sup>-5</sup>). This approach was particularly important considering the large numbers of unknown sequences in the library that under this flexible DNA/protein alignment could bring together paralogous genes or populational variants, revealing large groups that due to their abundance might indicate families of putative toxins. These alignments showed that a great number of those unknown sequences (140 ESTs, 18% of total transcripts) were grouped in a single family and most of clusters in this family showed a putative signal peptide-coding region, strongly suggesting that they might code for venom toxins. In fact, when some clusters of this family were translated, they showed to contain the N-terminus of isolated natterin as well as internal peptides of fraction MS2. This group of sequences was then named Natterin family.

### 3.4. cDNA cloning and sequence analysis of natterins

The sequences of Natterin family were further reassembled into more stringent parameters (> 90% identity and e-value 10<sup>-120</sup>) which allowed us to identify five sub-groups (GR1, GR2, GR3, GR4 and GR5) containing clusters closely related to each other.

The clone containing the longest insert of each group was isolated, and the inserted cDNA completely sequenced from 5' UTR to poly A tail. The product of each clone was named natterin 1, natterin 2, natterin 3, natterin 4 and natterin P, respectively, being the number a reference to the sub-group they came from. The complete sequences of these Natterins were submitted to the databases, but none of them showed any significant homology. The complete cDNA sequences and translated natterin sequences are shown in Appendix 1 and were deposited in the GenBank under accession numbers AAU11822 to AAU11826.

Amongst Natterin family of transcripts, natterin 1 and natterin 2 sequences contained the region coding for the N-terminal peptide of isolated natterin, indicating that this preparation may be composed of natterin 1, natterin 2 or both. Natterin 3 sequence contained the internal peptides of MS2 fraction which had been determined by MS/MS spectrometry. Natterins 4 and P sequences did not show any region coding for the peptides identified so far.

Fig. 2 shows the alignment of amino acid sequences predicted by the cDNAs described above. Natterins 1 and 2 are very close related, presenting 84% of identity. When comparing these two with natterins 3 and 4 they showed an identity around 40%. Taking natterin 1 as a reference sequence, natterin 2 presents a C-terminal extension of 20 residues including a hydrophobic–cationic motif that frequently appears in

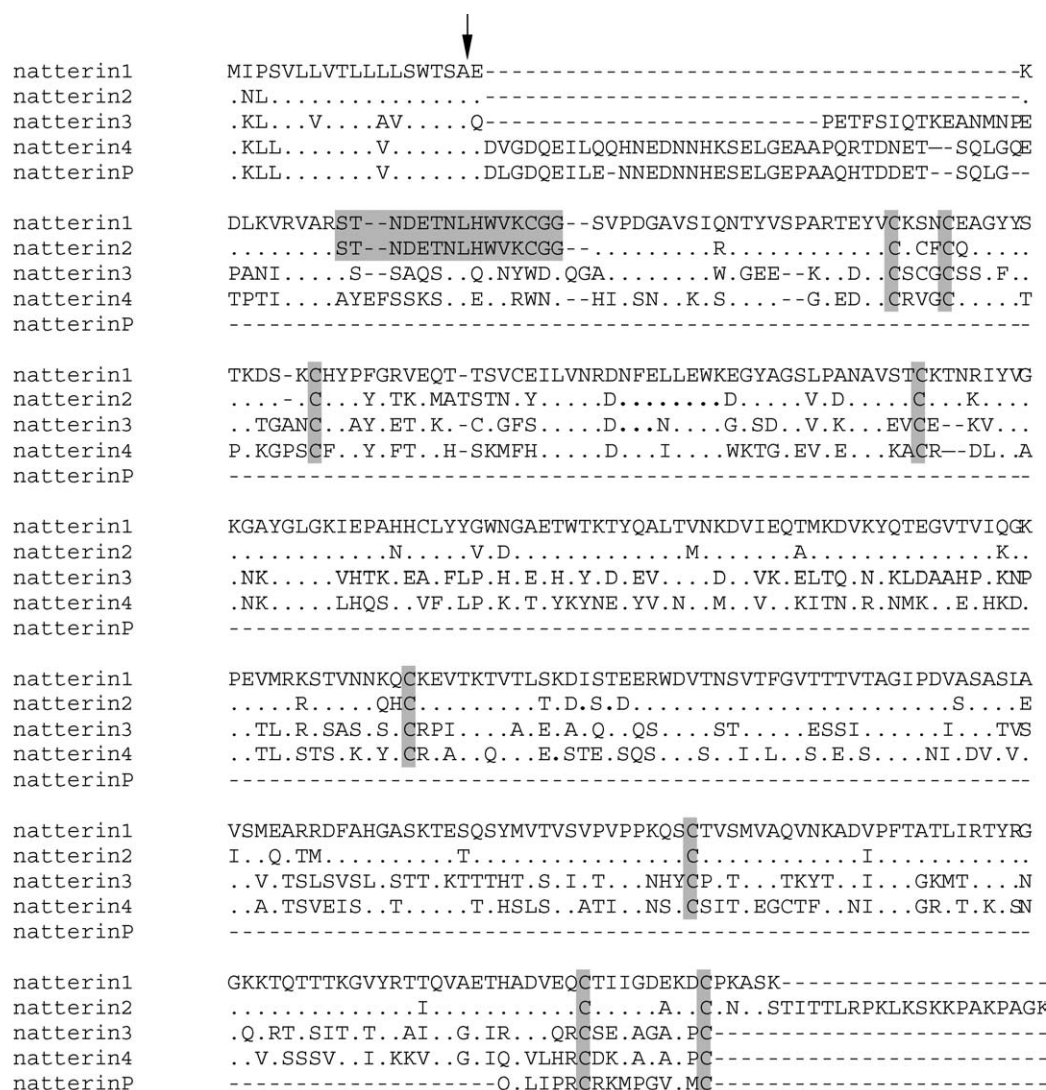


Fig. 2. Analysis of natterin sequences. Alignment of deduced amino acid sequences of natterins 1–4 and P. Dashes represent gaps to bring the sequences to better alignment. Amino acids residues identical to those of natterin 1 are indicated by dots. Conserved cysteins residues among the natterins as well as the N-terminal sequence found in isolated natterin are highlighted in gray. The first arrow indicates the putative signal peptide site of all natterins.

cytotoxins [26]. In opposition, natterins 3, 4 and P present inclusions at the N-terminal region of 15, 38 and 37 amino acid residues, respectively. Natterin P is the shortest among the natterins (71 amino acids long), and shows high homology mainly to the natterin 4 at the first 55 amino acids residues (84% of identity). The 16 last residues of natterin P align with the C-terminus of natterin 4, although with low identity.

The alignment between natterin 4 and natterin P cDNAs (data not shown) presents a clear deletion of 945 bp after nucleotide 165, followed by a number of point mutations that resulted in amino acid substitutions and the 3' UTR of these are highly conserved (93% of identity). Comparison of natterin 1 and natterin 2 cDNAs showed that the C-terminal extension of natterin 2 was due to an inclusion of 25 bp followed by a frame shift and an inclusion of a new stop codon 35 bp downstream. In both alignments, the number of non-synonymous mutation was higher than silent mutations, thus suggesting that the Natterin gene family may be suffering accelerated evolution as suggested for other families of toxins [27].

Using bioinformatics, all natterin sequences include a putative signal peptide of 18 residues (Fig. 2, arrow). However, the experimentally defined N-terminal sequence of isolated natterin was 10 residues downstream (Fig. 2, highlighted in gray). This difference could be attributed to a second processing site in natterins, between arginine and serine, in a similar way that occurs in hK1 tissue-kallikrein enzyme [28]. In the consensus regions of natterins, eight cysteine residues are conserved and might be involved in intra-chain S–S bonds, although other non-aligned cysteines are present. Table 2 includes the principal features of each natterin sequence and the abundance of their respective sub-group in the cDNA library.

### 3.5. Identification of natterin sequences in whole venom 2D-electrophoresis

Due to the fact that natterins 1 and 2 transcripts show the same N-terminal, high identity and close physicochemical

Table 2  
Principal features of natterins

	Mw <sup>a</sup>	pI <sup>a</sup>	Percent total transcripts <sup>b</sup>
Natterin 1	37054.77	8.52	8.5 (GR1)
Natterin 2	39370.71	8.90	3.5 (GR2)
Natterin 3	37905.31	6.77	0.8 (GR3)
Natterin 4	41374.28	7.73	1.7 (GR4)
Natterin P	5907.40	4.25	3.6 (GR5)

<sup>a</sup> The molecular weight (Mw) and isoelectric points (pI) were calculated theoretically considering the protein sequence after the site for signal peptidase enzyme.

<sup>b</sup> The % corresponds to the abundance of each group in the cDNA library.

properties (pI and Mw), we searched for the presence of natterin proteins in the whole venom from pooled specimens by 2D-electrophoresis followed by mass spectrometry of the major spots. As the most abundant natterins (Table 2) showed to be basic, a pH range between 6 and 11 was selected for the gel. The gel shown in Fig. 3 points out the most defined six spots that were picked up and subjected to MS/MS sequencing. Each one of the six spots contained peptides present in the sequences predicted by natterins 1 and 2 transcripts (Appendix 1, Figs. 1 and 2), although in different proportion, being sequences belonging to natterin 1 the most predominant. Both natterins did not show predicted sites to *N*-glycosylation, but showed, although with low score, sites to *O*-GalNAc-glycosylation, which might explain the difference in pI among the spots.

#### 4. Discussion

Biochemical characterization of major toxins from venom mixtures may lead to unknown components with important biological applications. Additionally, it is essential for understanding the mechanisms of action, structure/function relationships, cloning and production of recombinant proteins. In this paper, we used a combined approach of pharmacological, proteomic and transcriptomic techniques, which resulted in the description of natterins, a new class of kininogenases with completely distinct primary structure.

When isolated from the venom, natterin showed nociceptive, edema-inducing and kininogenase activity with release of kallidin from low molecular weight kininogen. Natterin sequence was further characterized and the N-terminal pep-

tide of isolated natterin was found in sequences of both natterins 1 and 2. Other recent studies have reported the presence of kallidin-releasing enzymes on venoms from the snakes *Bitis arietans* and *Trimeresurus elegans* [29,30], which showed the N-terminal sequence similar to other typical serine proteinases. A kininogenase was also isolated from mammal *Blarina brevicauda* venom that presented high sequence identity with human tissue-kallikrein [31]. Despite the kininogenase activity of natterin, its sequence similarity with kallikreins or any other serine proteinases was negligible. Furthermore, this is the first time that a toxin with such activity is described in fish venom. Therefore, natterin sequences are novel comparing to known kininogenases present in both physiological and venomous secretions. It is important to point out that the inhibition of natterin activity by conventional proteinase inhibitors followed the pattern expected to kallikreins but not to general serine proteinases. The best inhibition was observed after treatment with Trasylol, followed by specific inhibitors of tissue and serum kallikreins, respectively. This suggests that despite of the dissimilarity in primary structure, the catalytic residues of natterins may be presented in a similar conformational arrangement as in kallikreins, suggesting a convergence of structural assemblies and function in completely different protein structural families.

Other fraction (MS2) presented nociceptive and edema-inducing activity, although in lower levels when compared to natterin. This fraction had some internal peptide sequences determined, which allowed us to identify natterin 3. Considering that the nociceptive and edema-inducing activities of *T. nattereri* venom have been correlated to kininogenase activity [9] and the fact that natterin 3 share approximately 40% identity with natterins 1 and 2 we suggest that this natterin could also possess a kininogenase activity.

Kinins are mediators of inflammation which increases vascular permeability and also stimulate pain receptors [32]. Hence, the production of kinins by natterin might explain the edema and nociception. In addition to the release of kinins, natterin may also exert a direct cytotoxicity, as suggested by the presence of a hydrophobic-cationic motif that frequently appears in cytotoxins, thus explaining the whole venom cytotoxic activity to myoblasts, platelets and endothelial cells [7,8].

The discovery of natterins 1–3 transcripts, led us to identify and sequence natterins 4 and P from other clusters of Natterin transcript family. However, these two proteins were not yet detected in the venom secretion and their biological function needs to be confirmed. The mechanisms for generation of this new gene family are still undefined but certainly involve a large plasticity of the gene regions coding for the mature toxins. A number of non-synonymous mutations were observed in the alignments. Besides, inclusions or deletions of DNA sequences and the presence of retrotransposon-like transcripts in the library (manuscript in preparation) may also indicate shuffling of DNA material amongst different gene copies, indicating a mechanism to generate toxin diversity. Moreover, the complexity of natterins was also observed at

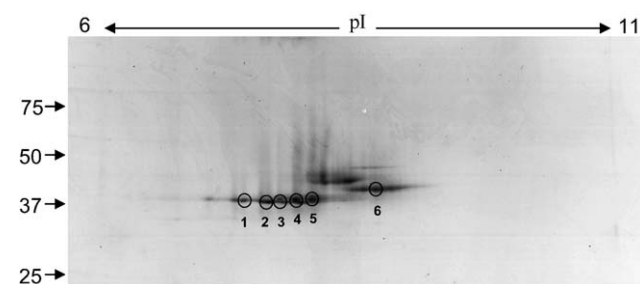


Fig. 3. 2DGE of crude *T. nattereri* fish venom separated on an IPG gel covering the pH range 6–11. Pooled venom from six specimens (80 mg). Circles point out six spots that were subjected to MS/MS spectrometry sequencing.

the protein level, when analyzing venom through 2DGE, indicating that post-translational modifications may also vary among the nascent proteins of this group.

Tissue kallikreins include 15 structurally related sequences, which degrade not only kininogens but also various proteins including growth factors, hormones, matrix metalloproteinases and produce bioactive compounds that interferes in cell proliferation, induction of apoptosis and inhibition of angiogenesis [33]. The effects of these enzymes can be noted in several physio-pathological conditions as hemostasis, breast, ovarian and prostate cancer and CNS injuries including Alzheimer's disease [28]. Their variable effects may be correlated to protein sequence variation, suggesting that each one of them interacts with specific substrate or a very restricted number of substrates to mediate specific biological events [28]. The identification of natterins, enzymes with tissue-kallikrein-like activity with unrelated primary structure, brings a new perspective in understanding the substrate specificity, and evolutionary parameters for generation of protein diversity, as

well as clues to understand the activity of other toxic kininogenases. Moreover, elucidating the sequences of major proteins from *T. nattereri* venom will help in elucidating the structural–function relationships and the mechanism involved in accidents with this fish.

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**Appendix 1.** Complete nucleotide and deduced amino acid sequence of natterins 1–4 and P. The highlighted amino acids residues correspond to the ones identified by MS/MS spectrometry or by Edman's degradation sequencing, as described in experimental procedures. The underlined nucleotides correspond to the probable polyadenylation signal



**NATTERIN 1**

ggtgcaccggcacaagatgattccttcagtgctgctcgtagaccctgctgcttctgtcctgg  
   M I P S V L L V T L L L L S W  
 accagtgcagagaaagatcttaaagtaaggggtggctcgatccaccaacgatgagaccaac  
 T S A E K D L K V R V A R S T N D E T N  
 ctacactgggtgaaatgtggaggatctgtgcctgatggagccgtttcgattcaaaacact  
 L H W V K C G G S V P D G A V S I Q N T  
 tacgtgagccccggctcgacagagtatgtctgcaaataattgcaagctgggtactac  
 Y V S P A R T E Y V C K S N C E A G Y Y  
 agcaccaaggattcaaagtgcactacccatttggagagtagaacagacaacctctgtc  
 S T K D S K C H Y P F G R V E Q T T S V  
 tgcgagatcctgggtaacagagataactttgagcttctggagtggagggttacgct  
 C E I L V N R D N F E L L E W K E G Y A  
 gggtcactgccagccaatgcagtcagtagcatgcaaaacgaataggatataatgtagggaag  
 G S L P A N A V S T C K T N R I Y V G K  
 ggtgcatacgggtctcgggaaaatagagcctgctcaccactgcttatattatgggttgaac  
 G A Y G L G K I E P A H H C L Y Y G W N  
 ggtgcagagacctggaccaagacgtaccaggctctgactgtgaataaagacgtgattgaa  
 G A E T W T K T Y Q A L T V N K D V I E  
 cagaccatgaaagatgtgaaataccagactgaagggttactgtcattcagggttaaacca  
 Q T M K D V K Y Q T E G V T V I Q G K P  
 gaagtcatgcgcaaatactaccgttaacaacaagcagtgcaagggaagtactaaaacagtt  
 E V M R K S T V N N K Q C K E V T K T V  
 accctctccaaagacatttcaacagaggaaagggtgggatgtcactaattcagtcacattt  
 T L S K D I S T E E R W D V T N S V T F  
 ggtgtcacaactactgtcacagctggatttctgacgttgcctctgcatcacttgcagtt  
 G V T T T V T A G I P D V A S A S L A V  
 agcatggaggcaaggagggttttctgctcagggagccagtaagactgagagtcagtcatac  
 S M E A R D F A H G A S K T E S Q S Y  
 atgggttactgtgtcagtgccgttccaccaaagcagtcctgtactgtcagtaggttgcg  
 M V T V S V P V P P K Q S C T V S M V A  
 cagggtgaataaagcagacgtccctttcacagcaaccttgatccgcacctaccgcggtggc  
 Q V N K A D V P F T A T L I R T Y R G G  
 aagaaaacacagacaacgaccaaagggtctataggacgacccaggttagcagaaaccac  
 K K T Q T T T K G V Y R T T Q V A E T H  
 gctgacgtcgaacaatgtaccatcatagggtgacgagaaagattgtccaaaggcttcaaaa  
 A D V E Q C T I I G D E K D C P K A S K  
**t**aaaattaaaaaagcctgttaaagtaactggaaaaaaggaaaaatatcgcttttctgtt  
 tcagcaaaatgcagaagtggtaacttattctgacttcttataaaagcacacatacagtta  
 cattcttacatttttctcactgcaggctgtcgaaaaatcaacttttgacatgttgcctcaa  
 acggcacattatccagtgaaataaaaaacatgttttttgaactctacattgaagaaaaatat  
 gaaaatattattgtcacttgtggttggaaattaacaaagtctgatgtggtttgtcgaata  
 caaatttttactgtactaaataatgataaaattattcacattctcatggtttgatataa  
 taagatgttgtcttctctcttgacaactgaattgaaatgaattgttttaaaaatatggaa  
 agtgttttaaatgatgtacctcagcatgaatttaaatatttgaatttttaattgtcagaata  
 gagattctgaaatgatccttcaaatgttttgaagggaagacatttcatctgtcgtcttttg  
 aagacacagtcagtttcaatgaatttgtttgaactttgttttgatttgatgataagaa  
 aagctgtgcaatgagaaagatgatgtcagagtgactgatgatgctgagcgaataagaaa  
 cctacacgtgtgtctccatctttggaaaccacaaacttaaaagccgggggcagggttca  
 ctttttgtcttttctctctgttggccttctatgttgtgagagattatgatgagtcgtt  
 tgtataactgactccttcttttgaataaaatcatttaaacatcaaaaaaaaaaaaaaaaaa

## A.2. Natterin 2

**NATTERIN 2**

ggtgcacccgcaaagatgaatctgtcagtgtctgctcgtagccctgctgcttctgtcctgg  
   M N L S V L L V T L L L L S W  
 accagtgcagagaaagatcttaaagtcaggggtggctcgatccaccaacgatgagaccaac  
 T S A E K D L K V R V A R S T N D E T N  
 ctacactgggtgaaatgtggaggatctgtgcctgatggagccgtttcgattcgcaacact  
 L H W V K C G G S V P D G A V S I R N T  
 tacgtgagtcggctcgacagagtatgtctgcaaagtgttttgccaagctgggttactac  
 Y V S P A R T E Y V C K C F C Q A G Y Y  
 agcaccaaggattcaaagtgcactacccttatggaacaaaagaaatggcaacttctacc  
 S T K D S K C H Y P Y G T K E M A T S T  
 aactgctatatcctgggaacagagataactttgagcttctggagtggaggacggctac  
 N C Y I L V N R D N F E L L E W K D G Y  
 gctgggttcagtgccagacaatgcagtcagtagcatgcaaacgaataagatatatgtaggg  
 A G S V P D N A V S T C K T N K I Y V G  
 aagggtgctgacgggtctcgggaaatagagcctgctaaccactgcttatattatgtttgg  
 K G A Y G L G K I E P A N H C L Y Y V W  
 gacgggtgcagagacctggaccaagacgtaccaggctctgactatgaataaagacgttatt  
 D G A E T W T K T Y Q A L T M N K D V I  
 gaacaggccatgaaagatgtgaaataccagactgaaggggttactgtcattaagggtaaa  
 E Q A M K D V K Y Q T E G V T V I K G K  
 ccagaagtcagtcgcagatctaccgtcaacaaccagcactgcaaggaagttactaaaaca  
 P E V M R R S T V N N Q H C K E V T K T  
 gttaccctcaccaaagacatttcaacagatgaaagggtgggatgtcactaattcagtcaca  
 V T L T K D I S T D E R W D V T N S V T  
 tttgggtgtcacaactactgtcacagctgggtattcctgacgtttcctctgcatcacttgag  
 F G V T T T V T A G I P D V S S A S L E  
 attagcatgcaggcaacgatggattttgtcatggagccagtaagactgagactcagtcg  
 I S M Q A T M D F A H G A S K T E T Q S  
 tacatgggttactgtgtcagtgccgttccaccaaacagtcctgtactgtcagtaggtt  
 Y M V T V S V P V P P K Q S C T V S M V  
 ggcgaggtgaacaaagcagacatccctttcacagcaaccttgatccgcacctaccgcggt  
 A Q V N K A D I P F T A T L I R T Y R G  
 ggcaagaaaacacagacaacgaccaaagggtctataggacgatccaggttagcagaaacc  
 G K K T Q T T T K G V Y R T I Q V A E T  
 cacgctgacgtcgaacaatgtaccatcataggtgacgcgaaagattgtccaaacgcttca  
 H A D V E Q C T I I G D A K D C P N A S  
 agcaccatcaccacacttcgcccaggttaaaatcaaaaaagcctgctaaccagctgga  
 S T I T T L R P K L K S K K P A K P A G  
 aaataaaaggaaaatatctcctttcttgtttcagcaaacgcagaagtggtaactaatt  
 K -  
 ttttcttcttataaaaagcacacatacagttacattcttacatttcatcactgcaggctgt  
 cgaaaagtcaactttgacaagttgcctcaaaccggcacattatccagtgaaaaaggcatgt  
 tttgactctacactgaacaaaaatatgaaaatgttattgtcacttgtggttataaaattaa  
 taaagtctgatgtggtttgtcaaatacaaatttttactgttgctaaataattatgaaatt  
 attcacattttttatgggtttggaacaataagatgttgcttcttgattattttaattgaatt  
 gaagtgttttaacaatatggaaagtgtttaaagtgtgtacatcagtatgaattaaaata  
 tttggatttttaatatcagaatagagattctgaaatgatacttcaaagtgttcgactgtcg  
 tcttttgaagatacagtaagtttcaatgaatttctttgaaactttgcttttgatttgatg  
 atgtaagaagctgtgcaatgagaacgatgatgtcagagtgtactgatgatacttagccaa  
 taagaaacctacacgtgtgtctccatctttggaaacgacataactaaaaagccggggcg  
 ggcttcactttttgctgttttctctgtcggccttctatgggtgtgagagtttatgatga  
 gtctgtttgtataactgactcctcctcttgaaataaacatttttaacatctgaaaaaaaaa  
 aaaaaaaaaa



## A.3. Natterin 3

**NATTERIN 3**

actgcaaagatgaagctgtcagttctcgttgtgaccctgctcgctgtgtcctggaccagc  
M K L S V L V V T L L A V S W T S  
gctcagcctgagacgttctccattcagaccaaagaagccaacatgaatccagaacctgca  
A Q P E T F S I Q T K E A N M N P E P A  
aatataagagttgctcggctctccagtgtcctcaaagcaacctgcagtggaactactgggat  
N I R V A R S S S A Q S N L Q W N Y W D  
ggtcaaggcgagtcctgatggagccgtttcaatttggatggcgaagaaaaacgaact  
G Q G A V P D G A V S I W N G E E K R T  
gactacgtctgtagctgtgggtgttcttctggcttctattccacaaaaacaggtgctaac  
D Y V C S C G C S S G F Y S T K T G A N  
tgtcactatgcctatggagaaacagagaagacttgcagtggttctccatcctggatgaac  
C H Y A Y G E T E K T C S G F S I L V N  
agagataaactttgaaaatcttgagtgaagggttctgatggttcagtaccaaagaac  
R D N F E N L E W K G G S D G S V P K N  
gctgtcgaggtttgtgagaaggtttatgtagggaagaacaagtatggcctcgggaaagt  
A V E V C E K V Y V G K N K Y G L G K V  
cataccaaacacgaagccttgttttggcatggcagcggaagagcactgggtacaaggat  
H T K H E A L F L P W H G E E H W Y K D  
tacgaagtccttactgttaatgatgatgtagtcaaacaggagctcactcaagtcaattat  
Y E V L T V N D D V V K Q E L T Q V N Y  
aaattggatgcagctcatcccatcaagaatcctccagagacctgcgcaggtcatctgct  
K L D A A H P I K N P P E T L R R S S A  
tcaaatagtcagtgccgtccaattacaaaaacagtcgcactcgagaaagcaattcagaca  
S N S Q C R P I T K T V A L E K A I Q T  
gagcaaagctgggatgtcaccagtagcgtcacgtttggtgttgatcttctatcactgca  
E Q S W D V T S T V T F G V E S S I T A  
gggatccctgatatcgctctgcaacagtttctgtcagtggtgagacatcattgtctgtc  
G I P D I A S A T V S V S V E T S L S V  
tctctgggatccactacgactaaaaacaaccacccacactgtgtcctgtgatagtcaccgtt  
S L G S T T T K T T T H T V S V I V T V  
ccaccaaaccactattgccccgtcactatgggtggctactaagtatactgctgacatccct  
P P N H Y C P V T M V A T K Y T A D I P  
ttcacaggaataatgaccgcagctaccgcaatgggcagaaaagaacaacatcaatcact  
F T G K M T R T Y R N G Q K R T T S I T  
gggacctacagggcgatccaggttgagaaaatccgagctgatgtgcaacgttggttcagaa  
G T Y R A I Q V G E I R A D V Q R C S E  
atagctggagctaaaccatgctaacttgatgattaaagtcagatgagatgtttgacctatgt  
I A G A K P C -  
agcatgtctgcatatgaaattaacatgtaacaatataagtcattgacatgttgaaataat  
gacttgatcatgttaaaactcacataaaattcatggagcataaaaaaaaaaaaaaa

## A.4. Natterin 4

**NATTERIN 4**

gagactgcagagatgaagctggtggtgctgctcgtcaccctgctggttctgtcctggacc  
M K L L V L L V T L L V L S W T  
agtgctgaagatgtcggagatcaagagatcttacaacaacacaatgaagacaacaaccac  
S A E D V G D Q E I L Q Q H N E D N N H  
aagtcagaacttggaagaagcagctcccaacgcacagataatgagacgtcacaaactcgga  
K S E L G E A A P Q R T D N E T S Q L G  
caggaaactccaacaataaggggtggtcgcagcttacgagtttagtttctaaatccaactta  
Q E T P T I R V A R A Y E F S S K S N L  
gaatgggttcggtggaatggccacattccaagtaacgcagttaaaattccaatacatat  
E W V R W N G H I P S N A V K I S N T Y  
gtaggtcgtgaagactacgtctgcagagtcggtgtgaagctggctactacactccaaa  
V G R E D Y V C R V G C E A G Y Y T P K  
aagggcccttctgtcttacccttatggatttacagaacaacacagtaaaatgttccac  
K G P S C F Y P Y G F T E Q H S K M F H  
atactggtgaacagagataactttgagattctggagtggaagtggaaaacaggtggtgaa  
I L V N R D N F E I L E W K W K T G G E  
gtgccagagaatgcagtcaggcctgcagagacctttatgtggcaaagaacaagtcaggt  
V P E N A V K A C R D L Y V A K N K Y G  
ctagggaaaactacaccaaagccatcatgttttctatcttccctggaaaggaacagagtat  
L G K L H Q S H H V F Y L P W K G T E Y  
aagtacaacgagtactatgtcctgaatgtcaacatggatgtagtagcagaaaaatcacg  
K Y N E Y Y V L N V N M D V V E Q K I T  
aatgtcagataacaacatgaaaggggtgaagttcataaagacaaaccagagaccctccgc  
N V R Y N M K G V E V H K D K P E T L R  
agcaccagcgtaaaaaactatcagtgctcggaagctaccaaaacaagttaccctcgagaaa  
S T S V K N Y Q C R E A T K Q V T L E K  
tcaactgagacgtcacaaagttgggacgtcagcaattctatcacacttggtgtctcgact  
S T E T S Q S W D V S N S I T L G V S T  
gaagtcagtgctgggatcccaacattgccgatgtgagtggtggcagtaagcgctgagacc  
E V S A G I P N I A D V S V A V S A E T  
tcagtggaatctcccatggaaccagtaagacggaatcaaccagtcactctctttccgctc  
S V E I S H G T S K T E S T S H S L S V  
tctgccaccattcctccaaacagctcctgtagtatcactatggagggttgacggttcaag  
S A T I P P N S S C S I T M E G C T F K  
gcaaacatcccggttcacaggacgcctgacccgcaagtacagcaatgggaaggtcacctct  
A N I P F T G R L T R K Y S N G K V T S  
tcatcggtcaaaggaatctacaagaaggtccaggtgggagaaattcaggcagtgctgcat  
S S V K G I Y K K V Q V G E I Q A V L H  
cgctgcgataagatagctgatgccaagcggtgctaatgttcagctgtttcacctttgtag  
R C D K I A D A K P C -  
gatgtctacatgtgaagtgctgatgtgtttcagtatgaaagaaaaatccagtcacatg  
tcaaaatctttacttgacatattaaagtgaaaaaaataaagtcatttagcatattaaaaaa  
aaaaaaaaaaaa

## A.5. Natterin P

**NATTERIN P**

ggtagactgcagagatgaagctggtggtgctgctcgtcaccctgctggttctgtcctgg  
M K L L V L L V T L L V L S W  
accagtgctgaagatctcggagatcaagagatcttggaacaatgaagacaacaaccac  
T S A E D L G D Q E I L E N N E D N N H  
gagtcagaacttggaagaaccagctgcccaacacacagatgatgagacgtcacaaacttgga  
E S E L G E P A A Q H T D D E T S Q L G  
caggcactgatacctcgctgcagaaagatgcctgggtgtcaagatgtgctaatgttcagct  
Q A L I P R C R K M P G V K M C -  
gtttcacctttttgtaggatgtcgacatgtgaagtgctgattttgtctcagtatgaaagaa  
aaaaatccagtcacgtgtcaaaatgttacttgacatattaaagtcaaaaaaataaagtaa  
ttcagcatattaaaaaaaaaaaaaaaaaaaa



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