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New view on crotamine, a small basic polypeptide myotoxin from South American rattlesnake venom

N. Oguiura[a,*], M. Boni-Mitake[b], G. Rádis-Baptista[c]

[a]*Laboratório de Herpetologia, Instituto Butantan, Av. Vital Brazil, 1500, São Paulo 05503-900, SP-Brazil*
[b]*Serviço de Radioproteção, Instituto de Pesquisas Energéticas e Nucleares, IPEN/CNEN-SP, Av.Lineu Prestes 2242, São Paulo 05508-000, SP-Brazil*
[c]*Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza 60455-760, CE-Brazil*

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Abstract

Crotamine is a toxin from the *Crotalus durissus terrificus* venom, composed of 42 amino acid residues and three disulfide bridges. It belongs to a toxin family previously called Small Basic Polypeptide Myotoxins (SBPM) whose members are widely distributed through the *Crotalus* snake venoms. Comparison of SBPM amino acid sequences shows high similarities. Crotamine induces skeletal muscle spasms, leading to spastic paralysis of the hind limbs of mice, by interacting with sodium channels on muscle cells. The crotamine gene with 1.8 kbp is organized into three exons, which are separated by a long phase-1 and short phase-2 introns and mapped to chromosome 2. The three-dimensional structure of crotamine was recently solved and shares a structural topology with other three disulfide bond-containing peptide similar to human β-defensins and scorpion Na^+ channel toxin. Novel biological activities have been reported, such as the capacity to penetrate undifferentiated cells, to localize in the nucleus, and to serve as a marker of actively proliferating living cells.

Keywords: Crotalus; Myotoxin; Crotamine; Cationic peptide; Structure; Cell penetrating peptide; Biological activity; Gene; Paralogous gene; Toxin

Contents

1. Introduction

Crotamine was first observed in the venom of Argentinean rattlesnakes (*Crotalus durissus terrificus*) by

* Corresponding author. Tel.: +55 11 3726 7222x2267; fax: +55 11 3726 7222x2167.
E-mail address: nancyoguiura@butantan.gov.br (N. Oguiura).

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Gonçalves and Polson (1947). Later on, this extremely basic toxin and inductor of spastic paralysis of the hind limbs of mice was found in other rattlesnake venoms from the southern region of Brazil (Gonçalves and Vieira, 1950). Gonçalves (1956) described an individual crotamine polymorphism in venoms after which Schenberg (1959) related this characteristic to geographic rattlesnake distribution. It was only at the end of the 70′s, that several toxins with similar chemical and biological properties were isolated from the venom of other rattlesnake species: myotoxin *a* from *C. viridis viridis* (Cameron and Tu, 1977; Fox et al., 1979), peptide C from *C. v. helleri* (Maeda et al., 1978), myotoxin I and II from *C. v. concolor* (Bieber et al., 1987), CAM toxin from *C. adamanteus* (Samejima et al., 1991), crotamine-Ile 19 from *C. d. ruruima* (Santos et al., 1993) and E toxin from *C. horridus horridus* (Allen et al., 1996). There is a high degree of amino acid conservation among these members, with scores varying from 83 to 98%. Ownby (1998) designated these toxins Small Basic Polypeptide Myotoxins (SBPM). Fig. 1 shows the amino acid sequences alignment and the evolutionary relationship of these toxins.

General knowledge concerning the SBPM is based on myotoxin *a* (Bieber and Nedelkov, 1997) and crotamine research. Interestingly, despite the fact that SBPM has been considered a homogeneous group due to the conserved amino acid sequences, research on toxin interactions with muscle cells was conducted by emphasizing distinct receptors. The works with myotoxin *a* were driven to Ca^{2+} influx via calsequestrin or Ca^{2+} ATPase (Bieber and Nedelkov, 1997), whereas the approach used to investigate crotamine interaction was with Na^{+} channels (Chang and Tseng, 1978; Pellegrini Filho et al., 1978; Matavel et al., 1998). In structural protein analysis, the solution structure of crotamine was resolved by Siqueira et al. (2002), and Nicastro et al. (2003). In molecular genetics, the crotamine gene was isolated, its structural organization determined and its chromosomal location mapped by Rádis-Baptista et al. (2003). Later, in the scope of cell biology, Kerkis et al. (2004) revealed new biological activities of crotamine by using mouse embryonic stem cells. In this work our objective is to present recent progress on crotamine research.

2. Crotamine polymorphism

Brazilian rattlesnake venoms present several levels of divergence in their composition, such as geographic variation (Schenberg, 1959), variation of crotamine content (Gonçalves and Arantes, 1956; Oguiura et al., 2000) and variation of crotamine sequence (Laure, 1975; Smith and Schmidt, 1990; Santos et al., 1993; Rádis-Baptista et al., 1999; Toyama et al., 2000).

Fig. 1 shows the evolutionary relationship of SBPM. We observed that primary sequences from *C. d. terrificus*, described by Smith and Schmidt (MYX 4_CRODU and MYX1_CRODU), are not in the same phylogenetic branch as crotamine (CXRSMT) and crotamine-Ile19 (MYX_CRODR), from *C. d. terrificus* and *C. d. ruruima*, respectively. Equivalent divergence was observed between myotoxin I and II, which were purified from pooled venom samples from *C. v. concolor* (MYX1_CROVC and MYX2_CROVC). This divergence might indicate that new isoforms of SBPM are emerging or have already evolved in the course of genetic diversification.

3. Crotamine structure

Crotamine is composed of 42 amino acids (Laure, 1975) and contains, for the most part, β-sheet, α-helix and random coil structures (Kawano et al., 1982; Beltran et al., 1985, 1990). Initially, some attempts were unsuccessful in solving the crotamine 3D structure based on protein crystallization and X-ray diffraction. Some of the difficulties observed in attaining crystals may be due to several crotamine isoforms, different conformations adopted by the toxin (Hampe, 1978; Endo et al., 1989) and the formation of aggregates (Beltran et al., 1985; Teno et al., 1990). However, Siqueira et al. (2002) proposed a theoretical 3D model for crotamine using computational calculation that was accomplished by homology modeling procedure and by intensive molecular dynamics simulations. They identified a fold common to β-defensin and antopleurine-B. Finally, with a practical approach of NMR spectroscopy, Nicastro et al. (2003) solved the solution structure of crotamine. The structure comprises a short N-terminal α-helix and a small antiparallel triple-stranded β-sheet arranged in a $\alpha\beta_1\beta_2\beta_3$ topology never before encountered among active toxins on ion channels. Interestingly, crotamine not only has disulfide bridges arrangement identical to members of the human β-defensin family, but also has similar structural fold conformation (Fig. 2).

4. Biological activity

Crotamine induces spastic paralysis in the hind limbs of mice, rats, rabbits and dogs (Gonçalves, 1956). Furthermore, pharmacological studies showed that myotoxin induces depolarization of membrane potential in skeletal muscle cells and influx of Na^{+}, and this effect is prevented by tetrodotoxin as well as by decrease of extracellular concentration Na^{+} (Chang et al., 1983). This suggests that crotamine acts on the Na^{+} channel of plasmatic membrane of skeletal muscle, thus inducing the increase of this cation (Pellegrini Filho et al., 1978; Tsai et al., 1981). The alterations of Na^{+} current were measured by loose patch clamp technique in frog skeletal muscle (Matavel et al., 1998). Other activities ascribed to crotamine are increase of the basal release of acetylcholine and dopamine in rat

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MYX_CRODR       YKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKGSG--- 42
MYX4_CRODU      YKQCHKKGGHCFPKEKICIPPSSDFGKMDCRWRWKCCKKRSGK-- 43
MYX1_CRODU      YKRCHIKGGHCFPKEKICIPPSSDFGKMDCPWRRKCCKKGSGK-- 43
CXRSMT          YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG--- 42
MYX1_CROVV      YKQCHKKGGHCFPKEKICIPPSSDLGKMDCRWKWKCCKKGSG--- 42
MYX_CROAD       YKRCHKKGGHCFPKTVICLPPSSDFGKMDCRWRWKCCKKGSVNNA 45
MYXA_CROAD      YKRCHKKGGHCFPKTVICLPPSSDFGKMDC--RWKCCKKGSVNNA 43
MYXC_CROVH      YKRCHKKGGHCFPKTVICLPPSSDFGKMDCRWKWKC       CKKGSVN-- 43
MYX1_CROVC      YKRCHKKEGHCFPKTVICLPPSSDFGKMDCRWKWKCCKKGSVN-- 43
MYX2_CROVC      YKRCHKKGGHCFPKEKICTPPSSDFGKMDCRWKWKCCKKGSVN-- 43
                **:** * ******  ** *****:*****  : ***** *
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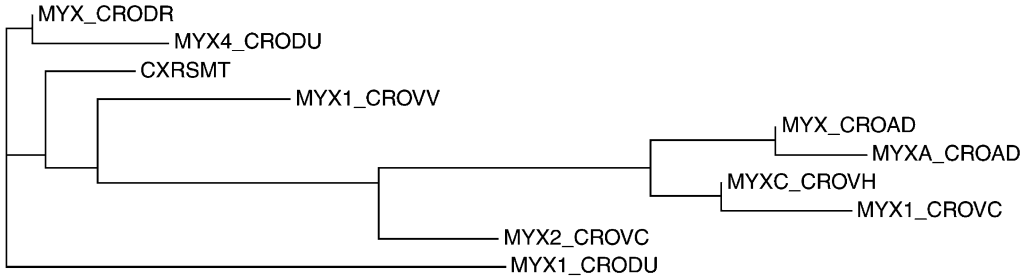

Fig. 1. Sequence alignment and evolutionary relationship of SBPM from North and South American rattlesnakes. Sequences were retrieved from protein data base at NCBI (http://www.ncbi.nlm.nih.gov) and multialigned with the program CLUSTAL W version 1.82 using default parameters from EBI sequence analysis toolbox (http://www.ebi.ac.uk/Tools/sequence.html). The Phylogram, an estimate of a phylogeny, where the branch lengths are proportional to the amount of inferred evolutionary change, was automatically generated after the alignment.

Sequences		Sources	Accession numbers
MYX_CRODR	Crotamine-Ile19	*C. d. ruruima*	gi|54036160|sp|P63327|
		C. d. terrificus	gi|52783150|sp|Q9PWF3|
CXRSMT	Crotamine	*C. d. terrificus*	gi|69518|pir|
		C. d. terrificus	gi|52783104|sp|O73799|
MYX4_CRODU	Myotoxin 4 precursor	*C. d. terrificus*	gi|127783|sp|P24334|
MYX1_CRODU	Myotoxin 1 precursor	*C. d. terrificus*	gi|127776|sp|P24331|
MYX1_CROVV	Myotoxin *a*	*C. v. viridis*	gi|127778|sp|P01476|
MYX_CROAD	CAM-toxin	*C. adamanteus*	gi|127786|sp|P24330|
MYXA_CROAD	Myotoxin	*C. adamanteus*	gi|228256|prf||1801364A
MYXC_CROVH	Toxic peptide C	*C. v. helleri*	gi|127785|sp|P01477|
MYX1_CROVC	Myotoxin I	*C. v. concolor*	gi|127777|sp|P12028|
MYX2_CROVC	Myotoxin II	*C. v. concolor*	gi|127780|sp|P12029

striatal tissues (Camillo et al., 2001) and increase of insulin secretion by pancreatic islets (Toyama et al., 2000). It is possible that these effects could be related to the interaction of crotamine with Na^+ channels.

The LD_{50} values of crotamine reported for mice reveal considerable disparity with a mean of 6,9 mg/kg, varying from 0.07 to 32.76 mg/kg (Moussatché et al., 1956; Cheymol et al., 1971; Giglio, 1975; Habermann and

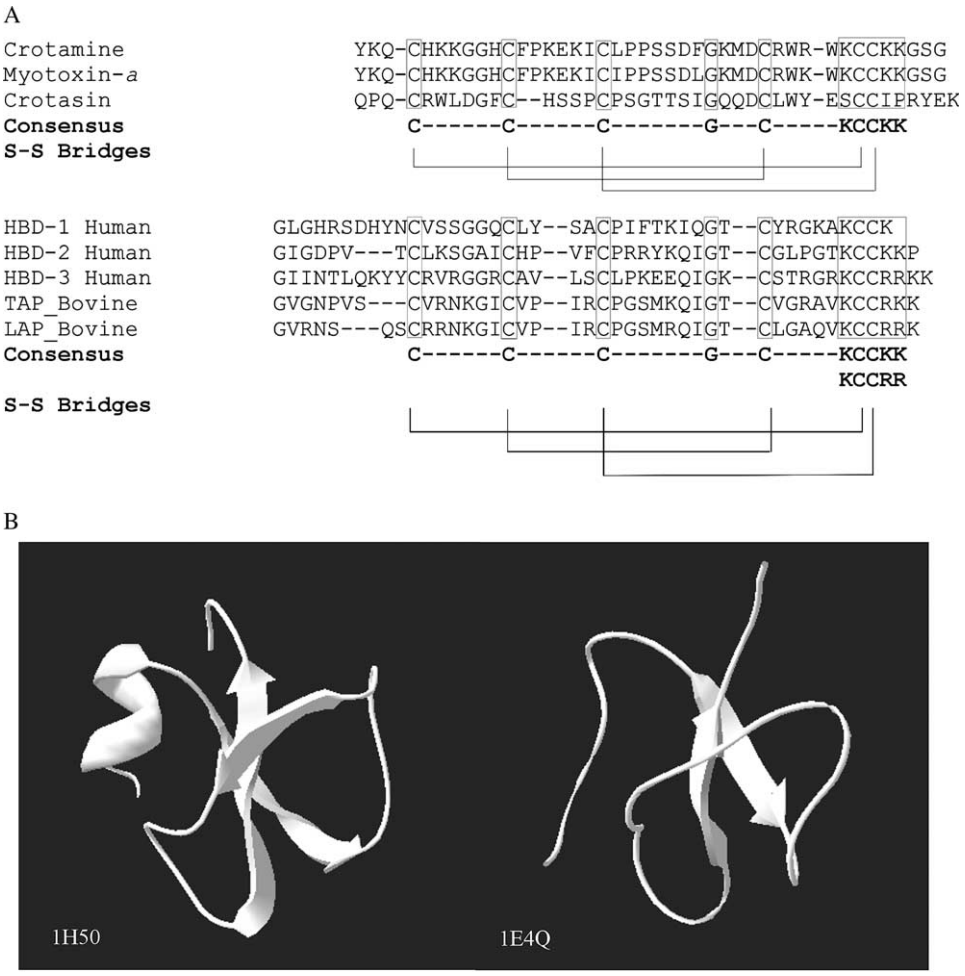

Fig. 2. Consensual scaffold of crotamine and myotoxin *a* with some mammalian defensin, and the tridimensional comparison of crotamine and human defensin 2. Disulfide bonds are represented by pairing lines (1–5, 2–4, 3–6). Positively charged –COOH terminal amino acids are also depicted. HBD 1–3, stands for human β-defensin 1, 2, and 3 (GenBank accession number, Q09753, 0152263, NP_061131); TAP, bovine tracheal anti-microbial peptides (P25068); LAP, bovine lingual anti-microbial peptide (Q28880) (as depicted in Rádis-Baptista et al., 2004). Tridimensional structures, crotamine (1H50) and human β-defensin 2 (1E4Q), were accessed by means of the structure explorer engine from the Protein Data Bank of The Research Collaboratory for Structural Bioinformatics (RCSB) (http://www.rcsb.org/pdb).

Cheng-Raude, 1975; Mebs et al., 1978; Vital-Brazil et al., 1979; Mancin et al., 1998; Boni-Mitake et al., 2001). Such differences could be attributed to the method used in determining LD_{50} values, such as type of mice, local of inoculation, solvent of toxin or the injection volume and crotamine purity.

In addition to most of the data regarding crotamine activity on ion channel, the remarkable finding of ground-breaking biological activity appears on the recent work of Kerkis et al. (2004). Using mouse embryonic stem cells and fluorescent-labelled crotamine, they reported that crotamine in micro molar concentrations penetrate into different cell types and in mouse blastocysts in vitro and in vivo. In both situations, crotamine strongly labels proliferating cells as evidenced by fluorescent confocal laser-scanning microscopy. They observed nuclear localization of crotamine in both fixed and unfixed cells and it was inferred that the capacity of the nuclear translocation of crotamine could be on account of two putative Nuclear Localization Signal (NLS) motifs existent in this toxin (amino acids 2–18 and 27–39). In the nucleus, this SBPM binds to the metaphase chromosomes. Crotamine specifically associates with centrosomes, when it is enabled to follow the process of centriole duplication and separation. The selectiveness for actively proliferating living cells, in vitro and in vivo, renders crotamine the characteristic of being a specific marker of these cells. The positive charge and the nuclear translocation capacity qualify the crotamine as a delivery system. This property has been rationally explored by Kerkis' group (personal communication) and it seems this

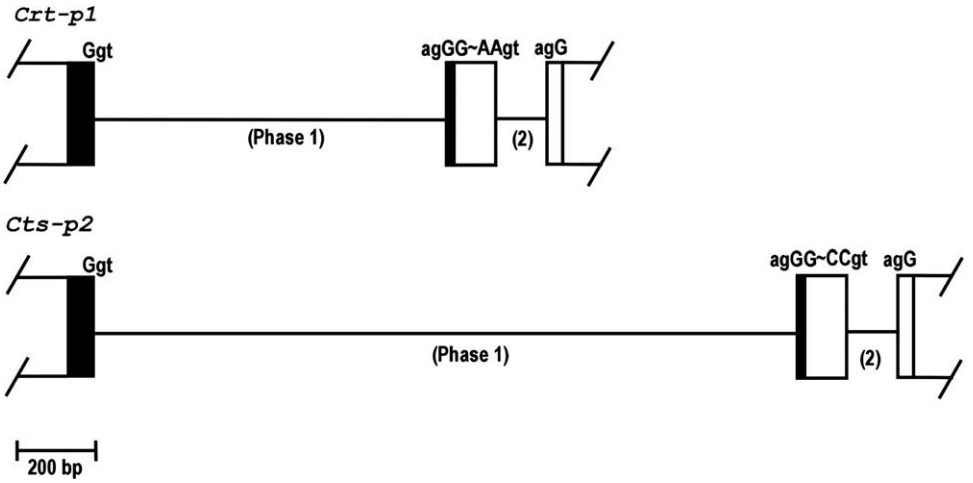

Fig. 3. Schematic structural organization of crotamine (*Crt-p1*) and crotasin (*Cts-p2*) genes from *C. durissus terrificus.* Boxes indicate the exons and lines the introns. The black boxes depict the nucleotide sequences that correspond to the signal peptide in both genes. Open boxes indicate the exons encompassing the sequences of mature toxins. The exon/intron junctions are indicated, as are the intron phases. The bar represents 200 base pairs.

activity is related to the small, condensed size, and positive charge of the crotamine.

5. Molecular genetics

The first sequences of crotamine cDNA were described by Smith and Schmidt (1990) and they demonstrated that the mRNAs encode a pre-crotamine of 65 amino acids (aa) which are comprised of a 22 aa signal sequence, a mature crotamine of 42 aa residues, and a carboxyl-terminal lysine residue, which is removed during post-translational modification of crotamine. The cDNA sequence of myotoxin *a*, described by Norris et al. (1997), showed a structure identical to crotamine cDNA. Rádis-Baptista et al. (1999) isolated various cDNA precursors from the venom gland library of a single *C. d. terrificus*, some coding for crotamine (Laure, 1975) and others for the crotamine-Ile 19 isoform (Santos et al., 1993).

Our group isolated and characterized the crotamine gene, *Crt-p1* (Rádis-Baptista et al., 2003). *Crt-p1*, with 1.8 kbp, which were organized into three exons separated by a long phase-1 (900 bp) and a short phase-2 (140 bp) introns (Fig. 3). Phase-1 interrupts the codon after the first nucleotide and phase-2 after the second one. Exon 1 includes the 5′-UTR and codes for the first 19 aa of the signal peptide. Exon 2 codifies 42 aa, three that belong to the signal peptide and 39 to the mature crotamine. Exon 3 codifies the last three amino acids of the mature toxin, the terminal lysine and the 3′-UTR. The crotamine gene was mapped to the end of the long arm of chromosome 2 and the intensity of signals contrasting between the two homologues. This result suggests the presence of a different number of gene copies that could explain the variation of crotamine quantity in the individual venoms of *C. d. terrificus* (Gonçalves and Arantes, 1956; Oguiura et al., 2000).

With the purpose of investigating a probable pseudogene corresponding to *Crt-p1* in crotamine-negative *C. d. terrificus*, Rádis-Baptista et al. (2004) identified a paralogous gene of crotamine, named crotasin, *Cts-p2*. This gene is found in both crotamine-positive and -negative rattlesnakes and codes for a peptide that might assume a β-defensin scaffold, as crotamine. This gene is circa of 2.5 kb and presents the same overall crotamine gene organization, that is, three exons separated by a long phase-1 and a short phase-2 introns, schematized in Fig. 3. The crotasin gene is 0.7 kbp longer than the crotamine gene. The difference in gene size is due to the length of intron I. Such gene organization was also observed in other small toxins, as for example the α-neurotoxins genes from Elapidae snakes that are organized in three exons and two introns with size differences mainly in intron I (Fujimi et al., 2003). The comparison of nucleotide sequences of crotamine and crotasin genes showed that the exon I, introns and UTRs are more conserved than the exon II. These data are similar to that found in other toxins that accumulate mutations in coding regions, mainly exon II (Fujimi et al., 2003). The crotasin gene is transcribed in several rattlesnake tissues, mainly in pancreas, but it is barely detected in venom glands. The physiological function of crotasin peptide in snake tissues and the pattern of expression of crotasin in other *Crotalus* species have not been determined up to the present moment.

Considering the absence of crotamine toxin in the venom glands of *C. d. terrificus* crotamine-negative, it has been argued that the absence of crotamine in the venom is due to the absence of the specific crotamine gene itself.

The presence of paralogous genes in the genome of two populations of *C. d. terrificus* gives rise to the question of how ancient the members of SBPM are — a toxin family thought to exist only in the venoms of *Crotalus* snakes. Regarding this, Bober et al. (1988) detected toxins antigenically related to myotoxin *a* in some venoms of Sistrurus snakes. Consequently, one can infer that SBPM evolved from a common ancestral gene that was present in the genome of a Crotalinae snakes before the division of the genera Crotalus and Sistrurus. Furthermore, the genes for members of this peptide family segregated and duplicated, giving rise to paralogous genes, and their advantageous gene structure was maintained to permit evolution of polymorphic peptides with subtle diversified biological functions.

6. Concluding remarks

Crotamine can be grouped by its scaffold and function. This SBPM, such as scorpion α-toxins and sea anemone toxins, changes the inactivation process of Na^+ channels that is inhibited by tetrodotoxin and potencialized by veratridine and grayanotoxin. Crotamine and scorpion toxin acting on sodium channel have scaffold in common with β-defensins. As the half-cystines are the only amino acids conserved in β-defensins family, it appears that the fold is more important than the amino acid sequence. The disulfide bonds permit exposition of some amino acids that are important for interaction with the target and for preservation of the tertiary structure. The amino acid residues that appear to be essential to function in β-defensins are lysines and arginines at the –COOH terminal of peptide and anionic residues at positions 15, 24 and 29 (Torres and Kuchel, 2004). These residues are observed in crotamine and are conserved in the SBPM family. The specificity concerning muscle membranes could be determined by the cationic region at residues 2–10 (+ 00+++00+) followed by hydrophobic site, a motif that also has myotoxic activity (Kini and Iwanaga, 1986) present in PLA_2 toxins. The 3D structure adopted by crotamine, its small size and its amino acid sequence bestow this peptide a structural configuration and surface charge distribution that reverberate in the manner it interacts with biological membranes. In addition, these structural characteristics confer to crotamine a unique penetrating property, that is, a cell penetration ability to localize and concentrate in the nucleus by means of a probable receptor-independent mechanism. In fact, structural topology resemblances and diversification of functional folds are common themes in animal venom peptides acting on ion channels as well as on other targets (Ménez, 1998; Mouhat et al., 2004).

The crotamine origin is still unknown, nevertheless the identification of crotasin gene, with similar structural gene organization, coding for polypeptides with the same three disulfide bridges arrangement but unrelated function, strengthen the relationship of crotamine with endogenous and non toxic protein, and could be another example of scaffold maintenance and diversification of biological function.

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