

ORIGINAL ARTICLE

In vitro antibacterial and hemolytic activities of crotamine, a small basic myotoxin from rattlesnake *Crotalus durissus*

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Crotamine, a myotoxin from the venom of South American rattlesnake, is structurally related to β -defensins, antimicrobial peptides (AMPs) found in vertebrate animals. Here, we tested the antibacterial properties of crotamine and found that it killed several strains of *Escherichia coli*, with the MICs ranging from 25 to 100 $\mu\text{g ml}^{-1}$. Time-kill and bacterial membrane permeabilization assays revealed that killing of bacteria by crotamine occurred within 1 h and reached the maximum by 2 h. Additionally, the anti-*E. coli* activity of crotamine was completely abolished with 12.5 mM NaCl. Furthermore, the three intramolecular disulfide bonds of crotamine appeared dispensable for its antibacterial activity. The reduced form of crotamine was active against *E. coli* as well. However, crotamine showed no or weak activity up to 200 $\mu\text{g ml}^{-1}$ against other species of Gram-negative and Gram-positive bacteria. Crotamine showed no appreciable hemolytic activity to erythrocytes. Our studies revealed that crotamine is also an AMP that kills bacteria through membrane permeabilization. However, crotamine appears to have a narrow antibacterial spectrum, distinct from many classical β -defensins, reinforcing the notion that crotamine originated from the β -defensin gene lineage, but has undergone significant functional diversification.

The Journal of Antibiotics (2011) 64, 327–331; doi:10.1038/ja.2011.10; published online 9 March 2011

Keywords: antimicrobial peptides; crotamine; β -defensin; snake venom; toxin

INTRODUCTION

Antimicrobial peptides (AMPs) constitute an important, phylogenetically conserved component of innate immunity in plants and animals.^{1,2} All AMPs share common features, such as small size (12–100 amino-acid residues), net positive charge and amphipathic structure. On the basis of structural similarities, AMPs can be broadly classified into two groups, that is, linear and cyclic peptides. Linear AMPs consist mainly of the peptides with an amphipathic α -helical structure or a flexible structure with a high proportion of certain amino-acid residues, whereas cyclic AMPs include primarily the peptides containing one or more disulfide bridges with a loop or β -sheet structure.^{1,2} Besides constituting the majority of cyclic peptides, defensins comprise cysteine-rich α - and β -defensins in vertebrates, two major groups that differ in the spacing and disulfide bonding pattern of six cysteines.^{3,4}

Besides a plethora of immunomodulatory activities, all defensins possess direct antimicrobial activities against a broad range of Gram-negative and Gram-positive bacteria, including antibiotic-resistant strains.^{3,4} Extensive studies on the mechanism of the antibacterial action of defensins have revealed that electrostatic interactions occur initially between the cationic peptides and the negatively charged bacterial membrane components, followed by peptide insertion,

channel formation and disruption of membranes.⁵ Because of the presence of cholesterol and mostly zwitterionic phospholipids on eukaryotic membranes, AMPs are generally much less lytic to eukaryotic cells, with the antibacterial concentrations being mostly 10- to 100-fold lower than cytotoxic concentrations.

Accumulating evidence indicated that defensins are phylogenetically conserved across a large spectrum of animal species. Polypeptides similar to β -defensins have been reported in the venoms of sea anemones, snakes and platypus, in which they display numerous pharmacological effects, including ion-channel inhibition and myonecrosis.⁶ Crotamine, a major myotoxin from the rattlesnake venom, is a 42-amino-acid peptide (4.9 kDa, pI 9.5).⁷ Similar to β -defensins in mammals and birds, crotamine consists of six conserved cysteine residues forming the same pattern of disulfide bonds (cys1–cys5, cys2–cys4 and cys3–cys6) with a net positive charge of +8, despite a low sequence homology (Figure 1). More importantly, crotamine is also structurally related to mammalian β -defensins by forming 2–3 stranded antiparallel β -sheets with or without an α -helix at the amino-terminus.^{6,8}

Because of structural similarity with β -defensins, it is conceivable that crotamine might function as an AMP, in addition to being a myotoxin. Consistent with this prediction, three crotalic venoms were

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Received 4 August 2010; revised 14 January 2011; accepted 22 January 2011; published online 9 March 2011

Table 1 MIC of native and reduced crotamine^a

Bacteria	ATCC no.	MIC ($\mu\text{g ml}^{-1}$)	
		Crotamine	Reduced crotamine
Gram-negative			
<i>Escherichia coli</i> O157:H7	700728	25	25
<i>E. coli</i> ML-35p		50	25
<i>E. coli</i>	25922	100	50
<i>Pseudomonas aeruginosa</i>	27853	>200	NT
<i>Salmonella typhimurium</i>	14028	>200	NT
Gram-positive			
<i>Staphylococcus aureus</i>	25923	>200	>200
<i>Listeria monocytogenes</i>	19115	>200	NT

Abbreviation: NT, not tested.

^aThe MIC values were determined by a modified microbroth dilution assay¹³ using native or reduced crotamine.

spectrum peptide antibiotic with preferences toward certain bacterial species.

To further study the kinetics of bacterial killing, $25 \mu\text{g ml}^{-1}$ of crotamine (or $1 \times \text{MIC}$ concentration) was incubated with *E. coli* ATCC 25922 for various times, and surviving bacteria were counted by serial plating, as described.¹³ Crotamine showed a modest 20% killing at 30 min following exposure to *E. coli* (Figure 2a). Approximately a 1-log reduction was observed 60 min upon exposure, with a dramatic 3-log reduction of *E. coli* occurring at 2 h (Figure 2a). Although crotamine is unable to completely suppress the growth of *S. aureus* ATCC 25923 after overnight incubation even at $200 \mu\text{g ml}^{-1}$ (Table 1), there was an obvious 1-log reduction in the bacterial counts following 2 h of exposure to $100 \mu\text{g ml}^{-1}$ of crotamine (Figure 2b). However, it is noted that killing of *S. aureus* by crotamine is slower than that of *E. coli* and did not proceed until 60 min. Furthermore, reminiscent of defensins,^{3,4} the antibacterial activity of crotamine was sensitive to salt, as inclusion of as low as 12.5 mM of NaCl failed to inhibit the growth of *E. coli* in a modified microbroth dilution assay (data not shown).

To examine the mechanism of action and confirm the bacterial-killing kinetics, $50 \mu\text{g ml}^{-1}$ (or $1 \times \text{MIC}$ concentration) of crotamine was incubated with *E. coli* ML-35p, which constitutively expresses cytosolic β -galactosidase, in the presence of a chromogenic substrate. The medium color change, proportional to the degree of permeabilization of bacterial cytosolic membranes due to the release of β -galactosidase, was monitored at $A_{420\text{nm}}$ every 2 min for up to 2 h. As compared with the control, no obvious membrane lysis occurred until 50–60 min following addition of crotamine to bacteria, and the membrane lysis became highly prevalent at 2 h (Figure 3), consistent with the earlier time-kill assay (Figure 2a). The results indicated that, like defensins, crotamine kills bacteria primarily through membrane permeabilization.

Impact of the disulfide bonds on the antibacterial properties of crotamine

To test the impact of the intramolecular disulfide bonds on the antibacterial activity, native crotamine was reduced with dithiothreitol. As shown in Table 1, reduced crotamine showed slightly enhanced antibacterial activity toward three *E. coli* strains, relative to the native crotamine. The time-kill assay further revealed that $25 \mu\text{g ml}^{-1}$ reduced crotamine gave a 1-log reduction in *E. coli* O157:H7 counts at as early as 15 min, and a nearly complete elimination of bacteria occurred at 60 min following incubation, with the bacterial counts

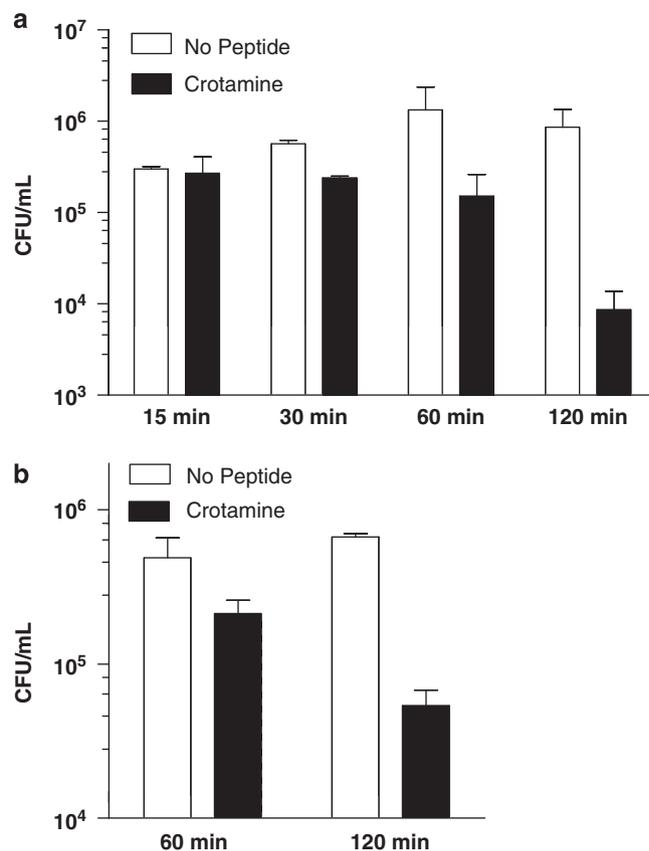


Figure 2 Kinetics of bacterial killing by crotamine. *E. coli* O157:H7 (a) or *S. aureus* ATCC 25923 (b) at $5 \times 10^5 \text{ CFU ml}^{-1}$ was incubated with or without $25 \mu\text{g ml}^{-1}$ (a) or $100 \mu\text{g ml}^{-1}$ (b) of crotamine for different times at 37°C , followed by bacterial enumeration. The results represent means \pm s.e.m. of three independent experiments.

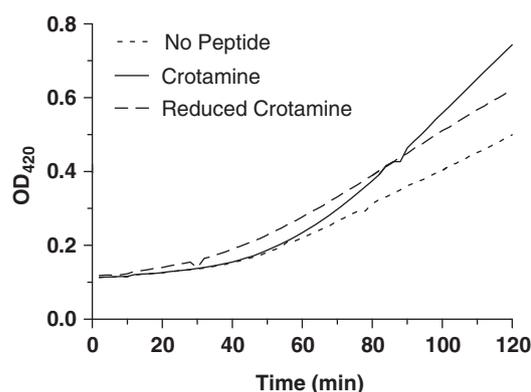


Figure 3 Inner membrane permeabilization capacity of crotamine. *E. coli* ML35p, which constitutively expresses β -galactosidase in the cytosol, was incubated with $50 \mu\text{g ml}^{-1}$ of native or reduced crotamine. Permeabilization of bacterial inner membranes as indicated by the release of cytosolic β -galactosidase to the cell culture medium was measured at $\text{OD}_{420\text{nm}}$ in the presence of a chromogenic substrate, *o*-nitrophenyl- β -D-galactopyranoside every 2 min for up to 2 h. The results represent means \pm s.e.m. of two independent experiments.

being below the limit of detection, which is 200 CFU ml^{-1} (Figure 4). This bacterial-killing kinetics of reduced crotamine was further confirmed by the inner membrane permeabilization assay. Enhanced release of β -galactosidase and therefore cytoplasmic membranes

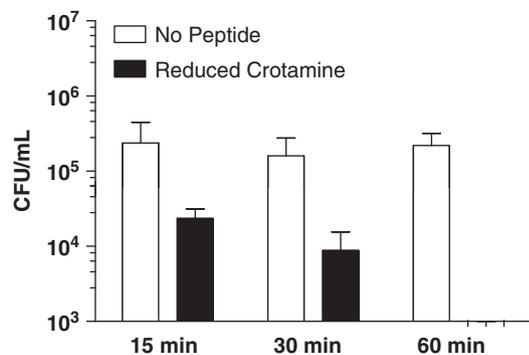


Figure 4 Kinetics of bacterial killing by reduced crotamine. *E. coli* O157:H7 at 5×10^5 CFU ml⁻¹ was incubated with or without 25 μ g ml⁻¹ of reduced crotamine for different times at 37 °C, followed by bacterial enumeration. The results represent means \pm s.e.m. of three independent experiments.

occurred 10 min following bacterial exposure to reduced crotamine (Figure 3). Furthermore, reduced crotamine showed slightly enhanced resistance to salt, as the anti-*E. coli* activity was not completely inhibited until the final NaCl concentration reached 50 mM, as opposed to 12.5 mM with native crotamine (data not shown).

Collectively, our results clearly indicated that the presence of intramolecular disulfide bonds is dispensable for the antibacterial activity of crotamine and even adversely affects its efficacy in membrane disruption. This may occur because of possible oligomerization of native crotamine,¹⁶ thereby reducing its effective concentration and efficacy in membrane disruption. However, SDS-PAGE under non-reducing conditions showed only one band in native crotamine, indicating no obvious aggregation in our hands (data not shown). Therefore, the enhanced activity of reduced crotamine might be explained by its altered structure, which may facilitate the interactions of the peptide with bacterial membranes.

Hemolytic activity of native and reduced crotamine

To further test their capacity to permeabilize mammalian cell membranes, both native and reduced forms of crotamine were tested for their hemolytic activity toward mouse erythrocytes with and without fetal bovine serum. No appreciable hemolysis was observed with native crotamine up to 1024 μ g ml⁻¹, the highest concentration tested (Figure 5), which is consistent with the cytotoxicities of defensins, regardless of serum. Only reduced crotamine presented a dose-dependent hemolytic activity in the presence of 10% fetal bovine serum, showing 35% hemolysis at 1024 μ g ml⁻¹ (Figure 5). It was consistent with earlier antibacterial assays that found that an increased membrane-lytic efficacy was associated with the reduced form of crotamine. Surprisingly, reduced crotamine exhibited virtually no hemolysis to erythrocytes in the absence of serum, implying that certain serum proteins might facilitate the interactions of reduced crotamine with membranes. This indirect hemolytic activity was observed in many snake venoms¹⁷ that also were not able to hemolysate in the absence of serum. Cadillo *et al.*¹⁸ and Jeng *et al.*¹⁹ suggested crotoxin, a phospholipase A₂ from rattlesnake venom, as the toxin responsible for this indirect activity. Crotamine shares with crotoxin a cationic region (+00+++00+) responsible for myotoxicity.²⁰

DISCUSSION

Because of the conservation of the cysteine-spacing pattern and spatial structure, it is speculated that crotamine, a well-known rattlesnake

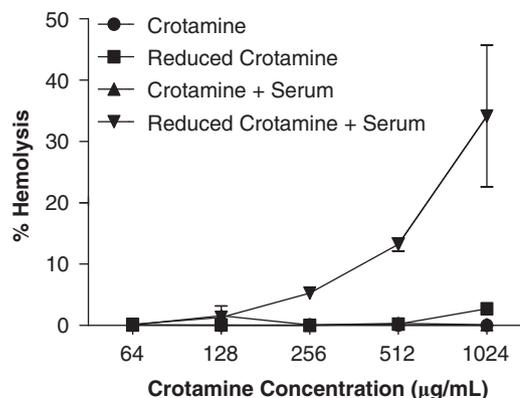


Figure 5 Hemolysis assay. Hemolytic activity of native or reduced crotamine with or without 10% serum (fetal bovine serum). The lysis of erythrocytes was monitored for the release of hemoglobin at A_{405 nm}, following incubation of blood with serially diluted crotamine.

venom myotoxin, may resemble vertebrate β -defensins by also acting as an AMP.²¹ Besides the modest anti-leishmanial activity seen with crotoxic venoms,⁹ no definitive evidence on the antibacterial activity of crotamine has been shown before the initiation of the present study. Following our comprehensive testing on the antibacterial and cytotoxic properties, we now can unequivocally conclude that crotamine is also a β -defensin-like AMP with a salt-sensitive antibacterial activity. To strengthen our conclusion, an independent study also demonstrated the antibacterial activity of crotamine²² during the preparation of this manuscript.

Similar to β -defensins, crotamine kills bacteria through membrane permeabilization that is independent of the integrity of disulfide bonding. In contrast, reduced crotamine even demonstrated an enhanced capacity in disruption of bacterial membranes (Figure 3). It is possible that the three intramolecular disulfide bridges of crotamine may prove beneficial for other biological functions such as myotoxicity and *in vivo* stability. Indeed, the lethality of unfolded crotamine was shown to be reduced by approximately 50%.¹²

Interestingly, crotamine appears to be active against only a narrow spectrum of bacteria, in contrast with most vertebrate β -defensins that are broadly active. This is perhaps not surprising, given a great degree of diversity in the primary amino-acid sequence between crotamine and β -defensins (Figure 1). In our study, among five Gram-negative and two Gram-positive bacterial strains that we tested, crotamine showed an obvious bactericidal activity only against *E. coli*, with a weak or no activity against all others in the MIC assay, although the time-kill assay revealed a weak activity against *S. aureus*. Consistent with our results, Yount *et al.*²² reported the antibacterial activity of crotamine against *E. coli*, but not *S. aureus*, using a radial diffusion method. In the same study, the activity against a Gram-positive (*Bacillus subtilis*) and fungal (*Candida albicans*) pathogen was also observed with crotamine.

The physiological significance of the narrow antimicrobial activity of crotamine remains to be studied, given the presence of a wide range of bacterial species including *Pseudomonas*, *Salmonella*, *Aerobacter*, *Bacillus*, *Citrobacter*, *Clostridium*, *Enterobacter* and *Streptococcus* in the mouth, fangs and/or venom of *Bothrops jararaca* and North and South American rattlesnakes.^{23,24,25,26} It is tempting to speculate that crotamine and isoforms²⁷ or paralogs such as crotasin²⁸ may have a complementary antimicrobial spectrum.

We revealed in this study that crotamine possesses antimicrobial activities. Reciprocally, it is interesting to see whether β -defensins also

behave like a myotoxin through activation of voltage-sensitive ion channels. Indeed, similar to crotamine, a β -defensin was shown to interact with K^+ channels, albeit with a different specificity.²² Although hBD-2, a human β -defensin, interacted with both prokaryotic and eukaryotic voltage-sensitive K^+ channels, crotamine only interacted with eukaryotic K^+ channels.²² The amino-acid sequence variation between crotamine and β -defensins may explain their difference in ion channel targeting.

Taken together, our results showing the antibacterial activity of crotamine reinforce the notion that crotamine evolved from the duplication of the β -defensin gene lineage. Coupled with its weak hemolytic activity, crotamine and other snake venom toxins may have potential for further development as therapeutics in the face of dwindling of effective antimicrobial drugs in the twenty-first century.

ACKNOWLEDGEMENTS

We are grateful to Poliana G Corrêa for her assistance with electrophoresis assays. This project was supported by a postdoctoral fellowship award (to NO) from FAPESP, S. Paulo, Brazil, and USDA CSREES grant (2008-35204-04544), Oklahoma Center for the Advancement of Science and Technology grants (HR07-113 and AR07.2-087) and Oklahoma Agricultural Experiment Station (H-2507).

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