BBA - Biomembranes 1859 (2017) 2340–2349

Contents lists available at ScienceDirect

BBA - Biomembranes

journal homepage: www.elsevier.com/locate/bbamem

Biophysical and biological properties of small linear peptides derived from crotamine, a cationic antimicrobial/antitumoral toxin with cell penetrating and cargo delivery abilities

C. Dal Mas[a,1], D.A. Pinheiro[b,1], J.D. Campeiro[a], B. Mattei[b], V. Oliveira[b], E.B. Oliveira[c], A. Miranda[b], K.R. Perez[b,*], M.A.F. Hayashi[a,**]

[a] Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, Brazil
[b] Departamento de Biofísica, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, Brazil
[c] Departamento de Bioquímica e Imunologia, Universidade de São Paulo (USP-RP), Ribeirão Preto, Brazil

ARTICLE INFO

Keywords:
Crotalus durissus terrificus
Venom
Crotamine
Linear cationic peptide
Antifungal
Snake toxin

ABSTRACT

Crotamine is a natural polypeptide from snake venom which delivers nucleic acid molecules into cells, besides having pronounced affinity for negatively charged membranes and antifungal activity. We previously demonstrated that crotamine derived short linear peptides were not very effective as antifungal, although the non-structured recombinant crotamine was overridingly more potent compared to the native structured crotamine. Aiming to identify the features necessary for the antifungal activity of crotamine, two linear short peptides, each comprising half of the total positively charged amino acid residues of the full-length crotamine were evaluated here to show that these linear peptides keep the ability to interact with lipid membrane model systems with different phospholipid compositions, even after forming complexes with DNA. Interestingly, the presence of cysteine residues in the structure of these linear peptides highly influenced the antifungal activity, which was not associated to the lipid membrane lytic activity. In addition to the importance of the positive charges, the crucial role of cysteine residues was noticed for these linear analogs of crotamine, although the tridimensional structure and lipid membrane lytic activity observed only for native crotamine was not essential for the antifungal activity. As these peptides still keep the ability to form complexes with DNA molecules with no prejudice to their ability to bind to lipid membranes, they may be potentially advantageous as membrane translocation vector, as they do not show lipid membrane lytic activity and may harbor or not antifungal activity, by keeping or not the semi-essential amino acid cysteine in their sequence.

1. Introduction

Nature is a generous source of compounds with the potential to treat diseases. Scientific research in natural products area has greatly contributed to the discovery of new compounds with several biological activities, including for the identification of compounds with antimicrobial activity [1]. Infectious diseases are one of the main causes of morbidity and mortality worldwide, and many infections caused by multi-resistant microorganisms often result in difficult to treat diseases. Novel therapeutic allies acting by alternative mechanism of action is highly desired for the treatment of such difficult to treat infections, and natural products bearing the potential to act as antimicrobial compounds by alternative mechanism of action, especially acting on lipid membranes, may strongly contributed to overcome the antimicrobial resistance threats [2,3,4,5].

Crotamine is a small cationic polypeptide originally found in the venom of the South American rattlesnake *Crotalus durissus terrificus* [6,7]. This native toxin is composed by 42 amino acid residues

Abbreviations: CPPs, cell-penetrating peptides; Fmoc, *N*-9-fluorenylmethoxycarbonyl; PTDs, protein translocation domains; ATCC, American Type Culture Collection; IOC, Oswaldo Cruz Institute Collection; MIC, minimal inhibitory concentration; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; CC_{50}, 50% cytotoxic concentration; LUVs, large unilamellar vesicles; CF, carboxyfluorescein; CD, circular dichroism; POPG, phosphatidylglycerol; POPC, phosphatidylcholine; AMPs, antimicrobial peptides; NTA, Nanoparticle Tracking Analysis; DLS, dynamic light scattering; DTT, dithiothreitol

* Correspondence to: K.R. Perez, Departamento de Biofísica, Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil.
** Correspondence to: M.A.F. Hayashi, Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP), Rua 3 de maio 100, Ed. INFAR, 3rd floor, CEP 04044-020 São Paulo, Brazil.
E-mail addresses: kdaghastanli@unifesp.br (K.R. Perez), mhayashi@unifesp.br (M.A.F. Hayashi).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbamem.2017.09.006
Received 21 February 2017; Received in revised form 15 August 2017; Accepted 5 September 2017
Available online 06 September 2017
0005-2736/

[YKQCHKKGGHCFPKEKICLPPSSDFGKM DCRWRWKCCKKGSG], among which 11 are basic residues (e.g. Arg or Lys), which together provide to this molecule a positive liquid net charge with a pI of about 9.5 [8,9]. In addition, this polypeptide also presents three disulfide bonds [Cys^{4}-Cys^{36}, Cys^{11}-Cys^{30} and Cys^{18}-Cys^{37}] that confer a structural stability and guarantee the exposure of all basic residues side chain on the surface of the native structured crotamine [8,9]. The amphipathic tridimensional structure of crotamine, which contains antiparallel beta-sheets and one single alpha-helix, is mainly warranted by the presence of two acidic and several neutral residues, in addition to the high content of basic residues, and all these residues are tied up by essential three intramolecular disulfide bonds [8,9]. Several biological functions of this polypeptide, including the antimicrobial and/or antitumoral activities, were shown to be mostly dependent on the ability of crotamine to target acidic surfaces or acidic cellular compartments, as lysosome vesicles [10,11,12,13,14]. Moreover, crotamine was also demonstrated to form complex with nucleic acidic molecules with no detriment to its selective translocation into highly proliferating cells [10,11,15]. All these properties was mainly predicted to be determined by the overall positive net charge distribution on the surface of crotamine, which was then characterized as a novel cell-penetrating polypeptide (CPP) nanocarrier with potential anticancer and biotechnological applications due to its peculiar specificity for highly proliferating cells [12,13,16]. Aiming to understand the molecular mechanism(s) of action underlying this selectivity, we conducted studies using membrane model systems (i.e., large unilamellar vesicles, LUVs, and giant unilamellar vesicles, GUVs), with different phospholipid compositions, showing that native crotamine presents a more pronounced lytic activity on negatively charged membranes compared to neutral membranes, containing or not cholesterol or ergosterol in their composition [14].

Crotamine was also suggested to be a member of the antimicrobial peptides (AMP) family, and we recently demonstrated its unique selectivity against fungi strains, in contraposition to the weak antibacterial activity against both Gram-positive or Gram-negative microorganisms [13]. In spite of the weak antibacterial activity of the native crotamine against both Gram-positive and Gram-negative bacteria, a pronounced antifungal activity against *Candida* spp., *Trichosporon* spp., and *Cryptococcus neoformans*, with remarkable no hemolytic activity against human erythrocytes, was described for this native peptide [13]. Seeking for potential medical and industrial applications for this natural peptide, the recombinant and synthetic analogs of crotamine were also produced and analyzed side-by-side showing that short linear peptides derived from native crotamine, with Ser replacing the Cys residues, were not very effective as antimicrobial, although the full-length non-structured recombinant crotamine showed overriding more potent activity than the structured native crotamine [13]. Therefore, we concluded that the significant loss of antimicrobial activity observed for these crotamine-derived short linear peptides was not determined by the lack of the tridimensional structure, since the non-structured recombinant crotamine expressed in bacteria [13], as well the linearized native crotamine reduced by DTT [17], showed both stronger antimicrobial activity compared to the native structured crotamine. Interestingly, other group also described enhanced antimicrobial activity of linear analogs of human alpha-defensin 5 [18]. Accordingly, unfolding itself was found to not suppress the antimicrobial activity of human beta-defensin-1 (hBD-1), and the hydrophobicity and also the free Cys residues located in its C-terminus were suggested to be important features for the antimicrobial activity [19].

In the present work, we evaluated the properties of two synthetic linear crotamine fragments, each comprising the half of the total positively charged amino acid residues of the full-length crotamine, on same membrane model systems and fungi strains employed in previous works [13,14]. Although similar peptides with Ser replacing the Cys residues showed no detectable antimicrobial activity [13], we showed here that these same peptides now harboring Cys residues present remarkable antifungal activity, suggesting the important role of the thiol-containing residues for the antimicrobial activity. These peptides were also demonstrated to form complexes with DNA molecules, with no prejudice to their ability to interact with lipid membranes, and with no noticeable lytic activity against lipid membranes, regardless of the phospholipid composition or total net charge on surface.

2. Materials and methods

The lipids POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 5(6)-carboxyfluorescein (CF) from Sigma-Aldrich (St Louis, MO, USA) was purified as originally described by Ralston [20]. Other chemicals and solvents were all purchased from Sigma (Deisenhofen, Germany or St. Louis, MO, USA). pCAG.GFP (Addgene plasmid # 11150) was a kind gift from Prof. Alysson Muotri (San Diego, CA, USA) [21].

2.1. Purification of native crotamine from snake venom

The crude venom of *Crotalus durissus terrificus* was extracted from rattlesnakes kept in the serpentarium of the Faculdade de Medicina de Ribeirão Preto, São Paulo University-Ribeirão Preto (USP-RP). The crotamine was prepared and purified according to the procedure described by Hayashi et al. [12], under authorization of access to genetic resources No. 010426/2010 COAPG/DABS/CNPq (term of concession No. 20100104268).

2.2. Synthesis and purification of crotamine fragments

Peptides were synthesized essentially as previously described by Yamane et al. [13]. Briefly, the synthesis of peptides with the original sequence of native crotamine, i.e. keeping the Cys residues C1 ($Crot_{2–18}$ [KQ**C**HKKGGH**C**FPKEKI**C**]) and C2 ($Crot_{27–39}$ [KMD**C**RWRWK**CC**KK]), and also the peptides with the modified sequence in which the Cys residues were replaced by Ser, namely P1 ($Crot_{2–18}$ [KQ**S**HKKGGH**S**FPKEKI**S**]) and P2 ($Crot_{27–39}$ [KMD**S**RWRWK**SS**KK]) (Fig. 1), were carried on using an automated PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan) by a stepwise solid-phase method using *N*-9-fluorenylmethoxycarbonyl (Fmoc) chemistry (Novabiochem-EMD Chemicals Inc., San Diego, CA, USA).

Crotamine	YKQCHKKGGHCFPKEKICLPPSSDFGKMDCRWRWKCCKKGSG
C1 (Present work)	KQCHKKGGHCFPKEKIC
P1 - Yamane et al. [13]	KQSHKKGGHSFPKEKIS
C2 (Present work)	KMDCRWRWKCCKK
P2 - Yamane et al. [13]	KMDSRWRWKSSKK

Fig. 1. Sequences alignment of crotamine and its derived linear fragments studied in the present work (C1 and C2) and described in Yamane et al. [13] (P1 and P2). The sequences of peptides are aligned under the primary sequence of crotamine. Cysteine residues in crotamine and also in C1 and C2 peptides are underlined, as well as the serine residues in P1 and P2 peptides.