Biomaterials 35 (2014) 8028–8039

Contents lists available at ScienceDirect

Biomaterials

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

Antimicrobial potency and selectivity of simplified symmetric-end peptides

Na Dong [a], Xin Zhu [a], Shuli Chou [a], Anshan Shan [a, *], Weizhong Li [a], Junguang Jiang [b]

[a] Laboratory of Molecular Nutrition and Immunity, The Institute of Animal Nutrition, Northeast Agricultural University, Harbin, PR China
[b] The State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, PR China

ARTICLE INFO

Article history:
Received 14 March 2014
Accepted 1 June 2014
Available online 19 June 2014

Keywords:
Symmetric-end peptides
Cell selectivity
Membrane
Bactericidal mechanism
Hemolysis

ABSTRACT

Because antimicrobial peptides (AMPs) are potentially useful for the treatment of multidrug-resistant infections, more attention is being paid to the structural modification and structure–function relationship of both naturally occurring and synthetic AMPs. Previous studies indicated that Protegrin-1 (PG-1), isolated from porcine leukocytes, exhibited considerable antimicrobial activity and cytotoxicity. The β-turn of PG-1 floated on the surface of bacterial membrane, while its β-strand inserted into the bacterial membrane and formed pores that were dedicated to producing cytotoxicity. For reducing cytotoxicity and improving cells selectivity, we designed a series of simplified symmetric-end peptides by combining the β-turn of PG-1 with simple amino acid repeat sequences. The sequence of designed symmetric-end peptides is $(XR)_nH(RX)_n$, ($n = 1,2$; X represents I, F, W and P; H represents CRRRFC). The symmetric-end peptides displayed antimicrobial activity against both gram-positive and gram-negative bacteria. In particular, $(XR)_2H(RX)_2$ (X here is I, F and W) showed greater antimicrobial potency than PG-1. Hemolysis activity and cytotoxicity, detected by using human red blood cells (RBCs) and human embryonic lung fibroblasts MRC-5 cells, were observably lower than the native peptide PG-1. $(IR)_2H(RI)_2$ (IR2), folded into β-sheet structures, displayed the highest therapeutic index, suggesting its great cell selectivity. The fluorescence spectroscopy, flow cytometry, and electron microscopy observation indicated that IR2 exhibited great membrane penetration potential by inducing membrane blebbing, disruption and lysis. Collectively, generating symmetric-end β-sheet peptides is a promising strategy for designing effective AMPs with great antimicrobial activities and cell selectivity.

1. Introduction

As multidrug-resistant bacteria, expecially 'superbug', emerge, antimicrobial peptides (AMPs) have been acknowledged as promising therapeutic alternatives to conventional antibiotics [1,2]. Although the precise mode of their action is not fully understood, most AMPs inhibit microbial growth by physical penetration to form pores or other types of lytic effect, thereby allowing the efflux of essential ions and nutrients [3–5], which is metabolically too costly for most microbes to promote resistance through mutation or through repair of membrane components [6]. Therefore, based on the above method of action, peptide-based biomaterials may aid in a wide range of bioengineering and biomedical applications, including regenerative medicines, biomimetic materials, therapeutic delivery, and antimicrobial agents [7–10].

Currently, more than 2000 AMPs originating from natural sources have been characterized [11]. Generally, these peptides contain both cationic and hydrophobic amino acids. The cationic charge provides electrostatic interactions between peptides and the negatively charged membranes and/or cell walls of bacteria, including lipopolysaccharide (LPS) [12], whereas the hydrophobic part presumably provides lipophilic anchors that ultimately induce membrane disruption [13]. By mimicking the amphiphilicity of these natural peptides, some synthetic materials such as derivative from natural peptide [14,15], de novo designed peptides [16,17] and polymers [18–20] have been engineered. However, systemic toxicity, *in vivo* stability and production costs are challenges for the further development of natural AMPs as therapeutic drugs and peptide-based biomaterials [21]. Therefore, numerous methods have been employed to eliminate these adverse effects. In particular, work has focused on increasing the activity against the pathogen of interest and decreasing toxicity at the therapeutic dose by modification and/or optimization of known AMP sequences.

* Corresponding author. Tel.: +86 451 55190685; fax: +86 451 5510 3336.
E-mail address: asshan@neau.edu.cn (A. Shan).

http://dx.doi.org/10.1016/j.biomaterials.2014.06.005
0142-9612/

However, there have been no organizing scientific principles in the design and/or optimization of AMPs [16].

Protegrin-1 (PG-1) is a cysteine-rich β-sheet peptide isolated from porcine leukocytes [22]. This peptide possesses broad-spectrum activity against gram-positive and gram-negative bacteria, fungi and some enveloped viruses [23]. Additionally, the peptide was more toxic to eukaryotic cells. PG-1 has eighteen amino acids, and two disulfide bonds constrain the molecule to a rigid β-hairpin fold. In the current study, we designed a series of simplified symmetric-end peptides by combining the β-turn of PG-1 with specific amino acid repeat sequences. The repeat sequences are composed of different types of hydrophobic amino acids including Ile (I), Phe (F), Trp (W), Pro (P) and the hydrophilic amino acids Arg (R). The average frequencies of Ile, Phe, Trp, Pro and Arg are 6.03, 4.01, 1.53, 4.71 and 5.50%, respectively, in 2211 antimicrobial peptides (APD: http://aps.unmc.edu/AP/statistic/statistic.php). The sequence of symmetric-end peptides is (XR)nH(RX)n, (n = 1,2; X represents I, F, W and P; H represents CRRRFC). All peptides were amidated to increase their stability. The adoption of the β-strand symmetrical method may improve the selectivity of the peptide. The peptides were first characterized for their secondary conformation in aqueous solution and in a simulated membrane environment by circular dichroism (CD). The antimicrobial properties of the peptides were evaluated using MIC measurement against a broad selection of threatening microbes, including *Escherichia coli*, *Salmonella Pullorum*, *Salmonella typhimurium*, and *Bacterium pyocyaneum* (gram-negtive), and *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, and *Bacillus subtilis* (gram-positive). We also measured the antimicrobial activity in the presence of monovalent and/or multivalent ions. Hemolytic activity and cytotoxicity were determined, and the peptide with the greatest cell selectivity was identified. The peptide–membrane interactions were evaluated using fluorescent, flow cytometric, and scanning and transmission electron microscopy assays. The overall aim of this study is to facilitate the development of peptide-based synthetic strategies to generate effective AMPs with great antimicrobial activities and cell selectivity.

2. Materials and methods

2.1. Materials

All peptides were synthesized by GL Biochem (Shanghai, China), and their fidelity was then identified via matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF MS, Linear Scientific Inc., U.S.A.), using α-cyano-4-hydroxycinnamic acid as the matrix. The peptides' purity was confirmed as greater than 95% with analytical reverse-phase high-performance liquid chromatography (RP-HPLC). To prevent the degradation of the peptides, each was dissolved in DI water at a concentration of 2.56 mM and was stored at −20 °C.

The test strains *E. coli* ATCC 25922, *S. enterica serovar* Typhimurium **C77-31,** *S. Pullorum* **C79-13,** *B. pyocyaneum* ATCC 27853, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *S. faecalis* ATCC 29212, and *B. subtilis* CMCC 63501 were obtained from the School of Veterinary Medicine, Northeast Agricultural University (Harbin, China). *E. coli* UB1005 was kindly provided by Prof. Q. S. Qi (State Key Laboratory of Microbial Technology, Shandong University, China). The bacteria were incubated in Mueller-Hinton Broth (MHB) media (beef infusion solids, 5 g/l, casein hydrolyzate, 17.5 g/l, soluble starch 1.5 g/l, pH 7.0 ± 0.2), purchased from AoBoX (Shanghai, China). Red blood cells (RBCs) used in the experiments were extracted from healthy blood donors. Sodium chloride, potassium chloride, ammonium chloride, zinc chloride, magnesium chloride, and ferric chloride were all analytical grade and purchased from Kermel (China). Human embryonic lung fibroblast MRC-5 cells were purchased from the Institute of Biological and Cell Biology, SIBS, CAS. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma–Aldrich Corporation (China), and DMEM supplemented with L-glutamine and 10% fetal calf serum was obtained from Invitrogen Corporation (U. S. A.).

Sodium dodecyl sulfate (SDS) micelles were obtained from Sigma–Aldrich (China) and used after dilution to 30 mM in DI water. Ethanol (analytical grade, 99%), acetone (analytical grade, 99%), tert-butanol (analytical grade, 99%) and glutaraldehyde (synthetic grade, 50% in H_2O) were obtained from Sigma–Aldrich Corporation (China). Glucose (analytical grade) was purchased from Zhiyuan (China). Triton X-100, N-phenyl-1-napthylamine (NPN), 3,3′-Dipropylthiadicarbocyanine iodide ($diSC_3$-5), HEPES and propidium iodide (PI) were obtained from Sigma–Aldrich (China).

2.2. Peptide design and analysis

We designed a series of simplified symmetric-end peptides by combining the β-turn of PG-1 with specific amino acid repeat sequences. First, the β-turn, the middle 9–12 residues of PG-1, was adopted because it included three Arg residues. Additional Arg residues are capable of participating in cation–π interactions, thereby facilitating enhanced peptide–membrane interactions. Additionally, a precious study found that the β-turn Arg11 of PG-1 is not involved in intermolecular hydrogen bonding and is located near the membrane surface, which suggests that it has more freedom of movement. Therefore, the β-turn exposed to the membrane surface and the β-strand immersed in the hydrophobic middle of the membrane produce the membrane damage by the previously proposed β-barrels method [24]. Using the Arg-rich β-turn sequence from PG-1, the structure and characteristic membrane insertion of the β-strand become principal elements for determining the degree of membrane insert, and they affect the antimicrobial properties and cell selectivity.

We replaced the unsymmetrical β-strand region containing residues 4–8 and 13–17 of PG-1 with alternately arranged hydrophilic amino acids (R) and hydrophobic amino acids (I, F, W, and P) and designed symmetric-end peptides: $(XR)_nH(RX)_n$, (n = 1,2; X represents I, F, W and P; H represents CRRRFC). The hydrophobic amino acid Ile is an aliphatic amino acid, Phe is an aromatic amino acids, Trp is a heterocyclic amino acids, and Pro is a heterocyclic imino acid; they represent different structural type of amino acid and possess the highest hydrophobic values for their respective types (Fig. 2.). The amino acid sequences of the symmetric-end peptides are listed in Table 1.

Primary sequence analysis of the peptides was performed with the bioinformatics program ProtParam (ExPASy Proteomics Server: http://www.expasy.org/tools/protparam.html). The mean hydrophobicity and relative hydrophobic moment were calculated online using CCS scale (http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html). The secondary content of the peptides was calculated online with K2D3 (http://www.ogic.ca/projects/k2d3/). The three-dimensional structure projection was predicted online with I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The helical wheel projection was performed online using the Helical Wheel Projections: http://rzlab.ucr.edu/scripts/wheel/wheel.cgi.

Table 1
Amino acid sequences, formula, molecular weights, charge, and the hydrophobicity values of the peptides used in this study.

Peptides	Sequence	Formula	TTheoretical MW[a]	Measured MW	Retention time (min)	H[b]	μHrel[c]	Net charge
IR1	IRCRRRFCRI-NH_2	$C_{57}H_{104}N_{26}O_{10}S_2$	1377.73	1377.74	10.71	−0.56	2.15	+6
IR2	IRIRCRRRFCRIRI-NH_2	$C_{81}H_{150}N_{36}O_{14}S_2$	1916.42	1916.44	16.34	−0.4	2.6	+8
FR1	FRCRRRFCRF-NH_2	$C_{63}H_{100}N_{26}O_{10}S_2$	1445.77	1446.78	12.67	−0.9	1.83	+6
FR2	FRFRCRRRFCRFRF-NH_2	$C_{93}H_{142}N_{36}O_{14}S_2$	2052.49	2052.51	17.22	−0.89	2.18	+8
WR1	WRCRRRFCRW-NH_2	$C_{67}H_{102}N_{28}O_{10}S_2$	1523.84	1523.85	13.79	−1.64	1.16	+6
WR2	WRWRCRRRFCRWRW-NH_2	$C_{101}H_{146}N_{40}O_{14}S_2$	2208.63	2208.66	15.51	−1.94	1.28	+8
PR1	PRCRRRFCRP-NH_2	$C_{55}H_{96}N_{26}O_{10}S_2$	1345.65	1345.66	10.61	−4.5	2.07	+6
PR2	PRPRCRRRFCRPRP-NH_2	$C_{77}H_{134}N_{36}O_{14}S_2$	1852.25	1852.27	10.74	−4.67	2.46	+8
PG-1	RGGRLCYCRRRF CVCVGR-NH_2	$C_{88}H_{151}N_{37}O_{19}S_4$	2155.61	2156.35	12.56	−2.55	2.40	+7

[a] Molecular weight (MW) was measured by mass spectroscopy (MS).
[b] The mean hydrophobicity (H) is the total hydrophobicity (sum of all residue hydrophobicity indices) divided by the number of residues.
[c] The relative hydrophobic moment (μHrel) of a peptide is its hydrophobic moment relative to that of a perfectly amphipathic peptide. This gives a better idea of the amphipathicity using different scales.