Int J Pept Res Ther
DOI 10.1007/s10989-014-9391-2

Enhancing Antibacterial Activity Against *Escherichia coli K-12* of Peptide Ib-AMP4 with Synthetic Analogues

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Accepted: 3 January 2014

Abstract A family of Ib-AMP4 peptide analogues was obtained by solid phase synthesis, modifying the net charge and hydrophobicity of C-terminal domain by replacing certain amino acidic residues by arginine and tryptophan. Additionally, disulfide bonds were eliminated by replacing the cysteine residues by methionine, which resulted in a decrease in the number of synthesis byproducts, and consequently diminished the subsequent purification steps. The obtained peptides were purified by RP-HPLC and their molecular mass was determined by MALDI-TOF mass spectrometry. The peptide analogues (IC_{50} between 1 and 50 μM) presented a higher antibacterial activity against *Escherichia coli K-12* than the native peptide ($IC_{50} >$ 100 μM). The hemolytic activity of the peptide with the highest antibacterial efficacy presented no degradation of erythrocytes for a concentration of 1 μM that corresponds to its IC_{50} value. The results show that the synthesized peptides are good candidates for the treatment of diseases caused by *E. coli*.

Keywords Antibacterial peptides · Ib-AMP · *Escherichia coli* · Modified peptides

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Introduction

The extensive and indiscriminate use of antibiotics has led to an increased pathogenic resistance which demands new strategies for treating of infection (Reddy et al. 2004). Antimicrobial peptides (AMPs) are a component of the immediate non-specific defense mechanism of almost all species during the infection process (Reddy et al. 2004; Marr et al. 2006; De Luca and Walsh 2000). Peptides isolated from plants inhibit a large variety of phytopathogens and bacteria as *Escherichia coli* (Selitrennikoff 2001; Pelegrini et al. 2008; Franco et al. 2006). For this reason the use of AMPs arise as a new strategy for the control of bacterial infections. Antimicrobial activity of AMPs is determined by its amphipathic structure and net charge, which must be greater than +2 (Epand and Vogel 1999; Kamysz et al. 2003; Bisht et al. 2007). Variations of these properties result in an increase or decrease of antimicrobial activity depending on the microorganism–peptide couple studied (Van der Kraan et al. 2005; Haney et al. 2009). Cerovský et al. (2008) synthesized analogues of mastoparan peptides isolated from the venom of *Polistinae* wasps and its antibacterial activity was tested against *E. coli*. The results showed that an increase in the positive charge leads to an enhancement of the antibacterial activity.

Tailor et al. (1997) isolated four closely related AMPs from seeds of *Impatiens balsamina* (Ib-AMPs). These peptides are highly basic and contain four cysteine residues which form two intermolecular disulfide bonds. Within this family, the Ib-AMP4 peptide inhibits a large variety of phytopathogenic fungal as *Fusarium culmorum*, *Botrytis cinerea* and *Mycosphaerella fijiensis* with the key advantage of not being cytotoxic to human cells (Thevissen et al. 2005; Vásquez et al. 2009). However, the values of half maximal inhibitory concentration (IC_{50}) obtained for

Published online: 16 January 2014

Gram–negative bacteria as *E. coli* were higher than 500 μg/mL. On the other hand, synthetic production of peptides as Ib-AMP4 is difficult and expensive due the formation process of disulfide bonds which generates unwanted isomers (Lee et al. 1999).

In this work we investigated the effect of removing disulfide bonds of peptide Ib-AMP4 by replacing cysteine for methionine residues. Additionally, we introduced arginine and tryptophan residues with the aim of modifying the net charge and hydrophobicity in the C-terminal domain. Antibacterial activity against *E. coli K-12* of the peptide Ib-AMP4 and its synthetic analogues was studied. We found five analogues that showed lower values of IC_{50} than the native peptide.

Materials and Methods

Strains and Growth Conditions

Escherichia coli K-12 was cultured aerobically in Müller-Hinton broth (MHB) at 37 °C by 18 h. After this time, the culture was diluted with MHB to achieve an inoculum of $\sim 1.6 \times 10^6$ colony forming units (CFU)/mL.

Peptide Synthesis and Peptide Cyclization

Peptides were prepared by the standard Fmoc-based solid-phase method on a solid support of Rink amide-AM resin using DIPEA (*N*,*N*-diisopropylethylamine) and TBTU (2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) as coupling reagents (Lee et al. 1999; Wang et al. 2009). The amino acids (5 equivalents) were preactivated with TBTU (5 equivalents) and DIPEA (10 equivalents) in 2 ml of *N*,*N*-dimethylformamide for 20 min followed by coupling for 2 h. The peptides were treated for 2 h at room temperature with the following cleavage reagents: trifluoroacetic acid (TFA):water:1,2-ethanedithiol:triisopropylsilane (92.5 %:2.5 %:2.5 %:2.5 %) as previously reported (Lee et al. 1999; Wang et al. 2009). The crude peptides were then purified by reverse-phase high-performance liquid chromatography (HPLC-Agilent 1100) with a C_{18} column using water-acetonitrile gradient (0–80 %) containing 0.05 % TFA in 110 min. The peptides were detected at 254 nm with a diode array detector (model G1315B). The purified linear products were oxidized using 0.1 M Tris–HCl (pH 8.5) containing 0.3 mM oxidized glutathione and 0.15 mM reduced glutathione for 4 h at room temperature. The molecular mass of these peptides was determined by MALDI-TOF.

Antimicrobial Assay

Antibacterial activity of peptides was evaluated using the macrodilution method. Samples of MHB containing serial dilution of the peptides and 1.64×10^6 CFU/ml of *E. coli K-12* were incubated at 37 °C with shaking (185 rpm) and the absorbance at 620 nm was monitored each 30 min spectrophotometrically. Antibacterial activity was estimated from the concentration that inhibited 50 % of bacterial growth. The bactericidal activity was evaluated using the viable cell count method and was defined as minimal bactericidal concentration (MBC).

Hemolytic Activity of Peptides

Hemolytic activity of the peptide Ib-M6 was tested according to protocols described previously (Thevissen et al. 2005; Helmerhorst et al. 1999). Erythrocytes from healthy individuals, who had given their informed consent, were collected in vacuum tubes. The erythrocytes were harvested by centrifugation for 10 min at $210 \times g$ at 20 °C and washed three times in isotonic buffer Tris (10 mM Tris/HCl, 150 mM NaCl, pH 7.4). Tris buffer was added to the pellet in order to yield 1 % (v/v) erythrocytes/Tris buffer suspension. Aliquots of the peptide Ib-M6 were incubated with erythrocytes solution for 1 h at 37 °C. After centrifugation (5 min, $473 \times g$) the absorbance was measured at 450 nm. A 0 % lysis control (without peptide) and a 100 % lysis control (fully lysed erythrocytes in water) was used to assess the hemolytic activity of the peptide. The percentage hemolysis was calculated according to the following formula:

$$\%\ \text{Hemolysis} = \frac{A_{450}\ \text{of the peptide treated sample} - A_{450}\ \text{of the buffer treated sample}}{A_{450}\ \text{of the water treated sample} - A_{450}\ \text{of the buffer treated sample}} \times 100$$

Results

Synthesis of Peptide Ib-AMP4 and its Synthetic Variants

The sequence of peptide Ib-AMP4 is shown in Table 1. Six linear variants of Ib-AMP4 were synthesized replacing Tyr residue 15 and Cys residues 6, 7 and 16 by Met residues in order to preserve the number of sulfur atoms in the structure.

Table 1 Antibacterial activity of native and synthetic variants of Ib-AMP4 against *Escherichia coli K-12* in medium MH

Peptides	Amino acids sequences	Charge	IC_{50}^{a} (μM)	Molecular mass	
				Calculated	Measured
Ib-AMP4	EWGRR**CC**GWGPGRRY**C**RRW**C**-NH_2	+5	>100	2538.94	2538.87
Ib-M1	EWGRR**MMGR**GPGRR**MMRWWR**-NH_2	+6	15	2649.24	2649.12
Ib-M2	EWGRR**MMGW**R**P**GRR**MMRWWR**-NH_2	+6	50	2778.40	2778.22
Ib-M3	EWGRR**MMGWGR**GRR**MMRWWR**-NH_2	+6	15	2738.33	2738.21
Ib-M4	EWGRR**MMGR**GPGRR**MM**RRW**W**-NH_2	+6	>100	2649.24	2649.33
Ib-M5	EWGRR**MMGW**R**P**GRR**MM**RRW**W**-NH_2	+6	15	2778.40	2778.45
Ib-M6	EWGRR**MMGWGR**GRR**MM**RRW**W**-NH_2	+6	1	2738.33	2738.39

[a] IC_{50} value is expressed as the mean of duplicate experiments

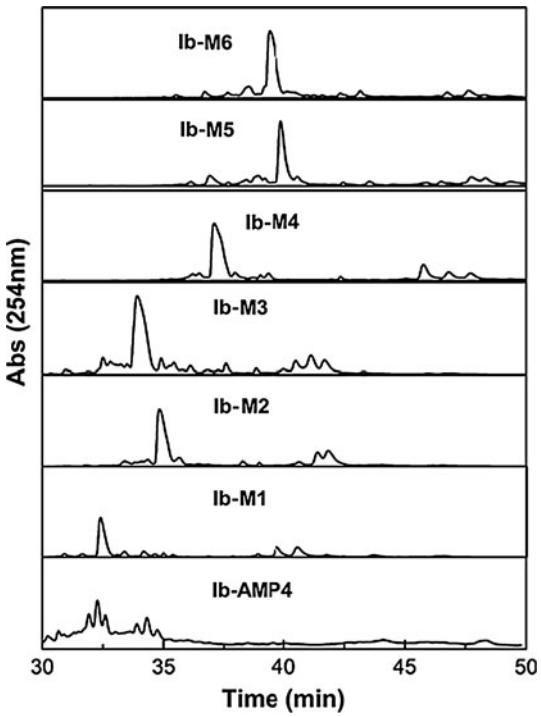

Fig. 1 Analytical RP-HPLC profiles of Ib-AMP4 and synthetic variants

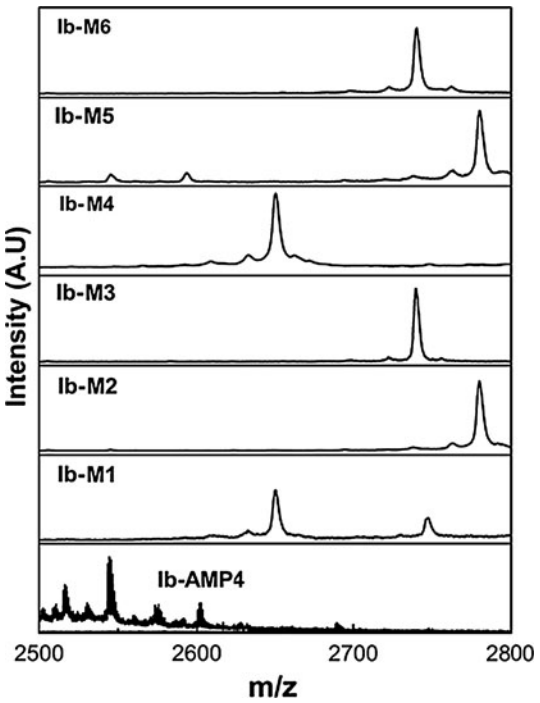

Fig. 2 Mass spectra of Ib-AMP4 and its synthetic variants

The peptides were classified into two groups in which the Cys-20 and Arg-18 residues were replaced by Arg and Trp, respectively, in group WR (peptides Ib-M1 to Ib-M3) and Cys-20 residue was replaced by Trp in group WW (peptides Ib-M4 to Ib-M6). In each group, Gly, Trp and Pro residues at positions 8, 9 and 10 were replaced, one at a time, by Arg (Table 1).

Peptides were synthesized by the solid phase method using Fmoc protected amino acids. The employed resin generated a C-terminal amide peptide after cleavage. Figure 1 shows the retention time of peptides on RP-HPLC. The peptides of group WW showed the highest retention time with 36.75 min for Ib-M4, 39.71 min for Ib-M5 and 39.05 min for Ib-M6 this is due to the high hydrophobicity of the C-terminal residues. Each of the obtained fractions was collected and its molecular mass was determined by MALDI-TOF mass spectrometry (Fig. 2).

Antibacterial Activity

The antibacterial activity of Ib-AMP4 peptide and its synthetic variants against *E. coli K-12* was determined using Müller-Hilton (MHB) culture medium. The values of IC_{50} obtained for each synthetic peptide and the corresponding to peptide Ib-AMP4 are shown for comparison in Table 1.

Removing the disulfide bonds and introducing Arg and Trp residues in the Ib-AMP4 sequence influenced the

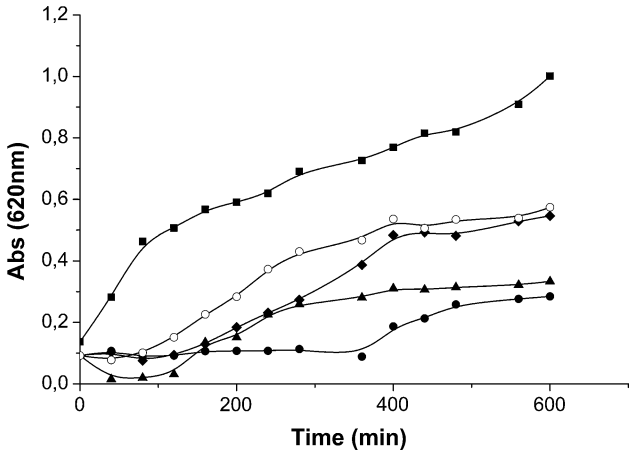

Fig. 3 Growth kinetics of *E. coli K-12* in the absence (*filled squares*) and in the presence of the peptide Ib-M6 at a concentration 5 μM (*filled circles*), 4 μM (*filled triangles*), 2 μM (*filled diamonds*) and 1 μM (*open circles*)

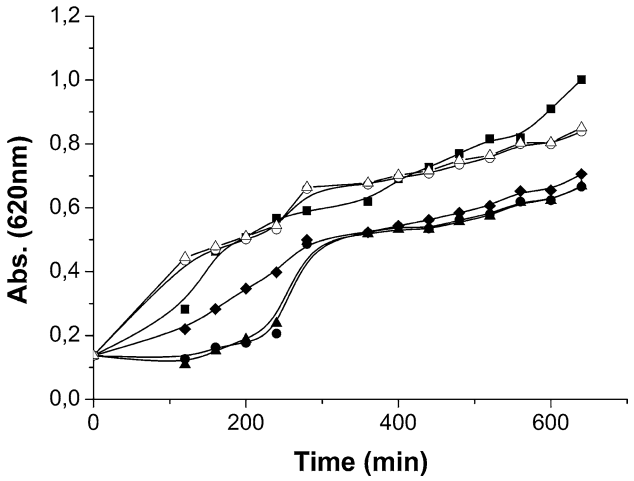

Fig. 4 Growth kinetics of *E. coli K-12* in absence (*filled squares*) and in the presence the peptide Ib-M4 at a concentration 100 μM (*filled circles*), 80 μM (*filled triangles*), 50 μM (*filled diamonds*), 20 μM (*open circles*) and 15 μM (*open triangles*)

antibacterial activity against *E. coli K-12*. With the exception of variant Ib-M4, each of the synthetic variants augmented the antibacterial activity. It was found that the peptide Ib-M6 exhibited the lowest IC_{50} value of 1 μM (Fig. 3).

Peptide Ib-M4, in which Gly-10 and Pro-11 residues are conserved, presented an IC_{50} value similar to the native peptide (i.e. higher than 100 μM). Nevertheless, in the presence of this synthetic peptide (from a concentration of 80 μM), *E. coli K-12* showed an increased lag time, starting its exponential phase at 120 min (Fig. 4). Although in peptide Ib-M1 Gly-10 and Pro-11 residues are also conserved, in this case the antibacterial activity was improved with respect to the native peptide ($IC_{50} = 15$ μM)) indicating that these residues are not involved in their antibacterial activity. However, the results show that not only the total charge (which corresponds to +6 for all analogues) but also the distribution of positive charges plays a role in peptide interaction with negatively charged bacterial cellular membrane. Supporting this hypothesis, Ikeda et al. (1990) found that introducing rigid spacers in polymeric quaternary ammonium salts augmented their antimicrobial activity.

Table 2 Hemolytic activity of peptide Ib-M6 on human erythrocytes

Peptide Ib-M6 concentration (μM)	Hemolysis (%)
1	Non detectable
2	0.9 ± 0.3
4	2.5 ± 0.3
5	3.0 ± 0.4
100	29 ± 1

Hemolytic Activity

Once defined the sequence of Ib-M6 as the best synthetic alternative, its hemolytic activity was determined on human erythrocytes. A 29 % of hemolysis was obtained for the highest concentration assayed (100 μM). However, for a concentration of 1 μM that corresponds to the IC_{50} value no degradation of erythrocytes was observed (Table 2).

Discussion

The polycationic charge is an important property in the activity of AMPs due to its role in peptide interaction with negatively charged bacterial cellular membrane (Alves et al. 2010). For this reason, one of the modifications done over the Ib-AMP4 sequence was the augmentation of positive charge introducing Arg residues. On the other hand, the C-terminal hydrophobicity was modified by means of Trp residues in positions 18 and 20, taking into account that the Trp residues play an important role in AMP activity (Chan et al. 2006). This is evidenced by the results of inhibition obtained for peptide Ib-M6, which showed the lowest IC_{50} value (1 μM). The contribution of Trp lies in the indol ring of its lateral chain, which promotes peptide′s insertion into the hydrophobic core of the cell membrane, favoring the association of peptides to bacterial membranes.

The modifications made of peptide Ib-AMP4 resulted linear analogs with a lower IC_{50} value with respect to the native peptide with the exception of Ib-M4. However, this last peptide causes an augmentation in *E. coli K-12* lag time, which goes to show that a temporal interaction with

the cell membrane exists and would be responsible for retardation in bacterial adaptation to the environment.

The simultaneous increment of the net charge and the hydrophobicity in the sequence of Ib-AMP4 antifungal peptide inherent to Ib-M6 analog resulted in an enhanced antimicrobial activity against *E. coli K-12*, presumably due to improved interaction with hydrophobic phospholipids of the bacterial membranes. In addition, this analog presented no hemolytic activity on human erythrocytes at a concentration of 1 μM which corresponds to its IC_{50} value, making it a therapeutic candidate for the treatment of diseases caused by *E. coli.*

Acknowledgments This work has been carried out with the financial support of Universidad Industrial de Santander (DIEF Ciencias, Project 5182), the University of Buenos Aires (UBACyT 20020100100636) and the National Research Council of Argentina (CONICET PIP 11220110101020). J.M. Flórez-Castillo thanks COLCIENCIAS for the doctoral scholarship in the frame of the program "Convocatoria nacional para estudios a nivel de doctorado en Colombia–año 2009". MP and MJ are Research Scientist of CONICET (Argentina).

Conflict of interest Johanna Marcela Flórez-Castillo, Mercedes Perullini, Matias Jobbágy and Herminsul de Jesús Cano Calle declare that they have no conflict of interest.

Ethical Standard All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Statement of Human and Animal Rights This article does not contain any studies with animal subjects performed by any of the authors.

References

Alves CS, Melo MN, Franquelim HG, Ferre R, Planas M, Feliu L et al (2010) *Escherichia coli* cell surface perturbation and disruption induced by antimicrobial peptides BP100 and pepR. J Biol Chem 285:27536–27544

Bisht GS, Rawat DS, Kumar A, Kumar R, Pasha S (2007) Antimicrobial activity of rationally designed amino terminal modified peptides. Bioorg Med Chem Lett 17:4343–4346

Čeřovský V, Slaninová J, Fučík V, Hulačová H, Borovičková L, Ježek R et al (2008) New potent antimicrobial peptides from the venom of *Polistinae* wasps and their analogs. Peptides 29: 992–1003

Chan DI, Prenner EJ, Vogel HJ (2006) Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. Membr Biophys Antimicrob Pept 1758:1184–1202

De Luca AJ, Walsh TJ (2000) Antifungal peptides: origin, activity, and therapeutic potential. Rev Iberoam Micol 17:116–120

Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta 1462:11–28

Franco OL, Murad AM, Leite JR, Mendes PAM, Prates MV, Bloch C (2006) Identification of a cowpea γ-thionin with bactericidal activity. FEBS J 273:3489–3497

Haney EF, Nazmi K, Lau F, Bolscher JGM, Vogel HJ (2009) Novel lactoferrampin antimicrobial peptides derived from human lactoferrin. Biochimie 91:141–154

Helmerhorst EJ, Reijnders IM, van't Hof W, Veerman EC, Nieuw Amerongen AV (1999) A critical comparison of the hemolytic and fungicidal activities of cationic antimicrobial peptides. FEBS Lett 449:105–110

Ikeda T, Yamaguchi H, Tazuke S (1990) Phase-separation in phospholipid bilayers induced by biologically active polycations. Biochim Biophys Acta 1026:105–112

Kamysz W, Okroj M, Lukasiak J (2003) Novel properties of antimicrobial peptides. Acta Biochim Pol 50:461–469

Lee D, Shin S, Kim D-H, Seo M, Kang J, Lee Y et al (1999) Antifungal mechanism of a cysteine-rich antimicrobial peptide, Ib-AMP1, from *Impatiens balsamina* against Candida albicans. Biotechnol Lett 21:1047–1050

Marr AK, Gooderham WJ, Hancock RE (2006) Antibacterial peptides for therapeutic use: obstacles and realistic outlook. Anti Infect Technol 6:468–472

Pelegrini PB, Murad AM, Silva LP, dos Santos RCP, Costa FT, Tagliari PD et al (2008) Identification of a novel storage glycine-rich peptide from guava (Psidium guajava) seeds with activity against Gram-negative bacteria. Peptides 29:1271–1279

Reddy KVR, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. Int J Antimicrob Ag 24(6):536–547

Selitrennikoff CP (2001) Antifungal Proteins. Appl Environ Microbiol 67(7):2883–2894

Tailor RH, Acland DP, Attenborough S, Cammue BPA, Evans IJ, Osborn RW et al (1997) A Novel Family of Small Cysteine-rich Antimicrobial Peptides from Seed of Impatiens balsamina Is Derived from a Single Precursor Protein. J Biol Chem 272(39): 24480–24487

Thevissen K, FranÃ§ois IEJA, Sijtsma L, Amerongen Av, Schaaper WMM, Meloen R, Posthuma-Trumpie T, Broekaert WF, Cammue BPA (2005) Antifungal activity of synthetic peptides derived from Impatiens balsamina antimicrobial peptides Ib-AMP1 and Ib-AMP4. Peptides 26(7):1113–1119

Van der Kraan MIA, van der Made C, Nazmi K, van't Hof W, Groenink J, Veerman ECI, Bolscher JGM, Nieuw Amerongen AV (2005) Effect of amino acid substitutions on the candidacidal activity of LFampin 265–284. Peptides 26(11):2093–2097

Wang P, Bang, J-K, Kim HJ, Kim J-K, Kim Y, Shin SY et al (2009) Antimicrobial specificity and mechanism of action of disulfide-removed linear analogs of the plant-derived Cys-rich antimicrobial peptide Ib-AMP1. Peptides 30(12):2144–2149