Peptides 37 (2012) 294–300

Contents lists available at SciVerse ScienceDirect

Peptides

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

Antimicrobial activity of recombinant Pg-AMP1, a glycine-rich peptide from guava seeds

Letícia Stephan Tavares[a], João Vitor Rettore[a], Renata Mendes Freitas[a], William Farias Porto[b], Ana Paula do Nascimento Duque[a], Júnya de Lacorte Singulani[a], Osmar Nascimento Silva[a], Michelle de Lima Detoni[a], Eveline Gomes Vasconcelos[a], Simoni Campos Dias[b], Octávio Luiz Franco[a,b,*], Marcelo de Oliveira Santos[a]

[a] Departamento de Biologia, Programa de Pós-Graduação em Genética e Biotecnologia, Universidade Federal de Juiz de Fora, Campus Universitário, 36036-900, Martelos, Juiz de Fora-MG, Brazil

[b] Centro de Análises Proteômicas e Bioquímicas, Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, SGAN, Quadra 916, Módulo B, Av. W5 Norte, 70.790-160-Asa Norte, Brasília-DF, Brazil

ARTICLE INFO

Article history:
Received 4 May 2012
Received in revised form 20 July 2012
Accepted 20 July 2012
Available online 27 July 2012

Keywords:
Pg-AMP1
Antimicrobial peptides
Heterologous expression
Psidium guajava

ABSTRACT

Antimicrobial peptides (AMPs) are compounds that act in a wide range of physiological defensive mechanisms developed to counteract bacteria, fungi, parasites and viruses. These molecules have become increasingly important as a consequence of remarkable microorganism resistance to common antibiotics. This report shows *Escherichia coli* expressing the recombinant antimicrobial peptide Pg-AMP1 previously isolated from *Psidium guajava* seeds. The deduced Pg-AMP1 open reading frame consists in a 168 bp long plus methionine also containing a His6 tag, encoding a predicted 62 amino acid residue peptide with related molecular mass calculated to be 6.98 kDa as a monomer and 13.96 kDa at the dimer form. The recombinant Pg-AMP1 peptide showed inhibitory activity against multiple Gram-negative (*E. coli*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermides*) bacteria. Moreover, theoretical structure analyses were performed in order to understand the functional differences between natural and recombinant Pg-AMP1 forms. Data here reported suggest that Pg-AMP1 is a promising peptide to be used as a biotechnological tool for control of human infectious diseases.

1. Introduction

In recent years, the number of bacteria with enhanced resistance to conventional antibiotics has dramatically increased [10]. Many hospital infections have been difficult to treat due to opportunistic bacterial infections. Many of these bacteria belong to regular microbial flora, making them a real challenge for immune-depressed patients. In general, treatment is expensive and inefficient, encouraging several research groups to screen novel antimicrobial compounds [9,40]. Among them, antimicrobial peptides (AMPs) have been focused since they are the first natural barrier against microorganisms from almost all living groups [40].

AMPs are constitutively expressed or induced by endogenous or exogenous elicitors, such as developmental stage or pathogen predation [32]. AMPs are small proteins, 20–50 amino acid residues long, and in some organisms constitute the primary innate host defense line, often have common properties such as the small number of amino acid residues, cationicity and amphipathicity [25]. Although various AMPs have been isolated in different kingdoms, several structural scaffolds are quite common and may be related to a single promiscuous class of peptides with multiple functions [8]. The mechanism of action include select electrostatic interactions that may induce lipid bilayer depolarization, permeability alterations and ion imbalance [28] that may lead to membrane disruption. Moreover, the presence of AMPs could

Abbreviations: AMPs, antimicrobial peptides; ATCC, American type culture collection; BL21-(DE3), bacterial expression systems; BSA, bovine serum albumin; CFU, colony-forming unit; GRPs, glycine rich proteins; GST, glutathione *S*-transferase; HDP, host–defense peptide; DOPE, discrete optimized protein energy; His6, hexa histidine tag; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl β-D-1-thiogalacto pyronoside; LAMP-1a, *Leymus arenarius* antimicrobial peptide 1a; LB, Luria–Bertani broth medium; LOMETS, local meta-threading-server; MBP, maltose binding protein; MIC, minimal inhibitory concentration; NusA, N-utilizing substance A protein; pBSK, expression and cloning plasmid; Pg-AMP1, *Psidium guajava* antimicrobial peptide 1; PR-39, proline-rich antibacterial peptide; PrDOS, protein DisOrder prediction system; ProSA, protein structure analysis; PT7, bacteriophage promoter; RBCs, human red blood cells; Trx, thioredoxin A.

* Corresponding author at: Centro de Análises Proteômicas e Bioquímicas, Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, SGAN, Quadra 916, Módulo B, Av. W5 Norte, 70.790-160-Asa Norte, Brasília-DF, Brazil. Tel.: +55 61 34487220; fax: +55 61 33474797.
E-mail address: ocfranco@gmail.com (O.L. Franco).

0196-9781/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2012.07.017

also lead to alteration of several gene expressions, improving protein synthesis and modifying enzyme activities [32]. In the last two decades a number of studies have shown that AMPs act synergistically to the immune response [10,23], making isolation, identification and characterization of natural AMPs an important tool for development of a new generation of drugs [11,23,39].

Among the AMPs, the glycine-rich proteins (GRPs) are a group of proteins that occurs in a wide variety of organisms. This group carries glycine-rich repeat domains [2,24] and their expressions in plants are normally modulated by abiotic and biotic stresses, showing defensive activity against fungi, bacteria and viruses [2]. Pelegrini et al. [28] demonstrated that a GRP isolated from guava seeds, denominated Pg-AMP1, showed activity against human pathogenic Gram-negative bacteria such as *Escherichia coli*, *Klebsiella* sp. and *Proteus* sp. In spite of the clear bactericidal activity observed, purification and yield of Pg-AMP1 was extremely low, reaching approximately 1 mg of peptide from almost 10 kg of total guava seeds [28]. This protein quantity was insufficient to allow novel experiments or to use these peptides as a biotechnological tool for infectious disease treatment. Furthermore, Pg-AMP1chemical synthesis, a peptide 55 amino acid residues long, is extremely expensive, therefore for Pg-AMP1 the strategy of recombinant protein production in a heterologous system is essential. However, few articles described heterologous expression of recombinant glycine-rich antimicrobial peptides. Feng et al. [7] expressed SK66-His and Sperstad et al. [37] expressed the GRP-denominated hystatin, that showed deleterious activity against Gram-positive and Gram-negative bacteria and yeasts. Furthermore, Shlyapnikov et al. [36] cloned and expressed a synthetic gene in a pET vector. This gene, named Ltc2a (latarcin 2a), was fused to 6 glycine residues and further demonstrated protective activity against *E. coli* and *Bacillus subtilis*.

The number of recombinant antimicrobial peptides expressed has greatly increased in recent years due to advances in molecular biology, allowing the establishment of strategies for expression such as host choice, promoter type and appropriate post-transcriptional modification features [33]. Bacterial systems remain highly attractive due to low cost, high productivity and rapid use. Although the bacterial systems seems to be useful for eukaryotic protein expression some limitations must be overcome, such as codon usage, incorrect folding, protein degradation by protease from host cell and host toxicity caused by heterologous protein [14]. Over-expression of heterologous proteins in *E. coli* often produces high quantities of insoluble protein. In order to overcome such limitations, the optimization of expression conditions such as temperature, growth media, induction parameters, promoters and *E. coli* expression strain maybe used [33]. Fusion tags as Trx, NusA, GST, MBP and His6 were able to increase protein solubility, improving the purification processes by decreasing production costs and increasing yield [27,31]. Although fusion tags maybe interesting since they facilitate peptide solubility and purification, these tags can also often interfere within protein structure and/or function. Consequently, it is commonly recommended that the tag be removed after the purification process [27], although Carson et al. [3] have shown that the His6 tag does not normally alter the structure of recombinant proteins. Tag removal can be performed by proteolytic cleavage, but this approach can be problematic due to non-specific and inefficient cleavage or loss of protein stability and solubility [27]. Another factor that impairs heterologous antimicrobial peptide expression in *E. coli* is cytotoxic AMP activity against the bacterial host. This leads to clear difficulties in scale-up and to low yields processing requires chemicals or expensive enzymes, and multistep purification of peptides reduces yield, thus making peptide production less cost effective [1]. In spite of all these problems, some studies describe the expression of AMPs in prokaryotic expression system as *E. coli* with yielding varying from 2 mg L^{-1} to 40 mg L^{-1} [18,19,33,38,41].

In the present work the guava GRP gene, named Pg-AMP1, was synthesized and further expression strategy was designed for production of the recombinant peptide in a prokaryotic system. Recombinant Pg-AMP1 was further evaluated for biological activity against human pathogenic bacteria. Moreover, theoretically structural studies comparing the native and recombinant Pg-AMP1 forms were also carried out to shed some light on structure–function relationship.

2. Materials and methods

2.1. Bacterial strains

Gram-negative bacteria *Escherichia coli* (ATCC 35218, ATCC 11229), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 13866) and *Salmonella typhimurium* (ATCC 14028) and Gram-positive bacteria *Staphylococcus aureus* (ATCC 29213, ATCC 25923), *S. aureus* MecA (ATCC 33591), *Staphylococcus epidermides* (ATCC 12228) were utilized in this report. Bacteria were cultured in Tryptone Soy Broth (TSB-Tryptone 5 g L^{-1}, yeast extract 2.5 g L^{-1}, Dextrose 1 g L^{-1} and sodium chloride 10 g L^{-1}). The induced *E. coli* bacteria (BL21-DE3) were cultured in Luria–Bertani broth medium (LB).

2.2. Gene synthesis and vector construction

The gene encoding Pg-AMP1, 168 bp long, was designed to be expressed carrying a His6 tag fused to C-terminal. The codon was optimized for *E. coli* expression and the cassette expression was synthesized by Epoch Biolabs and cloned into *SmaI* site of pBluescriptIISK(−). The expression cassette is composed of Pg-AMP1 gene under control of T7/*lac* promoter/terminator plus met codon His6 tag encoding a peptide with 62 amino acid residues (Fig. 1).

2.3. Expression of recombinant Pg-AMP1

Recombinant plasmid pBSK*Pg-AMP1* was used for transformation of *E. coli* BL21 (DE3) electrocompetent cells (Invitrogen, Carlsbad, CA). The induction was done according to the instruction manual His Trap FF crude (GE, Upsala), using IPTG as an inducer and ampicillin (100 μg mL^{-1}) as select agent. The IPTG induction (0, 0.5 and 1 mM) was done during 2, 4 or 6 h. Soluble and insoluble fractions were evaluated in each treatment.

2.4. Isolation of recombinant peptide

BL21 (DE3) cells were grown for 4 h from 500 mL of LB at 300 rpm. Pellet cells were obtained from 4500 × *g* at 4 °C after 15 min centrifugation. Pellets were resuspended in lysis buffer (1:10 v/v) containing 50 mM sodium phosphate (pH 7.8), 300 mM sodium chloride, 50 mM potassium chloride, 10% glycerol, 0.5% Triton X-100 and 10 mM imidazole. Enzymatic lysis was performed for 30 min at room temperature with 0.2 mg mL^{-1} lysozyme, 20 μg mL^{-1} DNAse, 1 mM MgCl and 1 mM phenylmethylsulfonylfluoride. Mechanical lysis was carried out by sonication on ice for approximately 10 min (in several short bursts). Suspension cells were disrupted by sonication (Sonics – Vibra Cell) 20 kHz 100% using the v188 probe on ice four times for 20 s separated by 1 min elapsed time. The suspension was centrifuged at 4500 × *g* at 4 °C for 30 min. Supernatant carrying soluble proteins were stored −20 °C for subsequent analysis. For each gram of pellet, 3 mL of lysis buffer containing 300 mM sodium chloride, 50 mM sodium phosphate (pH