BBA - Biomembranes 1860 (2018) 1425–1435

Contents lists available at ScienceDirect

BBA - Biomembranes

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

Novel bioactive peptides from PD-L1/2, a type 1 ribosome inactivating protein from *Phytolacca dioica* L. Evaluation of their antimicrobial properties and anti-biofilm activities

Elio Pizzo[a], Katia Pane[a], Andrea Bosso[a], Nicola Landi[b], Sara Ragucci[b], Rosita Russo[b], Rosa Gaglione[c], Marcelo D.T. Torres[e,f,g,h,i,j,k], Cesar de la Fuente-Nunez[e,f,g,h,i,j,k], Angela Arciello[c,d], Alberto Di Donato[a], Eugenio Notomista[a], Antimo Di Maro[b,*]

[a] Department of Biology, University of Naples 'Federico II', Via Cintia, I-80126 Napoli, Italy
[b] Department of Environmental, Biological and Pharmaceutical Sciences and Technologies (DiSTABiF), University of Campania 'Luigi Vanvitelli', I-81100 Caserta, Italy
[c] Department of Chemical Sciences, University of Naples 'Federico II' Via Cintia, I-80126 Napoli, Italy
[d] Istituto Nazionale di Biostrutture e Biosistemi (INBB), Italy
[e] Synthetic Biology Group, MIT Synthetic Biology Center, Massachusetts Institute of Technology, Cambridge, MA, United States.
[f] Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, MA, United States.
[g] Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, United States.
[h] Department of Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA, United States.
[i] Broad Institute of MIT and Harvard, Cambridge, MA, United States.
[j] Harvard Biophysics Program, Harvard University, Boston, MA, United States.
[k] The Center for Microbiome Informatics and Therapeutics, Cambridge, MA, United States

ARTICLE INFO

Keywords:
Antimicrobial peptides
Biofilm
Circular dichroism
Phytolacca dioica
Ribosome inactivating proteins

ABSTRACT

Antimicrobial peptides, also called Host Defence Peptides (HDPs), are effectors of innate immune response found in all living organisms. In a previous report, we have identified by chemical fragmentation, and characterized the first cryptic antimicrobial peptide in PD-L4, a type 1 ribosome inactivating protein (RIP) from leaves of *Phytolacca dioica* L. We applied a recently developed bioinformatic approach to a further member of the differently expressed pool of type 1 RIPs from *P. dioica* (PD-L1/2), and identified two novel putative cryptic HDPs in its N-terminal domain. These two peptides, here named IKY31 and IKY23, exhibit antibacterial activities against planktonic bacterial cells and, interestingly, significant anti-biofilm properties against two Gram-negative strains. Here, we describe that PD-L1/2 derived peptides are able to induce a strong dose-dependent reduction in biofilm biomass, affect biofilm thickness and, in the case of IKY31, interfere with cell-to-cell adhesion, likely by affecting biofilm structural components. In addition to these findings, we found that both PD-L1/2 derived peptides are able to assume stable helical conformations in the presence of membrane mimicking agents (SDS and TFE) and intriguingly beta structures when incubated with extracellular bacterial wall components (LPS and alginate). Overall, the data collected in this work provide further evidence of the importance of cryptic peptides derived from type 1 RIPs in host/pathogen interactions, especially under pathophysiological conditions induced by biofilm forming bacteria. This suggests a new possible role of RIPs as precursors of antimicrobial and anti-biofilm agents, likely released upon defensive proteolytic processes, which may be involved in plant homeostasis.

1. Introduction

Natural antimicrobial peptides (AMPs) are present in every life form, bacteria included. Their functionality is associated not only to their antimicrobial activity [1,2], but also to immunomodulation [3,4], angiogenesis [5], wound healing activity [6,7] and anti-tumor effects [8]. For this reason, they are more appropriately termed Host Defence Peptides (HDPs), in order to take into account their broad range of activities and their ability to stimulate the immune system in addition to being antimicrobial.

Abbreviations: CD, circular dichroism; CNBr, cyanogen bromide; LPS, lipopolysaccharide; Na/P, sodium phosphate buffer; SDS, sodium dodecyl sulphate; TFE, trifluoroethanol; Tris•Cl, Tris(hydroxymethyl)aminomethane-HCl buffer; SEM, standard error of the mean; LAL, *Limulus amebocyte* lysate; PI, propidium iodide; OD, optical density

* Corresponding author at: DiSTABiF, University of Campania 'Luigi Vanvitelli', I-81100 Caserta, Italy.
E-mail address: antimo.dimaro@unicampania.it (A. Di Maro).

https://doi.org/10.1016/j.bbamem.2018.04.010
Received 31 October 2017; Received in revised form 11 April 2018; Accepted 18 April 2018
Available online 22 April 2018
0005-2736/

HDPs have attracted considerable attention for their function in organism homeostasis and for their great potentialities in medical, agricultural and food industry [9]. In particular, HDPs are studied: 1) in medicine as an alternative source of new therapeutic agents able to counteract antibiotic resistance [10,11]; 2) in agriculture as efficient weapons to fight plant-diseases by taking advantage of their specificity, low toxicity and high biodegradability, that results in a low environmental impact with respect to chemical pesticides [12,13]; 3) in food industry as natural and effective food preservatives used alone or in combination with different preservation technologies, in order to replace the traditional chemical methods [14,15].

Although HDPs are characterized by differences in length and amino acid composition, they share some common features. In fact, they are small (12–50 amino acids), positively charged under physiological conditions (from +5 to +8), and are characterized by an unusual abundance of hydrophobic residues (≥30%). Their overall positive net charge is considered the main driving force of their selective electrostatic interaction with anionic microbial cell surface. HDPs are generally unfolded in aqueous solutions, but they fold into amphipathic conformations upon interaction with bacterial surface [16]; this allows their insertion into the membrane, with a consequent induction of cell death mediated by membrane disruption and/or inhibition of intracellular pathways/targets [17,18].

So far, > 2500 HDPs have been described, especially from mammals, insects and plants [9]. They can be classified into different classes on the basis of their structure: α-helical peptides (*e.g.*, LL-37, magainins and mellitin), β-sheets peptides stabilized by two to four disulfide bridges (*e.g.*, human and plant defensins); loop peptides with one disulfide bridge (*e.g.*, bactenecin) and peptides enriched in amino acids like proline, tryptophan, histidine or glycine without a well-defined structure (*e.g.*, indolicidin) [17].

Specific databases with increasingly accessible information [19,20] indicate that a number of antimicrobial peptides have been identified in proteins, known as HDP releasing proteins, not necessarily playing host defence activities. These proteins would release biologically active peptides upon proteolytic cleavage by bacterial and/or host proteases [21,22]. A few of these bioactive peptides were found in cationic proteins (with an isoelectric point > 8.0), such as lysozyme [23], RNase A [24], histone H2A [25] and lactoferrin [26], the latter with a high propensity to release unfolded peptides with a positive net charge.

In this framework, our research group identified an HDP in cationic PD-L4 enzyme, a type 1 ribosome inactivating protein (RIP) [27] from *Phytolacca dioica* L. [28], released by CNBr fragmentation of the native protein. RIPs are enzymes (3.2.2.22) identified in plants, fungi, algae, and bacteria. They are endowed with N-glycosilase activity, which leads to the cleavage of a specific adenine residue at a conserved site of the 28S rRNA, with a consequent inhibition of protein synthesis [29]. They are classified as type 1 or 2 according to their structure. Type 1 RIPs are single-chain proteins, with a size of approximately 30 kDa and a basic pI, whereas dimeric type 2 RIPs consist of two peptide chains: an A chain of about 30 kDa, endowed with enzymatic activity, linked to a B chain of about 35 kDa, endowed with lectin activity and capable of binding to oligosaccharides containing galactose moieties [30]. Although the biological role of RIPs has not been completely elucidated, some reports indicate that they may be involved in host defence activities [31,32].

To date, reports on HDPs derived from *P. dioica* L. are limited to our recent study [27] demonstrating that the identified peptide, named $PDL4_{(40–65)}$, exhibits structural and biological properties typical of Host Defence Peptides.

Recent evidence revealed that plant HDPs show antibacterial effects at micromolar concentrations not only against phytopathogens, but also against human pathogenic bacteria, such as enteric pathogens, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphyloccus aureus* [33]. As for HDPs derived from animals, also plant HDPs can be constitutively expressed in those tissues more vulnerable to microbial attacks, such as flowers, seeds and leaves, or their expression can be induced by specific stimuli, such as a mechanical injury [34].

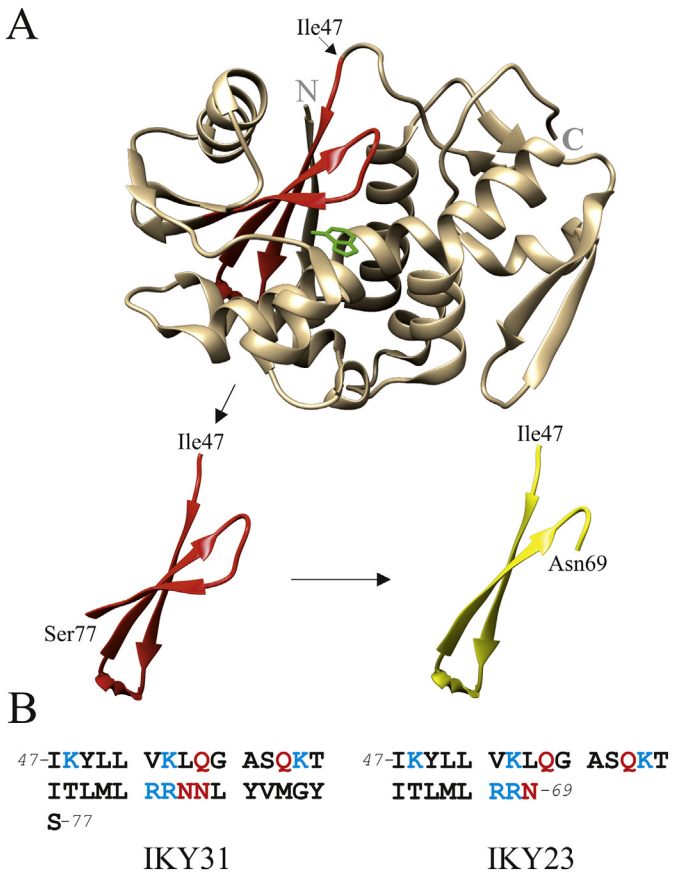

Fig. 1. PD-L1/2 structure and PD-L1/2 derived peptides primary structures. A. Crystallographic structure of PD-L1/2 (PDB code: 3H5K). IKY31 and IKY23 are highlighted in red and yellow, respectively; B. Primary structures of both PD-L1/2 derived peptides. Cationic residues are in blue; glutamine and asparagine are in red.

In order to evaluate the presence of novel bioactive peptides in other RIP members, such as PD-L1/2, dioicin1 and dioicin2 [35,36], here we used a novel bioinformatics approach, to identify potential cryptic antimicrobial peptides in protein precursors and to predict their strain specific antibacterial activity [37]. As a result of this screening, two promising HDPs have been identified in the N-terminal region of PD-L1/2 (amino acid position Ile50-Ser80, Fig. 1) [38], which were named IKY23 and IKY31.

The present study focuses on the characterization of the antibacterial and anti-biofilm activities of these two novel PD-L1/2 derived peptides. For this purpose, we analysed their effects on two relevant human pathogenic bacteria, such as *Pseudomonas aeruginosa* PAO1 and *Klebsiella pneumoniae*. We also analysed the structural features of the peptides upon interaction with membrane mimicking agents, biofilm extracellular matrix components and Gram-negative bacteria signature molecules, and evaluated their possible toxic effects on mammalian cells.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used in this study were: *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Klebsiella pneumoniae* ATCC 700603 (kindly provided by Dr. Eliana De Gregorio), wild-type *Pseudomonas aeruginosa* PAO1 (kindly provided by Dr. Donatella De