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iTRAQ-coupled 2-D LC–MS/MS analysis of cytoplasmic protein profile in *Escherichia coli* incubated with apidaecin IB

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ABSTRACT

Apidaecins refer to a series of proline-rich, 18- to 20-residue antimicrobial peptides produced by insects. Accumulating evidence that proline-rich antimicrobial peptides are not-toxic to human and animal cells makes them potential candidates for the development of novel antibiotic drugs. However, the mechanism of action was not fully understood. In this study, antibacterial mechanism of apidaecins was investigated. iTRAQ-coupled 2-D LC–MS/MS technique was utilized to identify altered cytoplasmic proteins of *Escherichia coli* incubated with one isoform of apidaecins — apidaecin IB. The production of the chaperonin GroEL and its cofactor GroES, which together form the only essential chaperone system in *E. coli* cytoplasm under all growth conditions, was decreased in cells incubated with apidaecin IB. The decreasing of the GroEL–GroES chaperone team was further found to be involved in a new antibacterial mechanism of apidaecins. Our findings therefore provide important new insights into the antibacterial mechanism of apidaecins and perhaps, by extension, for other proline-rich antimicrobial peptides.

1. Introduction

Antimicrobial peptides (AMPs) refer to a group of relatively short (less than 100 amino acids), positive charged peptides produced by a wide variety of organisms as part of their first line of defense [1]. These peptides possess broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria [2–4], fungi [2,5,6], protozoa [2,7,8] and viruses [9–11]. The mechanism of action of most of these peptides is based on their ability to interact with bacterial membranes and either form pores of the barrel-stave or wormhole types, or aggregates at the membrane surface that cause a cooperative permeabilization and loss of membrane integrity (carpet model) [12,13]. In contrast, a minority of AMPs, particularly the proline-rich group, have a mode of action that is completely devoid of any apparent membrane destabilization [14]. They can translocate across cell membrane, penetrate into the cytoplasm, and target essential cellular processes to mediate cell death [14]. Evidence is accumulating that these non-membrane-disruptive AMPs are not-toxic to human and animal cells, which makes them potential candidates for the development of novel antibiotic drugs [15]. Apidaecins, 18- to 20-residue peptides produced by insects, are the largest group of proline-rich AMPs known to date [15]. They are predominantly active against Gram-negative bacteria including a wide range of plant-associated bacteria and some human pathogens [16]. Previous studies suggested that the antibacterial mechanism of apidaecins is based on their ability to bind chaperone DnaK and inhibit its function of assisting the folding of polypeptides [17]. However, it is possible that these peptides inactivate bacteria by other mechanism not yet identified.

In this study, antibacterial mechanism of apidaecins was investigated. iTRAQ-coupled 2-D LC–MS/MS technique was

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utilized to identify altered cytoplasmic proteins of *Escherichia coli* incubated with one isoform of apidaecins — apidaecin IB. The production of the chaperonin GroEL and its cofactor GroES, which together form the only essential chaperone system in *E. coli* cytoplasm under all growth conditions, was decreased in cells incubated with apidaecin IB for both 1 h and 2 h. The decreasing of the GroEL/GroES chaperone team was further found to be involved in a new antibacterial mechanism of apidaecins. Our findings therefore provide important new insights into the antibacterial mechanism of apidaecins and perhaps, by extension, for other proline-rich AMPs.

2. Materials and methods

2.1. Bacterial strain and culture

The bacterial strain used in this work was *E. coli* ATCC25922 obtained from the American Type Culture Collection (Rockville, MD). Frozen *E. coli* stock was streaked on to Mueller–Hinton (MH) agar plates and grown at 37 °C. Cells from a single colony were inoculated into MH broth and cultured overnight at 37 °C with shaking at 225 rpm for subsequent experiments.

2.2. Cytoplasmic proteins isolation

E. coli cells (5×10^5 CFU/ml) were incubated with 1/10 MIC of apidaecin IB (AnaSpec, Inc., USA) for 1 and 2 h. The cytoplasmic proteins were isolated as described previously [18]. Briefly, the cells were harvested by centrifugation at $3000\times g$ for 10 min at 4 °C and lysed in lysis buffer (50 mM NaCl, 5 mM DTT, 1 mM PMSF and 50 mM Tris·Cl, pH 8.0) by intermittent sonication. Unbroken cells were removed by centrifugation at $3000\times g$ for 10 min at 4 °C. The supernatants containing the cytoplasmic proteins were collected by centrifugation at $15{,}000\times g$ for 30 min at 4 °C. The concentration of the proteins was determined by Bradford assay. Standard curves were made using γ-globulin as a control.

2.3. iTRAQ labeling

Proteins from each sample (100 μg) were precipitated by the addition of four volumes of cold acetone at −20 °C for 2 h. The precipitated pellets were reduced, cysteine blocked, digested and labeled with respective isobaric tags using iTRAQ reagent Multiplex kit (Applied Biosystems Inc., CA, USA) according to manufacturer's protocol. The sample labeling was as follows: iTRAQ tags 114, Control 2 h; iTRAQ tags 115, Apidaecin IB-incubated 2 h; iTRAQ tags 116, Control 1 h; iTRAQ tags 117, Apidaecin IB-incubated 2 h. Samples were then pooled for LC–MS/MS analysis.

2.4. LC–MS/MS analysis

iTRAQ-labeled peptide mixtures were analyzed as described previously with a slight modification [19–23].The analysis was performed on combination of an Agilent 1200 nanoflow LC system (Agilent Technologies Inc., USA) and a 6530 Q-TOF mass spectrometer (Agilent Technologies Inc., USA). In the first dimension 3 μl of the combined peptide mixture was loaded onto the PolySulfoethyl A SCX column (0.3×50 mm, 5 μm) and was eluted stepwise by injecting salt plugs of 10 different molar concentrations of 10, 20, 30, 40, 50, 60, 80,100, 300, and 500 mM KCl solution. The sequentially eluted peptides from the SCX column were trapped onto the enrichment HPLC chip and further eluted with buffer A (0.1% formic acid) and buffer B (a nanoflow gradient of 5–80% acetonitrile plus 0.1% formic acid) at a flow rate of 300 nl/min. For MS/MS analysis, survey scans were acquired from m/z 300 to 1500 with up to two precursors selected for MS/MS from m/z 100 to 2000 using dynamic exclusion, and the rolling collision energy was used to facilitate promoting fragmentation.

2.5. Mass spectrometric data analysis

The identification and quantification of the proteins were performed using Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Software Revision A.03.03.084 SR4). Each MS/MS spectrum was searched for species of *E. coli* against the UniProt_sprot_20070123 database. The searches were run using the following parameters: fixed modification of methylmethanethiosulfate-labeled cysteine, fixed iTRAQ modification of free amine in the amino terminus and lysine. Other parameters such as tryptic cleavage specificity, precursor ion mass accuracy, and fragment ion mass accuracy are built-in functions Spectrum Mill software. The protein profile results were filtered with a protein score greater than 11 and peptides score of at least 6, which gives a confidence value of 99%. Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and was the ratio of the areas under the peaks at 114, 115, 116 and 117 Da, which were the masses of the tags that correspond to the iTRAQ reagents. Sequence coverage was calculated by dividing the number of amino acids observed by the protein amino acid length. The following criteria were required to consider a protein for further statistical analysis: two or more distinct peptides had to be identified and the fold change had to be greater than 1.2 or less than 0.8.

2.6. Western blot analysis

Western blotting was performed as described previously [24]. Briefly, equal amounts of proteins were separated with 12% SDS-PAGE. The proteins were electro-transferred to PVDF membranes (Bio-Rad Laboratories Inc., USA), which were then probed with primary antibodies anti-GroEL and anti-GroES (Abcam, UK). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse antibodies (Santa Cruz Biotechnology Inc., USA) were used as the secondary antibody. The results were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., USA). The expression of DNA-directed RNA polymerase subunit alpha (RpoA) was used as a loading control. The densitometric intensity of protein bands was measured using Quantity One software (Bio-Rad Laboratories Inc., USA).

2.7. Growth kinetics of gene-overexpression strains in response to apidaecin IB incubation

Plasmids that permit controlled expression of GroEL–GroES and DnaK–DnaJ–GrpE chaperone teams separately (Takara,