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Purification, characterization and mode of action of plantaricin K25 produced by *Lactobacillus plantarum*

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ABSTRACT

The aim of this study was to elucidate the mode of action of a novel bacteriocin with low molecular weight that was isolated, purified and characterised from *Lactobacillus plantarum* K25 and found propagating in kimchi samples. This antimicrobial peptide, named plantaricin K25 was recovered from the producer cells by using adsorption-desorption method and purified by high-performance liquid chromatography. It exhibited a broad spectrum of inhibitory activity against both Gram-positive and Gram-negative bacteria. Plantaricin K25 retained bioactivity when exposed to high temperature (121 °C) and remained stable at pH values ranging from 2 to 8. This peptide was partially inactivated by proteinase and pronase and fully degraded by peptidase and trypsin. It remained active after being treated with lipase, amylase, catalase and certain detergents. Based on Tris-Tricine SDS-PAGE of purified bacteriocin, the molecular weight was estimated at 2 kDa. MALDI-TOF mass spectrophotometry showed that the precise molecular weight of the peptide was 1772 Da. Plantaricin K25 showed to be a pore-forming bacteriocin capable of permeabilising the cytoplasmic membrane of targeted bacterial cells. When viewed under SEM and TEM, cell membranes of *Bacillus cereus* appeared to collapse and disrupt after exposure to the bacteriocin. Plantaricin K25 inhibited the growth of *B. cereus* cultivated in *mul*-kimchi with reduction of viable cell counts compared to the control sample. Genome sequencing using Illumina MiSeq showed absence of virulence genes in strain K25 indicating its basic biosafety property. These results suggest that plantaricin K25 can be a suitable alternative to other preservatives used to improve the shelf life of many perishable food products.

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1. Introduction

Fermentation is one of the oldest methods of food preservation and is widely practiced in many Asian countries. Kimchi is a traditional fermented food that originated in Korea and mainly comprises of Chinese cabbages and radishes mixed with minor ingredients such as red pepper, green onions, garlic and fermented fish (Cheigh, Park, & Lee, 1994). The fermentation of kimchi involves a complex succession of microbial communities arising from naturally occurring microflora, especially lactic acid bacteria (LAB) originating from the raw materials used. During fermentation, LAB continuously produced various organic acids lowering the pH, thereby contributing to the good taste of kimchi (Lee & Lee, 2010).

LAB plays precise roles during fermentations of different food products and are commonly referred to as "Generally Recognised as Safe (GRAS)". Various natural metabolites were produced by these microorganisms during sugar fermentation such as lactic acid, diacetyl, hydrogen peroxide and bacteriocins that inhibit the growth of pathogenic and spoilage bacteria while giving the fermented foods distinctive flavours, textures, and aroma (Settanni & Corsetti, 2008). Bacteriocins are a heterogeneous group of ribosomally synthesised proteins with antimicrobial activities generated by selected bacteria during their growth. (Klaenhammer, 1993). Bacteriocins are classified into four major classes according to their structural characteristics: 1) lantibiotics (Class I), 2) small heat stable non-lantibiotics (Class II), 3) large heat-labile non-lantibiotics (Class II) and 4) cyclic peptides (Class IV).

The shelf life and quality of many food products were prolonged by antibiotic or food preservatives (for example, sulphur dioxide) in the past to inhibit or delay microbial growth. Long-term consumption of these artificial chemical preservatives may have an adverse impact on the health of consumers of such food products. The increasing awareness of food quality by consumers has led food

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industries to find alternative ways to preserving food by using natural and additive-free products. Bacteriocins have attracted much attention in food preservation because of their unique purity and non-toxic properties. The uses of bacteriocins produced by LAB as biopreservatives were studied since the last few decades to improve the quality, safety and freshness of food with minimal processing.

In this paper, we identified the bacteriocin-producing strain K25 and described its purification and characterisation. The antimicrobial activities of purified bacteriocin against food spoilage and foodborne pathogenic bacteria were evaluated. Furthermore, the mode of action of the bacteriocin against a target bacterial membrane was elucidated. *In vivo* performance of plantaricin K25 against *Bacillus cereus* in kimchi product samples was assessed. The preliminary safety assessment of strain K25 was studied by examining for the presence of any virulence genes within the genome and the susceptibility of the strain to a number of antibiotics.

2. Materials and methods

2.1. Screening bacteriocinogenic lactic acid bacteria (LAB)

2.1.1. Isolation of LAB

Some Kimchi sample was suspended and serially diluted in sterile peptone water and plated onto De man, Rogose and Sharpe (MRS) agar (Oxoid, England) for isolating LAB. All plates were incubated at 37 °C for 48 h under aerobic condition. Gram-positive, catalase-negative and oxidase-negative bacilli were chosen and screened for bacteriocin production. LAB isolates were cultured in MRS broth for 20 h at 37 °C and the cell-free culture supernatants (CFS) were obtained by centrifuging at 8,000 g for 10 min at 4 °C. The pH of supernatant was adjusted to pH 6 by 5 M NaOH.

2.1.2. Bacterial cultures

All indicator strains used in this study were stored at −80 °C in 30% (v/v) glycerol. *Lactobacillus plantarum* ATCC 8014, *Lactococcus lactis* ATCC 11454, *Streptococcus equisimilis* ATCC 12388, *Streptococcus pyogenes* ATCC 12344, *Micrococcus luteus* ATCC 10240 and *Bacillus cereus* ATCC 14579 were purchased from American Type Culture Collection (ATCC). *Listeria monocytogenes* NCTC 10890 was purchased from National Collection of Type Cultures (NCTC). Other indicator strains such as *Lactobacillus delbrueckii* M8, *Enterococcus faecium* C1, *Weissella confusa* A3, *Staphylococcus aureus* RF122, *Pseudomonas aeruginosa* PA7 and *Escherichia coli* UT181 were obtained from the culture collection of Microbial Biotechnology Laboratory, Division of Microbiology, Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. *L. plantarum*, *L. delbrueckii*, *L. lactis*, *E. faecium* and *W. confusa* were maintained at 37 °C in MRS agar. *Streptococcus equisimillis* and *S. pyogenes* were cultured in Todd Hewitt agar (Difco). Tryptic soy agar (Difco) was used to propagate *M. luteus* and *L. monocytogenes*. *B. cereus*, *S. aureus*, *P. aeruginosa* and *E. coli* were grown in nutrient agar (Oxoid).

2.1.3. Agar well diffusion assay

Screening of antimicrobial assay was performed by agar well diffusion method. Briefly, 20 ml of Mueller Hinton agar (1.7% w/v) was poured into a sterile plate and dried. 10 μl of overnight indicator strain culture (approximately 10^7 cells) was seeded onto the agar plate. Thereafter, 5 mm diameter wells were made in these agar plates and 40 μl of the CFSs were added into each well. These plates were incubated for 18 h at 37 °C and the antimicrobial activity was expressed as the diameter of zone of inhibition.

2.1.4. Identification of strain K25

Carbohydrate fermentation pattern of bacteriocin-producing strain was tested with the API 50 CHL identification system according to the manufacturer's instructions. The results were recorded after 24 h and 48 h of incubation. The sugar fermentation profile was recorded using APILAB Plus Software. Genotypic identification of bacterial strain was confirmed by 16S rRNA gene sequence analysis using 27F and 1492R primers.

2.2. Genetic characterisation and genomic draft of the pln locus of *L. plantarum* K25

Genomic DNA of *L. plantarum* K25 strain was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following manufacturer's instructions. The PCR reaction mixture was prepared in 25 μl of final volume containing EconoTaq® PLUS GREEN master mix (Lucigen), 0.1–0.5 μg of DNA template and 10 μg of each forward and reverse primers. The specific primers used in this study including plnR, plnL, plnK, plnJ, plnM, plnN, plnO, plnP, plnA, plnB, plnC, plnD, plnI, plnEF, plnG, plnH, plnS, plnT, plnU, plnV, plnW and *plNC8βα* and the PCR thermal profiles were set according to Sáenz et al. (2009). The PCR products were then analysed by agarose gel electrophoresis and visualised under UV after staining with GelRed™.

A genome draft was prepared by using Illumina MiSeq paired end sequencing. Briefly, library preparation was performed following NEXTflex™ DNA-seq Library protocol. Genomic DNA was sonicated to obtain 300–600 bp fragment size. Fragmented DNA was subjected to a series of enzymatic reactions and ligation. PCR (10 cycles) amplification of adaptor ligated fragments was performed. The prepared libraries were quantified using Qubit flourometer and validated for quality by running an aliquot on High Sensitivity Bioanalyzer Chip. The genome annotation was then performed using Rapid Annotations Subsystems Technology (RAST) system (Aziz et al., 2008). The genome analysis included the virulence assessment for *L. plantarum* K25 to study the presence of virulent determinants within the genome of the strain K25. The DNA sequences of *pln* genes were obtained from GenBank database and blasted against genome of strain K25.

2.3. Extraction of bacteriocin by adsorption-desorption method and purification of bacteriocin

Bacteriocin producer *L. plantarum* K25 was inoculated into 2 L of MRS medium. The bacteriocin adsorption method was conducted as previously reported (Yang, Johnson, & Ray, 1992) with some modifications. Briefly, *L. plantarum* K25 was grown to stationary phase (20 h) and the pH of the culture broth adjusted to pH 6.0. After centrifugation, the cells were harvested and washed with sterile 5 mM sodium phosphate buffer pH 6.0. The cell pellets were re-suspended in 200 ml of 95% methanol adjusted to pH 2.0 (by the addition of 2 ml of concentrated HCl). The mixture was stirred at 4 °C for 1 h followed by centrifugation at 8000 g for 10 min. The methanol was removed by subjecting the supernatant to rotary evaporation and the residue was dissolved in sodium phosphate buffer (pH 6.0). Bacteriocin activity was determined by agar well diffusion assay as described previously.

The bacteriocin extracted from producer cells was applied to a reversed-phase high performance liquid chromatography (RP-HPLC) on a RP-C18 column (Merck). Mobile phase A consisted of water with 2% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) while mobile phase B was 100% ACN with 0.1% TFA. The bacteriocin was eluted with a linear biphasic gradient of 2–40% ACN over 60 min at a flow rate of 1 ml/min. All peak fractions were pooled, vacuum concentrated and antimicrobial activities were determined. Antimicrobial activity of bacteriocin was expressed in

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