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Research article

Purification, characterization, mode of action, and enhanced production of Salivaricin mmaye1, a novel bacteriocin from *Lactobacillus salivarius* SPW1 of human gut origin

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ABSTRACT

Background: Emergence of antibiotic resistance among pathogenic and food spoilage bacteria such as *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pyogenes*, *Streptococcus sanguinis*, *Streptococcus mutans*, *Bacillus cereus,* and *Listeria monocytogenes* triggered the search for alternative antimicrobials. An investigation aimed at purifying, characterizing, elucidating the mode of action, and enhancing the production of salivaricin from *Lactobacillus salivarius* of human gut origin was conducted.
Results: Salivaricin mmaye1 is a novel bacteriocin purified from *L. salivarius* isolated from human feces. It is potent at micromolar concentrations and has a molecular weight of 1221.074 Da as determined by MALDI-TOF mass spectrometry. It has a broad spectrum of antibacterial activity. Salivaricin mmaye1 showed high thermal and chemical stability and moderate pH stability. The proteinaceous nature of salivaricin mmaye1 was revealed by the complete loss of activity after treatment with pepsin, trypsin, α-chymotrypsin, protease, and proteinase. Salivaricin mmaye1 is cell wall associated, and adsorption–desorption of the bacteriocin from the cell wall of the producer by pH modification proved successful. It exhibited a bactericidal mode of action mediated by pore formation. Its biosynthesis is regulated by a quorum sensing mechanism. Enhanced production of salivaricin mmaye1 was achieved in a newly developed growth medium.
Conclusions: A novel, cell wall adhering, highly potent bacteriocin with a broad spectrum of inhibitory activity, membrane-permeabilizing ability, and enhanced production in a newly constituted medium has been isolated. It has a quorum sensing regulatory system and possesses interesting physicochemical characteristics favoring its future use in food biopreservation. These findings pave the way for future evaluation of its medical and food applications.

1. Introduction

There are increasing reports on antibiotic resistance among pathogens [1,2]. Furthermore, some pathogens have developed multidrug resistance to commercially available antibiotics [3,4,5,6]. Furthermore, consumers' preference for food products containing less chemical preservatives continues to increase [7,8,9]. The use of natural products as antibiotics and food preservatives is receiving increasing attention because they do not significantly alter the microbiota of the human gut unlike synthetic drugs and chemical preservatives [8,10,11].

One of the classes of natural products that have been receiving increasing attention recently is bacteriocins. Bacteriocins are low-molecular-weight peptides that exhibit either a narrow or broad spectrum of inhibitory activities against other microbiota [8]. Bacteriocins are one of the inherent defense systems of bacteria required for effective niche competition [12]. They differ from most antibiotics in that they are ribosomally synthesized and are generally harmless to humans and the environment. Bacteriocins are gaining more interest owing to their novel modes of action and inhibitory activities against several pathogenic multiresistant bacteria. Recent studies have shown that they possess a huge potential in the treatment of multiple human and animal infections, and their potential use as biopreservatives and probiotics are becoming major areas of scientific interest [13]. The fact that bacteriocins are quick acting and potent even at nanomolar concentrations reduces chances of resistance development [13].

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Although bacteriocins are produced by different bacteria, those from lactic acid bacteria (LAB) are generally recognized as safe [14]. Furthermore, the bacteriocins are heat stable and inactivated by gut proteases; their biosynthetic gene cluster are plasmid encoded, thereby facilitating the use of genetic engineering approaches in improving production [15,16]. Heterologous expression of genes encoding bacteriocins in genetically amenable and more robust hosts such as *Escherichia coli* is one of the approaches that can be used to enhance bacteriocin production [17]. Enhanced production of pediocin PA-1 and bactofencin A was achieved by expressing their structural and transporter genes in *E. coli* [17]. The yield and antimicrobial activity of enterocin A when heterologously produced in *Kluyveromyces lactis* and *Pichia pastoris* were higher than those in its native producer (*Enterococcus faecium*) [18].

Lactobacillus salivarius is one of the promising LAB strains with potential probiotic applications. It is mostly found in human, avian, and porcine gastrointestinal tracts, the majority of which are bacteriocinogenic [10,15]. It is suggested that bacteriocin production is an essential attribute of the probiotic *L. salivarius* [10]. To date, *L. salivarius*-derived bacteriocins from mammalian sources are as follows: the two-peptide bacteriocin ABP-118, made up of ABP-118α and ABP-118bβ (4096.69 and 4333 Da, respectively) [19,20]; the two-peptide bacteriocin salivaricin P, made up of Sln1 and Sln2 (4096 and 4283 Da, respectively) [20]; the two-peptide bacteriocin salivaricin T, constituted of SalTa and SalTb (5656.25 and 5270.51 Da, respectively); the one-peptide bacteriocin salivaricin L (molecular weight not determined) [10]; the two-peptide bacteriocin salivaricin CRL 1328, made up of Salα and Salβ (4096.14 and 4333.12 Da, respectively) [21]; bacteriocin LS1 (10 kDa) [22]; bacteriocin-like substance (5 kDa) [22]; bacteriocin LS2 (4115.1 Da) [23]; two-peptide bacteriocin-like inhibitory substance (5655.58 Da and 5269.02 Da) [23]; and bactofencin A (2782 Da) [24]. The bacteriocins isolated from the avian intestine are as follows: bacteriocin FK22 (4331.70 Da) [19], OR-7 bacteriocin (5123 Da) [25], bacteriocin L-1077 (3454 Da) [26], and bacteriocin SMXD51 (5383.2 Da) [27].

Despite the prospects of the bacteriocinogenic *L. salivarius* as a future probiotic, only limited research efforts have been put to unravel its bacteriocins. The mechanism by which it regulates bacteriocin production has not been elucidated. Moreover, modes of action of its bacteriocins remain largely unknown. Furthermore, optimization and scale-up studies are quite limited. In this study, a novel bacteriocin (salivaricin mmaye1) produced by *L. salivarius* SPW1 of human fecal origin was purified and characterized. Its mode of action was investigated. Enhancement of salivaricin mmaye1 production through modification of growth media was studied.

2. Materials and methods

2.1. Bacterial strains and culture media

All streptococci (except *Streptococcus mutans*), enterococci, *Bacillus cereus*, *Micrococcus luteus,* and *Lactococcus lactis* were obtained from American Type Culture Collection (ATCC). *Listeria monocytogenes* NCTC 10890 was obtained from National Collection of Type Culture (NCTC). *Staphylococcus aureus* RF122, *S. mutans* GEJ11, *Pseudomonas aeruginosa* PA7, *Corynebacterium* spp. GH17, *Escherichia coli* UT181, *Lactobacillus plantarum* K25, *Lactobacillus agilis* Yanat1, and *Lactobacillus pentosus* CS2 were taken 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. salivarius* SPW1, *L. plantarum* K25, *L. agilis* Yanat1, and *L. pentosus* CS2 were maintained on De Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany). All streptococci were maintained on Todd-Hewitt agar (Difco, Le Pont de Claix, France). *M. luteus* ATCC 10240, *B. cereus* ATCC 14579, *S. aureus* RF122, *P. aeruginosa* PA7, *Corynebacterium* spp. GH17, and *E. coli* UT181 were maintained on Mueller-Hinton agar (Merck, Darmstadt, Germany). *E. faecium* ATCC BAA-2127 and *E. faecium* ATCC 349 were maintained on Tryptic soy agar (Merck, Darmstadt, Germany), whereas other enterococcal strains and *L. monocytogenes* NCTC 10890 were maintained on Brain Heart Infusion agar (Merck, Darmstadt, Germany). *L. lactis* ATCC 11454 was maintained on M17 agar (Merck, Darmstadt, Germany) supplemented with 5% glucose (Merck, Darmstadt, Germany).

2.2. Isolation, identification, and screening of LAB for bacteriocin production

MRS broth (Merck, Darmstadt, Germany) was inoculated with human feces from a healthy adult human subject and incubated aerobically for 24 h at 37°C. The culture was serially diluted in peptone water (Merck, Darmstadt, Germany), and LAB was isolated by growing on the MRS agar plate (Merck, Darmstadt, Germany) at 37°C. MRS broth was inoculated with single colonies from a 24 h old MRS agar LAB culture and incubated aerobically at 37°C for 24 h. Screening of LAB for bacteriocin production was carried out using cell-free supernatant (CFS) by a well diffusion assay. MRS agar used for well diffusion assay was supplemented with 0.1% $CaCO_3$ (Friedemann Schmidt Chemical, Germany). *S. aureus* RF122, *M. luteus* ATCC 10240, *Streptococcus pyogenes* ATCC 12344, *Streptococcus sanguinis* ATCC 10556, *S. mutans* GEJ11, *B. cereus* ATCC 14579, and *L. monocytogenes* NCTC 10890 were used as indicators because they are the bacterial targets of interest.

Molecular identification of LAB was conducted by amplifying 16S rRNA gene by PCR using the universal primers 27F (5′-AGAGTTTGATC (A/C)TGGCTCAG-3′) and 1492R (5′-ACGG(C/T) TACCTTGTTACGACTT-3′). The 16S rRNA gene was sequenced, and a similarity search was performed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3. Purification, determination of molecular weight, and molar extinction coefficient of bacteriocin

L. salivarius SPW1 was grown in the MRS medium for 18 h in a bioreactor (Sartorius Stedim, Germany) at 37°C, with an impeller agitation speed of 150 rpm; the medium was then centrifuged (10,000 rpm for 20 min at 4°C) to collect the supernatant, which was subsequently filtered using a 0.22 μm filter to obtain a CFS. This was subjected to hydrophobic interaction chromatography in which acetonitrile (Merck, Darmstadt, Germany) gradient (20%, 40%, 60%, and 80% v/v) was used for elution of the peptides adsorbed onto the surfaces of amberlite XAD-16 particles (Sigma-Aldrich, St. Louis, USA) packed in a glass column. Fractions were evaporated, and activity was determined by a well diffusion assay. The active fraction was subjected to reverse-phase high-pressure liquid chromatography (RP-HPLC). The mobile phase consisted of two solvents: solvent A (95% Milli-Q water [Millipore, USA] and 5% acetonitrile [Merck, Germany]) and solvent B (100% acetonitrile). Elution was performed using a biphasic gradient of 20–80% (v/v) acetonitrile at a flow rate of 1 ml/min for 90 min. Fractions were collected and evaporated using a centrifugal vacuum evaporator (Thermo Fisher Scientific, Vantaa, Finland), and activity was tested. The molecular weight of the bacteriocin was determined by subjecting the active HPLC fraction to matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. To ascertain the molar extinction coefficient, twofold dilutions of the bacteriocin were prepared, and the bacteriocin concentration was expressed in molar units. Absorbance at 280 nm was measured, and a standard curve was generated and used for determining the molar extinction coefficient.