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Characterization of Antimicrobial Peptide Produced by Probiotic *Lactobacillus plantarum* DM 69 Isolated from Yogurt Sample of Odisha, India

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A B S T R A C T

The aim of this study was to characterize the antimicrobial peptide (bacteriocin) produced by a probiotic *Lactobacillus plantarum* strain DM 69 isolated from yogurt sample of Odisha. Total DNA of *L. plantarum* DM 69 was extracted and tested for the presence of bacteriocin genes. PCR-amplified DNA showed a single band in gel which partially confirmed the presence of Plantaricin gene responsible for showing antimicrobial activity. The purified antimicrobial peptide was found to be stable at 60 ^{0}C, 80 ^{0}C up to 60 min and 100 ^{0}C for 30 min, pH 2-8, 1-8% NaCl and in presence of digestive enzymes such as α-Amylase, trypsin, lipase and lysozyme.

Introduction

Antimicrobial peptides produced by *Lactobacillus* species are small and thermo-stable which are known as bacteriocin. It is categorized in three classes (Cotter *et al.*, 2005). Class I bacteriocins are named as Lantibiotics. They are small (<5 kDa) and thermostable peptides with residues of lanthionine and methyl lanthionine (thioether amino acids). Furthermore, non-lantibiotics are considered as class II bacteriocin which are small (≤10 kDa), thermo-stable and non-lanthionine containing peptides. Classes III are thermo-labile sometimes hydrophilic proteins or protein complexes consisting of phospholipids and/or carbohydrates. Deegan *et al.*, (2006) divided Class I bacteriocins into subclasses Ia, composed of long, flexible and positively charged peptides, which act on the cytoplasmic membrane by pore formation, and subclass Ib, composed of spherical, rigid, and neutral or negatively charged peptides.

The class II was also divided into two subclasses: IIa, which contains bacteriocins presenting the N-terminal consensus sequence YGNGVXCXXXXCXV and IIb, which is composed of bacteriocins that require two peptides for antimicrobial activity. Research has focused mostly on bacteriocins belonging to class I and IIa, since they are the most

abundant and have potential for industrial application.

Some studies done in the past have suggested that certain strains of *L. plantarum* are capable to produce more than one peptide with synergistic antibacterial effect, possibly class IIb bacteriocins (Maldonado *et al.*, 2003). Since then, there was a great advance on the knowledge on properties of bacteriocins and this study contributes with the description of molecular identification of a antimicrobial peptide produced by a *L. plantarum* strain isolated from yogurt sample of Odisha.

Materials and Methods

Bacterial culture and growth condition

A probiotic strain *Lactobacillus plantarum* DM 69 (Accession no KT716348) was isolated from yogurt sample of local region of Odisha. It showed antimicrobial property against several pathogens like *E. coli* ATCC 25922, *Bacillus cereus* ATCC 10702, *Salmonella typhi* MTCC 3216, *Salmonella enterica* ATCC 35640, *Staphylococcus aureus* ATCC 29213 and *Staphylococcus aureus* MTCC 902.

Molecular identification of antimicrobial gene present in *L. plantarum* DM 69

It was carried out in 3 steps such as

Genomic DNA isolation
Primer design
PCR detection of targeted gene

Genomic DNA isolation

The bacterial culture was inoculated into freshly prepared sterile MRS broth and incubated at 37 ^{0}C for 24 h. After confirmation of purity, 2 mL aliquot of active log phase culture from this broth was then used to isolate genomic DNA. To the 2 mL culture, 2–3 µL of a sodium salt of ampicillin solution (50 mg/mL) was added and the mixture incubated at 37 ^{0}C for 1 h and centrifuged at 5000 rpm for 5 min in a refrigerated centrifuge. The media supernatant was decanted and the pellet washed thrice with 1 mL of NaCl EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0). The washed bacterial pellet was resuspended in 100 µl of NaCl-EDTA (30 mM NaCl, 2 mM EDTA, pH 8.0) and 100 µL of freshly prepared lysozyme solution (concentration 10 mg/mL in NaCl-EDTA) was added to it and mixed. The mixture was incubated at 37 ^{0}C for 1 h with intermittent shaking. To remove RNA, 4 µl of Rnase-A solution (Stock 10 mg/mL, working concentration 100 g/mL) was also added to the mixture before incubation. The volume of the mixture was then made up to 500 µL with additional NaCl-EDTA and 50 µL of a 10% SDS solution followed by 10 µL of proteinase K solution (20 mg/mL) were added to the mix. The contents were mixed thoroughly and incubated at 55 ^{0}C for 1 h. After incubation, an equal volume of Tris-saturated phenol (pH 8.0) was added and mixed thoroughly. The resultant mixture was centrifuged at 10000 rpm at 22 ^{0}C for 10 min and the upper aqueous phase was separated without disturbing the interphase containing cell debris and proteins. This step was repeated once with a fresh aliquot of Phenol-Chloroform mixture (1:1) and the supernatant was collected in a sterile eppendorf tube. DNA in the supernatant was precipitated out with 0.8 volumes of Isopropanol in the presence of 0.3 M Sodium Acetate (pH 5.2). The precipitated DNA was pelleted by centrifugation at 10000 rpm at 4 ^{0}C for 5 min. The supernatant was discarded and the DNA pellet was washed once with freshly prepared 70% ethanol and air-dried. The final pellet thus obtained was dissolved in 50 µL Tris-EDTA (10:1, pH 8.00) and stored frozen at -20 ^{0}C till further analysis (De *et al.*, 2010).

Primer used for this study

PlanS-F5'GCCTTACCAGCGTAATGCCC3' and PlanS-R5' CTGGTGATGCAATCGTTA GTT T 3' is the pair of primer which was used in this study to amplify the target antimicrobial gene (Plantaricin) having expected size approximately 450 base pair (bp) (Stephens *et al.,* 1998; Barbosa *et al.,* 2016).

PCR detection of targeted gene

Total DNA was extracted and submitted to amplification in a reaction mixture (20 ml) containing approximately 25 ng/ml of extracted DNA, PCR buffer, 100 mM $MgCl_2$, 200 mM dNTPs, 0.025 U Taq polymerase and 1 pmol each primer.

Amplification was achieved in 35 cycles using a DNA thermocycler Master Cycler® PCR (Eppendorf Scientific, Germany). PCR amplified DNA was separated by 0.8%-2.0% (w/v) agarose gel electrophoresis and band size was determined.

Effect of different treatment on stability of purified antimicrobial peptide

The stability of purified antimicrobial peptide was determined by exposing it to different ranges of temperature, pH, salt (NaCl) concentrations and enzymes (Tiwari and Srivastava, 2008; Francois *et al.,* 2013).

Effect of heat treatment

To estimate the thermo stability of purified antimicrobial peptide, 200 µL of purified sample was tested by heating it at various temperatures. It was exposed to heat treatment in a water-bath at 60 ^{0}C, 80 ^{0}C and 100 ^{0}C for 15, 30, 45, 60 and 90 min. The residual antimicrobial activity was assayed by the well diffusion method against test pathogens.

Effect of pH treatment

The antimicrobial peptide was re-suspended in 1:1 ratio of different buffer solutions ranging from pH 2.0-10.0 (50 mM acetate buffer, pH 2.0-5.0; 50 mM potassium phosphate buffer, pH 6.0 and 7.0; Tris-Cl, 50 mM, pH 8.0-10.0) and incubated for 2 h at room temperature to examine the sensitivity of the compound towards several pH values. The antimicrobial activity was detected.

Effect of salinity treatment

Susceptibility of the antimicrobial peptide to various salt (NaCl) concentrations of 2.0%, 4.0%, 6.0%, 8.0% and 10.0% was performed by using NaCl salt. After 5 h of incubation at room temperature the activity was determined.

Effect of enzymes

In a separate experiment, the purified antimicrobial compound was treated with α-Amylase (in 0.05 M phosphate buffer, pH 8.0), trypsin (in 0.05 M Tris-HCl buffer, pH 8.0), lipase (in 0.05 M phosphate buffer, pH 7.0) and lysozyme (in 0.05 M phosphate buffer, pH 7.0) at 1 mg/mL final concentrations, and incubated at 37 ^{0}C for 2 h. The residual antimicrobial activity was assayed by the same well diffusion method.

Results and Discussion

Molecular identification of antimicrobial gene

Bacteriocinogenic strain *L. plantarum* DM 69 was analyzed in PCR reactions for the presence of known bacteriocin gene (Plantaricin) responsible for the antimicrobial activity. A primer pair (5' GCCTTACCAGCGTAATGCCC 3'; 5'CTGG TGATGCAATCGTTAGTTT 3') which has been used in the study, amplify the Plantaricin

gene. The amplification was achieved with this set of primer by obtaining an amplicon having size approximately 450 bp by using a gradient PCR. This size was quite similar with expected amplicon size. The single band in gel partially confirmed the presence of a Plantaricin gene responsible for showing antimicrobial activity. Annealing temperature was varied in between 45 and 55 ^{0}C to see the effect of temperature on the specific amplification. The annealing temperature was found to be best at 46.6 ^{0}C for the amplification of bacteriocin encoded gene of *L. plantarum* DM 69. Our results were also in accordance to the report on the presence of bacteriocin encoded gene in *L. plantarum* LPCO10 (Stephens *et al.,* 1998). Again, similar set of primer has been used in Song *et al.,* (2014) to amplify the same Plantaricin gene where expected amplicon size was 450 bp. This study was in accordance of our result. This gene not only has high activity against Gram-positive bacteria, but also has strong activity against Gram-negative bacteria, such as *Escherichia coli* and *Salmonella* spp.

Estimation of stability of purified antimicrobial peptide (bacteriocin) against several physico-chemical factors

Estimation of stability of purified antimicrobial peptide against several factors was described briefly in table 1. Results of the effect of temperature revealed that antimicrobial compound produced from *L. plantarum* DM 69 showed heat stability after treatment at 60 ^{0}C, 80 ^{0}C up to 60 min and 100 ^{0}C for 30 min by showing zone of inhibition.

The zone of inhibition having more than 10mm was considered as positive result. Again, antimicrobial peptide was found to retain its activity in pH 2.0-8.0. Additionally, stability of antimicrobial peptide of *L. plantarum* DM 69 was observed in 2.0%, 4.0%, 6.0% and 8.0% NaCl concentrations. The inhibitory peptide was fully inactivated by proteolytic enzyme trypsin. But α-amylase, lysozyme and lipase had no effect on the activity.

Similar result was observed in Francois *et al.,* (2013). As per their study, bacteriocin from *L. plantarum* 29V (P29V) showed strong heat stability. It could still be recorded upon treatment at 100 ^{0}C for 120 min and 121 ^{0}C for 30 min respectively. It was also active in pH 2.0-10 and NaCl range of 1-7%. As per the reports of Srinivasan *et al.,* (2013), the purified bacteriocin was found to be heat-stable since it was showing the inhibitory activity even after boiling at 100 ^{0}C for about 15 min. The activity of this bacteriocin was also found to be retained in the pH range of 2.0-8.0 during 24 h of incubation. The purified bacteriocin was found to be stable over a pH range of 2.0-8.0. Certain enzymes like amylase, lipase, and catalase could not destroy the bacteriocin activity. As per Amina *et al.,* (2015), Plantaricin MZ was found to be heat resistant to 121 ^{0}C for 15 min. At temperatures of 60, 70 and 80 ^{0}C for 30 min, 100% activity was retained and stable. Stability of the compound was also found in pH range from 2-10. It was sensitive to pepsin, trypsin, chymotrypsin and proteinase-K but insensitive to catalase confirming that inhibition was due to proteinaceous molecule (bacteriocin). Its activity was not reduced by lipase indicating that there was no structural modification by lipid moiety. All these reports corroborate our result and proved that antimicrobial compound produced by *L. plantarum* DM 69 is stable under certain adverse condition.

The experimental results showed that probiotic *L. plantarum* DM 69 produces the promising novel bacteriocin possibly, plantaricin, which exhibits the antimicrobial activity against *E. coli*, *Bacillus cereus*, *Salmonella typhi*, *Salmonella enterica* and *Staphylococcus aureus.*

Table.1 Stability of the antimicrobial peptide (bacteriocin) produced by *L. plantarum* DM 69

Sl no	Parameters	Conditions	Incubation period	Zone of inhibition
1.	Temperature	60 ^{0}C	15 min	+ve
			30 min	+ve
			45 min	+ve
			60 min	+ve
			90 min	-ve
		80 ^{0}C	15 min	+ve
			30 min	+ve
			45 min	+ve
			60 min	+ve
			90 min	-ve
		100 ^{0}C	15 min	+ve
			30 min	+ve
			45 min	-ve
			60 min	-ve
			90 min	-ve
2.	pH	2.0-8.0	2 hr	+ve
3.	NaCl	2.0%	5 hr	+ve
		4.0%	5 hr	+ve
		6.0%	5 hr	+ve
		8.0%	5 hr	+ve
		10.0%	5 hr	-ve
4.	Digestive enzymes	α-Amylase, Lipase and Lysozyme	2 hr	+ve
5.	Proteolytic	Trypsin	2 hr	-ve

This peptide was found to be stable in wide range of pH, heat and NaCl concentrations *i.e.*, it exhibits antimicrobial activity against indicator pathogens in such adverse conditions. Based on these characteristics, it is considered that antimicrobial peptide (plantaricin) can be used in food and drug industries.

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