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Assay of a Novel Antimicrobial peptide Curvacin A produced by *Lactobacillus curvatus* isolated from fermented food products and its Molecular characterization

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Samples of dairy origin and other fermented vegetable samples were screened for the presence of Lactic acid bacteria specifically for *Lactobacillus curvatus*. The collected samples were tested for antagonistic activity through well diffusion assay. The supernatant was partially purified and concentrated through ammonium sulphate precipitation and the same was used as a sample for determining antibacterial activity of bacteriocin against selected human gastro intestinal pathogens adopting Kirby Bauer disc diffusion assay. Bacteriocin curvacin A was found to inhibit a wide range of bacteria. Resistant nature of commercial antibiotic against lactic acid bacteria was also screened. As a part of molecular characterization of curvacin A, peptide sequence was also done using LCMS – IT- TOF mass spectrophotometry. A short peptide with 6257 dalton mass with 59 amino acid residue was obtained.

Antimicrobial peptide / Curvacin A

Probiotic cultures have been associated historically with cultures of milk and dairy products, from which there is a substantial evidence for positive effects on human health and general well being. Several in vitro and in vivo experiments on antagonism of different *Lactobacillus* strains against *Helicobacter spp. Clostridium spp,. Campylobacter jejuni* and *E. coli* were made (Cornelium et al. 2002). All tested human *Lactobacillus* strains were able to inhibit the growth of all strains of facultative anaerobic human gastrointestinal pathogens.

In recent years, numerous reports have been published on antimicrobial peptides like bacteriocins of proteins from lactobacilli at different environment. However only two reports mention bacteriocin production in *Lactobacillus curvatus* isolates which were obtained from Japanese grass leaves and from a human vaginal samples (Brink et al.1994).

Bacteriocins are secreted oligo peptides proteins or protein complexes with anti microbial activitiy against strains taxonomically related to the producer organism. In recent years numerous reports have been published on antimicrobial peptides produced by Gram positive bacteria including LAB and bacteriocins of lactobacilli from different environments have been described. Bacteriocin production in LAB isolated from animal fecal samples in order to determine their spectrum of action including human pathogens and their significance in the ecology of gastro intestinal environment was detected.

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Bacteriocins are ribosomally synthesized single polypeptides or post translational modified ones that are usually inhibitory only to closely related bacterial species (Reddy et al. 1984). Many LAB produce a high diversity of different bacteriocins. In recent years lab bacteriocins have generated interest due to their potential as a safe biopreservatives that could at least partially replace chemical preservatives. Though these bacteriocins produced by LAB are found in numerous fermented foods and other resources, nisin A is currently the only bacteriocin widely used as a food preservative (Graciela et al.1995). Many LAB bacteriocins have been characterized biochemically and genetically, while many aspects of these compounds are still unknown.

Usage of bacteriocins as a natural food preservative is increasing during last decades. But the in depth research on antibacterial properties of lactic acid bacteria have not been fully carried out. Maximal production of curvacin A, a type of bacteriocin of *Lactobacillus curvatus* could be obtained when culture medium is supplemented with growth regulating factors such as carbon and nitrogen sources, vitamins and also regulating pH of the medium and growth temperature.

Much of the work has not been done on a novel peptide curvacin A, produced by *Lactobacillus curvatus*. *Lactobacillus curvatus* is invarialbly found in raw cattle milk and also in fermented milk products. It is curve and bean shaped rods with rounded ends (Shiou et al. 2006). It occurs in pairs and short chains; closed rings of usually four cells or horse shoe forms frequently observed. They are at first motile; lost motility on subculture. The basis of the present study is to evaluate the efficiency of bacteriocin curvacin A produced by *Lactobacillus curvatus*, and also to find out the antagonistic activity of antimicrobial peptide against common human pathogenic bacteria. Partial purification and molecular characterization of curvacin A was also proposed in the work.

Material and Methods

Bacterial strains and media

The strains of *Lactobacillus curvatus* were isolated from fermented milk products like curd and cheese and sour dough (Daba et al.1991). They are identified based on morphological, cultural and biochemical characteristics described by Bergy's Manual of Systematic Bacteriology. The pattern of fermentation of carbohydrate was also performed to identify the isolates at species level. Lactobacilli specific medium de Mann Rogosa and Sharpe (MRS) broth and agar media was used in the study for isolation and maintenance of Lactobacilli strains.

Bacteriocin assay

The supernatant fluid of bacteriocin producing *Lactobacillus cuarvatus* was obtained from 24 h grown MRS broth culture media and a two fold dilution was made. The pH of the supernatant was adjusted to 7.0. The antibacterial activity in each dilution was determined by agar well diffusion method against respective sensitive indicator strain of *Lactobacillus curvatrus.* The titre was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in arbitrary units (AU) per ml (Fricourt et al.1994).

Antibacterial susceptibility test

Antibacterial susceptibility test was performed based on Kirby Bauer's disc diffusion method. LAB was grown overnight in MRS broth at 37º C under aerobic conditions. 20µl of supernatant of the culture broth was impregnated into 6mm dia filter paper disc. All the disc were kept for 10 min for absorbance and placed carefully on the surface of MRS agar plates swabbed with *Staphylococcus aureus* culture. Commercial antibiotic disc were also simultaneously used for comparison. Amikacin (10µg), chloromphenicol (20µg), gentamycin (20µg), penicillin G (10U), tetracycline (20 µg) and erythromycin (30µg) were also used for inhibition test. All the culture plates were incubated at 37º C for 24 h. The diameter on zone of inhibition were measured and recorded.

Partial purification of curvacin A

Curvacin A producing *Lactobacillus curvatus* isolates were cultured in 500 ml of MRS broth for 24h at 37º C. Supernatant was separated using centrifugation (10000 x g, 10 min 4º C) and were precipitated with 70 % Ammonium sulphate solution. The precipitate was collected by centrifugation (10000 x g, 1hr, 4º C) and the pellet was re suspended in 10 ml ammonium acetate buffer (pH 6.5).

Sequencing of curvacin A gene

The complete amino acid sequence of curvacin A was elucidated using Shimadzu LCMS-IT-TOF Mass spectrometer which combines IT (ion trap) and TOF (time of flight).The peptide was run on a native poly acrylamide gel and the peptide in the gel was subjected to liquid chromatogram and spectroscopic analysis (Piard et al.2006).

Results

Isolation of Lactobacilli from samples

In the present study, totally 156 samples were collected from the retail outlets of Tiruchengode region of Namakkal district, Tamilnadu, India during a period of 2 years from 2009 to 2010. The samples collected for the screening of presence of Lactobacilli ranges from milk, curd, fermented fruits, fermented vegetables and chicken meat. Out of 156 samples, 51 (33%) showed positive results for lactobacilli (Table -1).

Table - 1: Distribution of Lactic Acid Bacteria in samples collected

S. No	Sample Source	Total No. of Samples collected	Total No. of Lactobacilli isolated	% of occurrence of Lactobacilli
01	Milk	42	20	47.6
02	Curd	38	12	31.5
03	Fermented fruits	34	09	26.4
04	Fermented vegetables	22	06	27.2
05	Chicken meat	20	04	20.0

Table- 2: Effect of antibiotics on the isolates of *Lactobacillus curvatus*

S. No.	Antibiotics	LC01	*LC04*	LC05	LC07	LC09
01	Tetracycline (10ug)	R	*R*	R	R	R
02	Gentamycin(20ug)	R	*R*	R	R	R
03	Penicillin (10U)	R	*R*	R	R	R
04	Erythromycin (20ug)	S	*S*	S	S	S
05	Amikacin (10ug)	R	*R*	S	R	R
06	Chloromphenicol (20ug)	R	*R*	R	S	S

R – Resistant; S – Sensitive

Table- 3: Peptide sequence of Curvacin A Produced by *Lactobacillus curvatus*

Length	Mass (Dalton)	Peptide sequence
59aa	6257	MNNVKELSMTELQTITGGARSYGNGV YCNNKKCWVNRGEATQSIIGGMISGWASGLAGM

Antibiotic susceptibility of isolates

Antibiotic susceptibility of isolates was tested using Kirby Bauer disc diffusion technique. All the isolates were resistant to gentamycin, penicillin and tetracycline and the isolates showed sensitivity towards other antibiotics tested (Table -2).

Antagonistic activity

The antagonistic effect *of Lactobacillus curvatus* against *Escherichia coli* was done on MRS agar by well diffusion method. Wide inhibitory zone was observed in strains isolated from milk and curd. Other strains showed less antagonistic activity against test pathogen (less than 6 mm).

Peptide sequencing of caurvacin A

The complete amino acid sequence of curvacin A was elucidated using Shimadzu LCMS IT-TOF mass spectrophotometer. It contains 59 amino acid residues. The molecular mass of the peptide was found to be 6257 daltons (Table 3).

Discussion

Milk and dairy products provide a rich source of valuable proteins, minerals and vitamins. The nutritional significance of proteins includes macronutrient as well as physiological and functional aspects. Besides bioactive proteins, dairy products may also provide peptides (Tina et al.2006). These are formed by enzymatic digest of intact proteins, which themselves are not necessarily bioactive. If enzymatic activation is required to generate bioactive peptides, proteolysis has to be considered the major event of value addition in dairy products (Jimenez et al. 1993). This study reports the presence of potential antibacterial peptide, curvacin A in *Lactobacillus curvatus* isolated from different food sources.

Lactic acid bacteria are widely naturally present in many dairy products, which enhances the value of the products in terms of nutrition. They also confer natural immunity to human body against gastro intestinal pathogens. It is due to the synthesis of natural peptides by selective lactic acid bacteria (Joerger and Klaenhammer, 1980). The isolates LC01, LC04, LC05, LC07 and LC09 were able to inhibit the pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli* in plates treated with the supernatant *of Lactobacillu curvatus* after 24 h. Resistance to the commercial antibiotics like gentamycin., chloromphenicol, penicillin erythromycin and amikacin are appeared to be plasmid borne and thus these traits are transferable (Biswas *et al.*1991). But synthesis of curvacin A was not found to be plasmid borne and hence the trait is non transferable.

Antimicrobial peptides from food protein deserve attention due to their mechanism of activity, which makes microbial resistance improbable and for increasing the functional values of foods. Milk proteins are currently the main source of bioactive peptides. Most of these bioactivities in milk are encrypted within the primary structure of milk proteins, requiring proteolysis for their release from precursors. Proteolysis may release the biogenic peptides during gastrointestinal transit or during food processing (Messaoudi *et al.* 20113).

Compared with other bioactivities (eg. Anti hypertensive peptides), only a few reports have considered the enzymatic release of antimicrobial peptides in milk and dairy products. Among various peptides studied, curvacin A is a heat stable and low molecular mass peptide bacteriocin. Curvacin A displayed a concentration dependent bactericidal effect towards a bacteriocin sensitive strain without causing concomitant cell lysis of the indicator organisms. Amino acid composition analysis of curvacin A revealed a strongly hydrophobic peptide. However, N terminal amino acid sequencing was not possible, because the N terminal end was blocked (Tagg et al.1976). This may indicate the presence of modified amino acids, for instance as a result of post translational modifications. It was further found that curvacin A activity is stable at wide range of pH (4.0 – 10.0). Taking all these biochemical characteristics, curvacin A produced by *Lactobacillus curvatus* appears to belong to the class II lactic acid bacterium bacteriocins according to the classification of Klaenhammer (Balasubramanyan and Varadaraj,1998).

Conclusion

The bacteriocin curvacin A can be used as a bio preservative agent to enhance the nutritional and microbiological quality of dairy products and thus they act as a natural probiotic agents even for infants.

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