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Studies on the Mode of Action of Bacteriocin Produced by *Lactobacillus fermentum* CrT21

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Abstract: ***Lactic acid bacteria (LAB) are very important preservative microflora involved in the fermentation of food products due to their ability to produce organic acids, bacteriocins and other bioactive antimicrobial metabolites. LAB in this study were isolated from cassava waste and retting, screened for their ability to produce bacteriocin and the bacteriocin was subsequently characterized. Lactobacillus fermentum CrT21 produced bacteriocin with crude and purified activity units of 1280 and 2560 AU/ml respectively; purification was done by ammonium sulphate precipitation and through 1, 10 and 20 KDa ultrafiltration membranes. The bacteriocin retained its inhibitory activity over a wide range of temperature (40^0-100^0C), pH (2-10) and in presence of metals, surfactants and enzymes (lipase and lysozyme); the activity was further characterized to cleave the peptide bond between D-glutamic acid and L-lysin along the peptide stem chain of L. fermentum CpL08 peptidoglycan wall.***

Keywords: Lactic acid bacteria, bacteriocin, activity units, *Lactobacillus fermentum*

1. Introduction

Most known Lactic acid bacteria (LAB) are non-pathogenic, low-GC containing, acid-tolerant, and generally recognized as safe (GRAS) microorganisms. They are generally gram-positive, non-spore forming, cocci or rods, and produce lactic acid as the major end product of carbohydrate fermentation [1], they include the genera *Lactococcus, Streptococcus, Leuconostoc, Pediococcus* and *Lactobacillus;* however, some taxonomic submissions on LAB classification included the genera: *Enterococcus, Carnobacterium, Aerococcus, Tetragenococcus, Weissella* and *Vagococcus* [2].

Improving intestinal health through the activities of bacteriocins of dairy product associated LAB is extremely important to expand the potential application of these food products to better improve human health. Bacteriocins, including those produced by lactic acid bacteria, have been defined as ribosomally synthesized primary or modified bioactive peptides, with bactericidal or bacteriostatic effect on other (usually closely related) species [3].

LAB produce a wide range of proteinaceous bacteriocins and bacteriocin-like extracellular metabolites [4], their bactericidal mechanisms vary and many include pore formation on the cell wall of sensitive cells [5]. Bacteriocins produced by LAB strains have the potentials to provide an additional platform for controlling several food spoilage and many pathogenic microorganisms. Bacteriocins with broad spectrum activities have been reported; among these are bacteriocins, particularly from *Bacillus* and *Pseudomonas* species, with inhibitory activities against spoilage fungi of agricultural products [6]. *Lactobacillus fermentum* is frequently isolated from mucosal surfaces of healthy humans and fermented foods [7] and based on its probiotic properties (especially, an array of bacteriocin production), it has been characterized to have beneficial effects on the gastrointestinal tract and has also been used as an alternative treatment for urogenital infection.

So far, studies on bacteriocins have concentrated on their genetic features (coding and reading), process of production, transportation, expression, immunity, susceptibility, mode of action of individual bacteriocin on susceptible strains, as well as the identification and characterization of novel bacteriocins from microorganisms that are not previously characterized as bacteriocin producers or the establishment of new bacteriocins from known producers [8]. This current work is as such geared towards designing and establishing sensitive procedural steps to screen for and characterize bacteriocins from lactic acid bacteria and infer the results of such characterization.

2. Materials And Methods

2.1 Isolation of Lactic Acid Bacteria

Lactic acid bacteria were isolated from cassava waste and retting generated from a fabricated cassava processing machine. 20 g sample in each case was weighed into filtered stomacher bags and subsequently mixed with 200 ml of presterilzed peptone water (0.1% v/v). The resulting mixture was blended at 280 rpm for 5 minutes and serially diluted; 80µL of each dilution was pour-plated onto de Man, Rogosa and Sharp (MRS) agar. Plates were incubated both under aerobic and anaerobic conditions at 37°C for 48 hours, pure cultures were characterized through identification manuals and preserved in equal volume of MRS broth in sterile glycerol (30% v/v) until they were required for analysis.

2.2 Screening and production of bacteriocin from LAB

The agar diffusion bioassay described by Yanagida [9] was used to screen for bacteriocin producing LAB. One ml of each indicator cell suspension (5×10^5 cfu ml^{-1}) was inoculated into 15 ml of MRS soft agar maintained at 50°C and then poured into a petri dish. After solidification, this method was then modified by cutting three 5 mm diameter wells in the agar and each well was thereafter filled with

50µl of either treated or untreated cell-free supernatant (CFS) from each producer LAB isolate.

To prepare the CFS, one ml (0.5 McFarland) of the LAB isolate was cultured overnight in 20 ml MRS broth, cells were then removed by centrifugation at 10,000 g for 5 min. The supernatant was thereafter filtered through a sterile 0.22µm syringe filter, this was then divided into three fractions; one left unadjusted, the second fraction was adjusted to pH 6.0 with 1M NaOH in order to neutralize the possible effects organic acids within the metabolite while the third fraction was neutralized with 1M NaOH and then treated with 1 mg ml^{-1} of catalase at 25°C for 30 min to eliminate the possible effects of both organic acid and the antimicrobial effect of H_2O_2 respectively. These fractions were filtered and 5.0 µl each of the unadjusted and the adjusted CFS aliquots were added separately to each of the bored wells. The prepared plates were replicated and incubated at 37°C for 24 h. Inhibition zones were measured and scored positive if inhibition against the indicator lawn was equal to or greater than 2mm [10]. However, if inhibition exists against indicator lawn from the NaOH and catalase adjusted metabolite well, the metabolite is scored positive for possible bacteriocin content for further characterization.

2.3 Purification and Characterization of bacteriocin

The first purification step of cell free bacteriocin from *Lactobacillus fermentum* CrT21 was done by protein precipitation with the addition of solid ammonium sulphate to 0% - 70% saturation followed by 70% - 90% saturation and centrifuged after storing overnight at 4°C [11]. The mixture was stirred for 2 h at 4ºC and later centrifuged at 20,000 rpm for 1 h (4ºC). The centrifuged precipitates were pooled, re-suspended in of 0.05 M potassium phosphate buffer (pH 7.0) to the initial volume of culture filtrate; the resulting solution was thereafter dialyzed against 0.05M potassium phosphate buffer (pH 7.0) in a tubular cellulose membrane (spectra por number four dialysis tube) for 16 hours at 4°C for further purification. Assay of the bacteriocin activity was thereafter carried out in both the precipitate and supernatant [12].

The partially purified bacteriocin fraction collected from the ammonium sulphate precipitation procedure was then further purified through ultrafilteration technique. The Fraction was resuspended in 50mM potassium phosphate buffer (1:30 v/v, pH 7.0). 1ml aliquot was then ultrafiltered through 1, 10 and 20 KDa molecular exclusion sizes of filtron membranes. Bacteriocin activity was determined in eluted fractions [12], while protein concentration of each purification fraction was determined by the Bradford method [13].

2.4 Effect of various chemicals, temperature, pH and enzymes on purified bacteriocin

To determine the effect of temperature on the activity of purified bacteriocin from *L. fermentum* CrT21, 400µl of this metabolite was exposed to various heat treatments: 40, 80, 100 and 121ºC for 60 minutes [14], cooled to room temperature and then assayed for residual bacteriocin activity. Effect of pH on the partially purified bacteriocin was determined by adjusting the pH of the bacteriocin to 2.0, 4.0, 6.0, 8.0 and 10 using hydrochloric acid (HCl) and sodium hydroxide (NaOH). The treated metabolite in each case was incubated for 60 minutes at room temperature, then neutralized and assayed for residual activity [15].

To examine the effect different enzymes on the bacteriocin, 1 mg/mL final concentration of enzymes (trypsin, lysozyme and lipase) were added, at room temperature, to the bacteriocin for 60 minutes. The effect of surfactants on the purified bacteriocin was determined by adding Triton X-100, Tween 20 and Tween 80 to the bacteriocin to a final concentration of 1% (v/v), while 0.1g/mL Ethyleledіaminetetraacetic acid (EDTA) and Sodium dodecyl sulphate (SDS) were added at the final concentration of 10% (v/v) [11], Urea was added at 6M final concentration. The effect of metal ion on bacteriocin activity was determined by adding $CaCl.2H_2O$, $FeSO_4.6H2O$, $MgSO_4.6\ H_2O$, $ZnSO_4.7H_2O$ or $CuSO_4$ at 2mM final concentration [16]. Bacteriocin treatment was allowed to stay for 60minutes before the analysis of residual activity; untreated bacteriocin was used as a positive control while treatment without bacteriocin (replacing bacteriocin with sterile distilled water in each case) was used as negative control.

2.5 Effect of bacteriocin on purified cell wall of indicator cell

2.5.1 Extraction and digestion of *Lactobacillus fermentum* CpL08 cell wall

Lactobacillus fermentum CpL08 (0.5 McFarland) was grown overnight in MRS broth, then centrifuged at 10000 rpm for 15minutes to separate the cells from the growth medium and other metabolites. The collected cells were washed twice with 20 mM phosphate buffer (pH 7.0) and resuspended in same buffer for ultrasonication, which was carried out at 20 kHz for 20 minutes. To remove unbroken cells, the resulting solution was heated at 95 °C for 15 minutes, followed by centrifugation at 2000rpm for 15minutes. Nucleases, DNAse and RNAse (1 mg each), were added and allowed to stand at room temperature for 4hours, 1mg trypsin was also added and each added enzyme was removed by centrifugation (20000 rpm for 30 minutes), the recovered cell wall was lyophilized till it is used for subsequent analysis [17]. Purified cell wall of *L. fermentum* CpL08 (0.5 mg in 10 mM phosphate buffer, pH 7.0) was added at 30% (v/v) to 1280 AU/mL diluted purified bacteriocin of *L. fermentum* CrT21 and digestion was allowed to proceed for 14hours, while undigested cell wall was thereafter removed at 16000 rpm for 15 minutes.

2.5.2 Extraction of generated N and C-terminal amino acids

Eighty microliter of potassium tetraborate ($K_2B_7O_4$) and 10 µl fluorodinitrobenzene reagent was added to the bacteriocin digested cell wall of *L. fermentum* CpL08, this was incubated at 60 °C for 30 minutes and the resulting mixture was acidified with 50 µl concentrated HCl, the N-dinitrophenyl (DNP) derivatives of free amino acids within the digested cell wall mixture was then extracted with ether. Residual ether was removed at 37 °C with gentle agitation and the extracted amino acids were resuspended in 0.05 M

NH_3. For liberated C-terminal amino acids, the digested cell wall of *L. fermentum* CpL08 was resuspended in 50 µl hydrazine at 60 °C for 16 hrs, benzaldehyde (25 µl) was thereafter added and mixed intermittently for 1hr. 100 µl of the resulting mixture was added to another 25 µl benzaldehyde and also mixed for 1hr; extracted C-terminal amino acid was then recovered with ether as earlier described [18]. In each case, standard amino acids (alanine, aspartic acid, glutamic acid and lysine) generally found in LAB cell wall [19] were extracted with each procedure and used as control reference for subsequent thin layer chromatography.

2.5.3 Thin layer chromatography of amino acid samples

To determine each free amino acid released by the bacteriocin digestion of *L. fermentum* CpL08 cell wall, the extracted amino acids (both standard and digested) were spotted on silica gel coated aluminum sheets of the chromatograph and developed with isopropanol at room temperature. The preparation was visualized under UV light to determine the distance traveled by each amino acid.

3. Results and Discussion

In all, 28 strains of several species of LAB were isolated from cassava waste and retting, they all produced inhibitory metabolites against other LAB and non-LAB isolates. Having produced extracellular metabolite inhibitory to indicator lawn from the adjusted metabolite well (with both NaOH and catalase), *Lactobacillus fermentum* CrT21 was characterized to produce bacteriocin against other LAB (Table 1), including *Lactobacillus fermentum* CpL08 (1280 AU/mL) which was selected as indicator strain for further characterization of bacteriocin produced by *L. fermentum* CrT21

3.1 Purification of bacteriocin from *L. fermentum* CrT21

Inhibitory activity was detected, after ammonium sulphate purification, only in the salt precipitate (protein content of the metabolite) and not in the supernatant. Gradual increment in specific activity of bacteriocin was recorded with the purification steps applied (Table 2). Maximum bacteriocin elution and specific activity (8533.3) was recorded with the 10 KDa molecular exclusion pore size through ultrafiltration procedure; this might correspond with the average molecular size of the bacteriocin produced by *L. fermentum* CrT21. Increments in bacteriocin activity, achieved through precipitation and ultrafiltration procedures, have been similarly reported by Novak and Elayaraja [20; 21].

3.2 Characterization of purified bacteriocin

Part of the fascinating reported characteristics of bacteriocins is their ability to retain a significant fraction of their inhibitory properties over a wide range of conditions [22], bacteriocin from *L. fermentum* CrT21 retained all or most of its inhibitory activity (1280-640 AU/ml) at temperature range of 40-80 ^{0}C, pH 2-8 as well as in the presence of various metal ions and enzymes (Table 3). However, increment in activity was observed in the presence of Tween 80, Tween 20 and SDS but loss of activity was recorded at 121^{0}C and in the presence of Trypsin.

Table1: Antimicrobial activity of *L. fermentum* CrT21 bacteriocin

Lactic Acid Bacteria	Activity Unit (AU/ml)
Lactobacillus casei CrT11`	1280
Lactococcus lactis Y02	320
Lactobacillus fermentum CpL08	1280
Lactobacillus plantarum CpL16	640

3.3 Effect of bacteriocin on purified cell wall of indicator cell

The extracted amino acids resulting from the purified cell wall digestion of *L. fermentum* CpL08 by the bacteriocin of *L. fermentum* CrT21, run on the thin layer chromatographic sheet, showed the presence of derivatized C-terminal glutamic acid and N-terminal lysine when compared to standard amino acids (Figure 1). This indicates that the bacteriocin cleaved the peptide bond between D-glutamic acid and L-lysin along the peptide side chain of the peptidoglycan wall of *L. fermentum* CpL08 (Figure 2). This could lead to loss in the maintenance of cell integrity and internal turgor pressure [23], thereby depleting *L. fermentum* CpL08's ability to mediate interactions with both biotic and abiotic components.

Figure 1: Thin Layer Chromatographic sheet of standard and extracted amino acids

Spots 1, 2, 3, 4, 5 and 6 are standard Glutamic acid, standard Lysine, standard Aspartic acid, standard Alanine, extracted N-terminal lysine and extracted C-terminal Glutamic acid respectively.

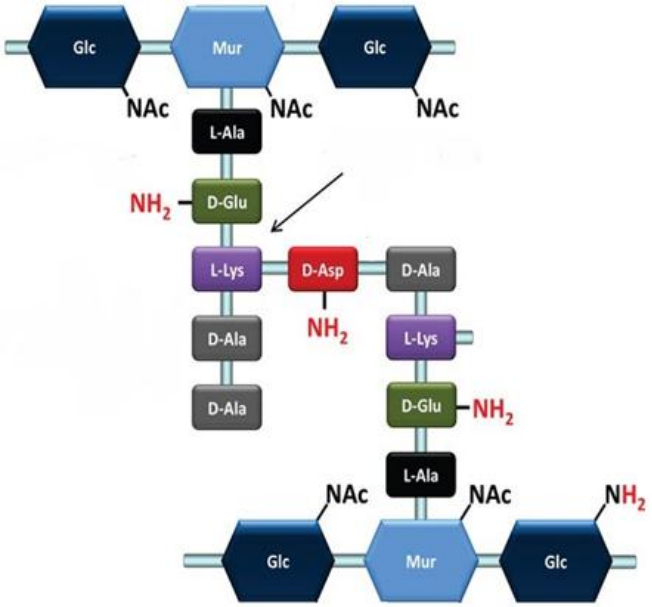

Figure 2: Cleaving site of *L. fermentum* CrT21 bacteriocin on *L. fermentum* CpL08 cell wall. Adapted: Chapot-Chartier and Kulakauskas (2014).

Table 2: Purification of bacteriocin from *L. fermentum* CrT21

Purification step	Volume (ml)	Activity Unit (AU/ml)	Total activity (AU/ml)	Protein (mg/ml)	Specific activity	Purification fold	Recovery (%)
Crude	500	1280	640000	1.5	853.3	1	100
Ammonium sulphate	30	2560	76800	1.2	2133.3	2.5	80
1 KDa*	5	320	1600	0.06	5333.3	6.3	4
10 KDa*	4	2560	10240	0.3	8533.3	10	20
20 KDa*	6	1280	7680	0.5	2560	3	33.3

*Each filtration was done with a 10ml batch from the ammonium sulphate precipitate solution.

Activity unit = Reciprocal of the highest bacteriocin dilution with at least 2mm inhibition diameter.

Total activity=Activity x volume; Specific activity=Activity unit/protein content; Purification fold=Specific activity/crude specific activity; Recovery=Protein concentration /Crude protein concentration.

Table 3: Effect of various treatments on the inhibitory activity of *L. fermentum* CrT21 bacteriocin

Condition and Treatments	*Residual activity (AU/ml)*
Temperature	
40^0C	1280
80^0C	1280
100^0C	640
121^0C	0
pH	
2	1280
4	1280
6	1280
8	1280
10	640
Surfactants/metals	
Triton X-100	1280
Tween 80	5120
Tween 20	5120
Urea	1280
SDS	2560
EDTA	1280
$CaCl.2H_2O$	1280
$FeSO_4.6H2O$	1280
$MgSO_4.6\ H_2O$	1280

$ZnSO_4.7H_2O$	1280
$CuSO_4$	1280
Enzymes	
Trypsin	0
Lipase	1280
lysozyme	1280
Control	1280

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