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Research Article

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Purification and partial characterization of mesenterocin produced by the locally isolated *Lc. mesenteroides* NEF42

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Abstract This study involves the isolation and identification of the lactic acid bacterium *Leuconostocmesenteroides*from dairy product (cheese) and variable plant material by the colony morphology and routine confirmative biochemical reactions. From sixty isolates were collected, 19 isolates were belonged to *Leuconostoc*species and only 10 of them were *Lc. mesenteroides subsp. mesenteroides.* All isolates were bacteriocin producer, and the inhibitor activity is tested against *Listeria monocytogenes.*The purification of the bacteriocin from the selected isolates was carried out by two steps including cation exchange chromatography by using CM-Sepharose CL-6B and gel filtration by using Sepharose 6B followed with HPLC. The purified bacteriocin was named bacteriocin NEF42 according to the isolate NEF42. For Characterization of purified bacteriocin the molecular weight was determined by gel filtration on Sepharose 6B column, and it was about (1724.6) Daltons.

Keywords *Leuconostocmesenteroides*; mesenterocin;CM-sepharose, gel filtration, molecular weight

Introduction

Mesenterocin 5 and mesenterocin Y105 were the first identified bacteriocin or bacteriocin-like substances produced by *Lc. Mesenteroides* subsp. *mesenteroides*. Both had a narrow spectrum of inhibition and were found to be active mainly against *Listeria* species, including *L. monocytogenes* [1]. However, their modes of action are different. Mesenterocin Y105 was shown to exhibit a bactericidal mode of action [2] while mesenterocin 5 was found to be bacteriostatic[1]. Bacteriocin or bacteriocin-like substances have been identified in *Lc. mesenteroides*. *Lc. mesenteroides subsp*. *Mesenteroides* FR52 can produce mesenterocin 52A and 52B [3], which are identical to mesenterocin Y105 and mesenterocin B105 respectively [4]. *Lc. Mesenteroides* TA33a was found to produces leucocins ATA33a and C-TA33a, two anti-Listeria active peptides, and leucocin B-TA33a with activity against *Leuconostoc/Weisella*genera [5]. Within the *Leuconostoc* genus, some strains have been recently shown to produce more than one bacteriocin. In addition to mesenterocin 52A, which is identical to mesentericin Y105, *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 produces the 32-mer polypeptide mesenterocin 52B. The same bacteriocin has also been detected in culture extracts of *Lc. mesenteroides* Y105 [6]. *Lc. mesenteroides* strain TA33a actually produces three different bacteriocins: class IIaleucocin A-TA33a, leucocin B-TA33a, and leucocin C-TA33a, a new anti-Listeriabacteriocin [5]. Class IIabacteriocins are mostly secreted from their producing cell by dedicated ATP-binding cassette (ABC) transporters and their accessory proteins, with concomitant cleavage of their N-terminal leader sequence [6].

Besides bacteriocins, LAB produces many other inhibitory compounds, such as organic acids, free fatty acids, ammonia, diacetyl, hydrogen peroxide, and enzymes. However, bacteriocins have unique applications in food

processing and food safety because of their heat stability and sensitivity to proteolytic enzymes. Nisin, a bacteriocin synthesized by *L. lactis*, has been used in several countries to extend the shelf life of food products.
According to the forthcoming presentation, this study achieves the following aim is purification and partial characterization of a bacteriocin produced by one of *Leuconostoc mesenteroides* isolates.

Materials and Methods
Bacteriocin was extracted from the selected local producer isolate of *Lc.mesenteroides* by centrifugation, applied on an 18-24 hrs cultured at 28°C of modified MRS broth, at 8000 rpm for 20 min at 4°C for the removal of cells from the bacterial culture medium. The cell-free supernatant fluid was decanted and used as crude extract [7].

Purification of Bacteriocin by Ion Exchange Chromatography:
The exchanger CM-Sepharose CL-6B was prepared and packed into a column according to Guyonnet *et al*. [9] and Uteng *et al*.[8]as follows:. 20 mM phosphate buffer pH 5.8 contained 0.2M NaCl after that pour the slurry in to the column 2.5x15 cm, then the column equilibrated with the same buffer overnight. Partially purified concentration GTF 60 ml were separately passed after loaded onto the column carefully. Then 100 ml of 20 mM phosphate buffer pH 5.8 was added. Flow rate estimated as 50 ml / hr. proteins were eluted by using 200 ml of a gradient from 0.2-1 M sodium chloride. Fractions of 5ml were collected and absorbency was monitored at 280 nm. The bacteriocin was determined by inhibition growth of the test strains by an antagonism well diffusion method [10].
Purification of Bacteriocin by Gel Filtration Chromatography
Sepharose 6B column 75 x 1.5cm was prepared and packed according to Stellwagen[11], the column was equilibrated with 0.25M Tris-HCl buffer pH 8.0 at a flow rate of 50 ml / hr. A 10 ml sample of each concentrated partially purified bacteriocin was added to the column. Elutions of proteins were done with the application of 150 ml of 50 mM phosphate buffer PH 7.5. A 5 ml fraction was collected for each bacteriocin then protein concentration was estimated by measuring the absorbency at 280 nm. The beaks for each bacteriocin were determined by plotting the absorbency of protein fraction versus the elution volumes. bacteriocin activity was determined for each fraction of the major peaks.
Isolation by Preparative HPLC
The peptide or bacteriocin produced by *Lc .mesenteroides* was further purified to homogeneity by using reversed-phase chromatography (HPLC) of the active fractions 1M NaCl obtained in the cation-exchange chromatography step and were monitored for absorbance at 280 and for activity against the indicator strain *L. monocytogens*. The active fraction 1M NaCl was applied directly to a C18 cartridge (column) and bacteriocin was eluted in the 80% acetonitrile as solvent system at a flow rate of 0.5 ml/min after concentrated by sucrose and dialyzed against 0.02 M phosphate buffer, pH 5.8 overnight. To prevent fouling of the reverse-phase column, samples applied to the column were cleared of gross contaminants by centrifugation at about 4,000 rpm for 10 min and separation was carried out by using a water-acetonitrile solvent system.
Determination of Molecular Weight of by Bacteriocin Gel Filtration Chromatography:
1. Determination of the Void Volume of the Column:
It was prepared by using Sepharose -6B column 70 ×1.5 cm and peaked according to the recommended of the manufacturing company. The column was equilibrated with 50 mM phosphate buffer pH 7.5and with a flow rate of 48 ml / hr. blue dextran 2000 solution, was passed through the column then 200 ml of 50 mM phosphate buffer pH 7.5 was added to the column. Fractions of 5 ml were collected. The absorbency at 280 nm for each fraction was measured. The void volume (Vo) was determined by the estimation of total volume of fraction as characterized with start point movement of the blue dextran to that of climax of absorbency of the blue dextran.
2-Determination of Bacteriocin Elution Volumes (Ve):
A 5 ml of partial purified GTFs samples were passed separately through Sepharose-6B column 70 ×1.5 cm and 200 ml of 50 mM phosphate buffer pH 7.5 with a flow rate 48 ml / hr. were passed through the column. Fractions of 5 ml were collected. The elution volumes (Ve) were estimated separately for each separated and dissolved fractions of purified Bacteriocin by following the absorbency at 280 nm.

3-Mesurment of Standard Proteins Elution Volumes (Ve)

It was prepared by using several standard proteins showed in table (1) were passed through Sepharose 6B column, and then eluted with (50 mM) phosphate buffer (pH 7.5) with a flow rate of (48 ml / hor.)Table (1):

Molecular Weight of Some Standard Protein

Standard Proteins	Molecular Weight (Dalton)
Inulin (Sugar)	5000
Bovine Serum Albumine	67000
Alkaline phosphatase	168000
Catalase	232000

The elution volume was calculated for each standard protein by following the Bacteriocin absorbency for the separated fraction at wave length 280 nm then the ratio of (Ve/Vo) was calculated for each standard protein and for the dissolved and separated fractions of partial purified , then standardization was done, by plotting the elution volume of each standard protein to the void volume of the blue dextran 2000 versus the log of each standard protein molecular weight [11] and thus the molecular weight of bacteriocin were accordingly calculated.

Results and Discussion

Lc. mesenteroides NEF42 has been shown to produce a proteinaceous substance with antibacterial activity. This bacteriocin, named Mesenterocin NEF42, demonstrated a bactericidal effect on tested bacteria over an incubation period of 18 hrs. The size of bacteriocins ranges from small peptides to large proteins complicated with lipid and/or carbohydrate moieties. The majority are small cationic and hydrophobic molecules. The hydrophobic nature of the bacteriocins has complicated the purification process because bacteriocins tend to aggregate and sometimes nonspecifically adhere to materials that are employed during purification procedures. In some cases, bacteriocins are co-purified with other cellular proteins [7].

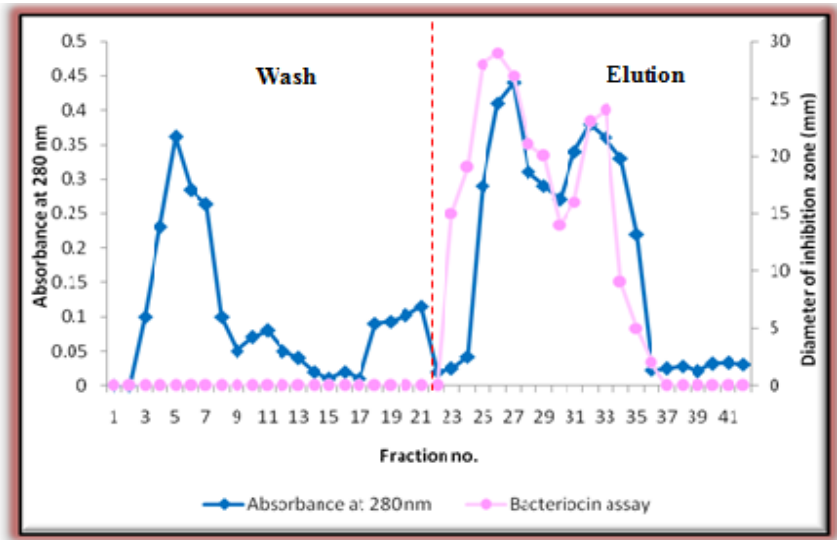

Figure 1: Purification of bacteriocin produced by the locally isolated Lc. mesenteroides NEF42 using ion exchange chromatography technique

In the present study, Ion – exchange chromatography has been carried out by using the Fast Flow cation exchange column (2.5×18 cm) where An overnight culture supernatant of *Lc. mesenteroides*NEF42, propagated at 28°C in MRS broth (the productive media) was applied on a 25-ml CM-Sepharose 6B which was equilibrated with 20 mM sodium phosphate (phosphate buffer), pH 5.8. The column was subsequently washed with 150 ml of the same phosphate buffer to remove unadsorbed protein, and 100 ml of the phosphate buffer containing 0.2M NaCl, and the adsorbed proteins (bacteriocin) was then eluted with 100 ml of the phosphate buffer containing 1M NaCl. Absorbance of washing and elution fractions was read at 280 nm. Curve was plotted between the absorbance and

fraction number which gave protein peaks (Figure 1). All peaks appeared in the elution step where gave inhibitory activity in the fractions (25-29, 31-33). Ganzle*et al*. [12] demonstrated that ecological factors, such as protein content, pH, salinity, or/and salt composition of the medium, modulate the activity of bacteriocins (nisin, sakacin A and P); for example, divalent cations inhibited bacteriocins, whereas acidic pH and NaCl increased their activity.

Moreover, the surfactant Tween 80 may form micelles with the proteins in the growth medium and was described to interfere with bacteriocins, extending or diminishing their apparent activities according to the peptide tested [13]. Therefore, this compound, being present in the culture supernatant, must still be present in subsequent fractions tested in order to minimize the error in the activity determination [9]. Thus, all the critical dilution assays were made by using a solution of 0.01% Tween 80 as the diluting solvent.

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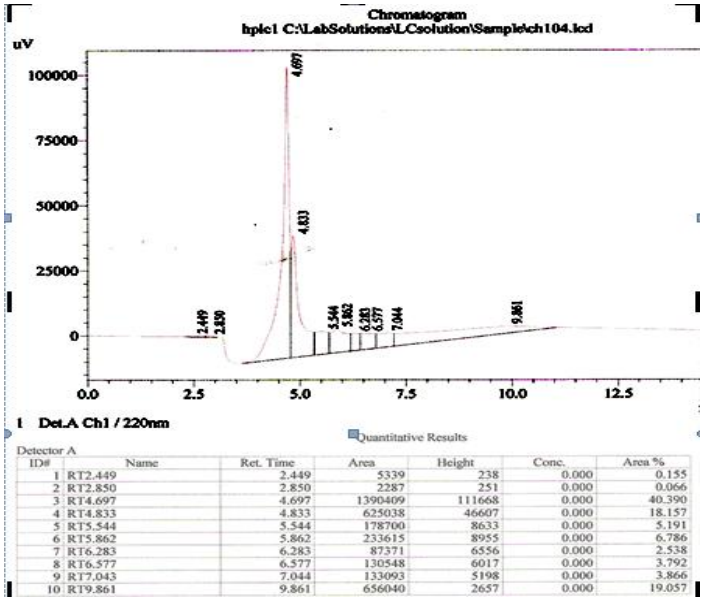
After concentration (dialyzed against sucrose), the active fractions from ion-exchange chromatography was applied directly to a C18 analytical reverse-phase (HPLC) column at a flow rate of 0.5 ml/min and mesenterocin NEF42 was eluted in the 80% acetonitrile fraction. The elution profile, recorded at 220 nm, is presented in Figure 2. The bacteriocin NEF42 was appeared at 4.697 min. The bacteriocin appeared as a major absorbance peak upon reverse-phase chromatography in the second purification step [8].

High pressure liquid chromatography (HPLC) is a versatile, robust, and widely used technique for the isolation of natural products. The main difference between HPLC and other modes of column chromatography is that the diameter of stationary phase particles is comparatively low (3-10µm), and these particles are tightly packed to give a very uniform column bed structure. The low particle diameter means that a high pressure is needed to drive the chromatographic solvent (or "eluent") through the bed [14].



ID#	Name	Ret. Time	Area	Height	Conc.	Area %
1	RT2.449	2.449	5339	238	0.000	0.155
2	RT2.850	2.850	2287	251	0.000	0.066
3	RT4.697	4.697	1390409	111668	0.000	40.390
4	RT4.833	4.833	625038	46607	0.000	18.157
5	RT5.544	5.544	178700	8633	0.000	5.191
6	RT5.862	5.862	233615	8955	0.000	6.786
7	RT6.283	6.283	87371	6556	0.000	2.538
8	RT6.577	6.577	130548	6017	0.000	3.792
9	RT7.043	7.044	133093	5198	0.000	3.866
10	RT9.861	9.861	656040	2657	0.000	19.057

Figure 2: HPLC Technique for bacteriocin purification from the local isolate Lc. mesenteroides NEF42 after ion exchange chromatography and dialyses against sucrose.

The concentration of the metabolite in the crude source (e.g., fermentation broth) might be isolated using a high–capacity, low-resolution technique such as ion exchange chromatography or adsorption-elution from a polymer resin, with preparative HPLC applied as the final purification step, if required [14].

Purification through Gel Filtration

After concentration (dialyzed against sucrose), the active fractions (bacteriocin NEF42 containing fractions) were pooled from ion-exchange chromatography was applied directly to gel filtration chromatography. The sample was loaded over Sepharose 6B column and each eluted 5ml fraction read at 280 nm and the curve was plotted between the absorbance fraction numbers which gave two protein peaks (Figure 3).

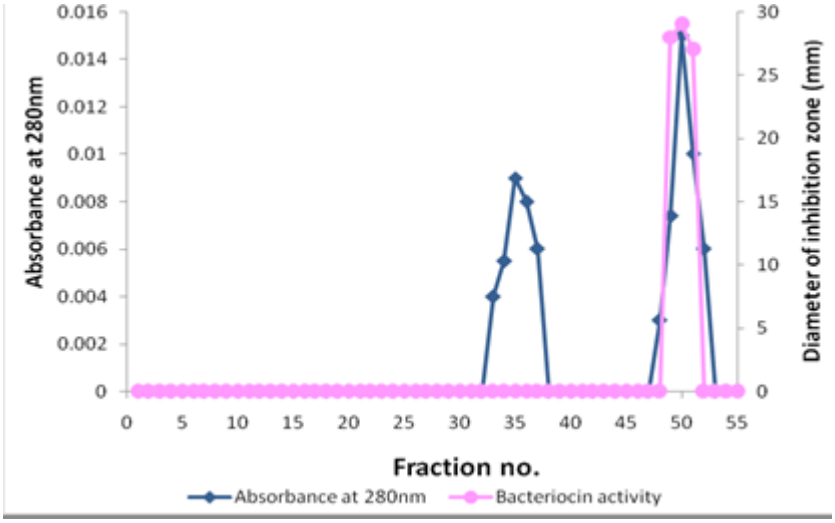


Figure 3: Gel filtration chromatography for the purification of bacteriocin NEF42 isolated from Lc. mesenteroides NEF42 by using Sepharose 6B column which was equilibrated with phosphate buffer, pH 8.0. The bacteriocin eluted with the same buffer and flow rate was adjusted to give 0.5 ml/min, and 5 ml for each fraction was collected.

All peaks appeared in the elution step where gave inhibitory activity in the fractions (49-51) when assayed with *L. monocytogenes*. These fractions were collected then concentrated and applied to HPLC. Gel filtration is performed using porous beads as the chromatographic support and it is unique in that fractionation is based on the relative size of protein molecules. A good resolution of different sizes of proteins could be obtained by using this technique , if some criteria follow such as volume of matrix to volume of samples, low flow rate, appropriate column diameter with high length, quality of sample application, and absence of any denaturizing agents in elution buffer [11].

Characterization of Purified Bacteriocin

The molecular weight is determined by gel filtration in Sepharose 6B based on the standard curve made by standard proteins (Figure 4). The Blue Dextran 2000 was used as a guide for assessing the correct column packaging and the estimation of void volume [15]. In this study the curve plotted between absorbance at 600 nm and fraction number and the resolution of one identical peak indicated the correct column packaging while the void volume was estimated to be equivalent to 64ml.

Five ml of the concentrated active fractions by sucrose obtained in the ion-exchange chromatography step and were monitored for absorbance at 280nm and for activity against the indicator strain *L. monocytogenes* was used for estimation of molecular weight of bacteriocin NEF42 by gel filtration chromatography with the aid of fractionation of standard proteins showed that the resolute protein had (1724.6 Daltons) molecular weight this was closer to the molecular weight of Mesenterocin Y105 which had already been reported by Stiles, [16]. The molecular mass was estimated to be 2.5 to 3.0 k Dalton based on SDS-PAGE analysis but was calculated as 3666.6 Dalton based on the amino acid sequence. Whereas the molecular weight of Mesenterocin 5 about 4.5 kDa estimated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [17].

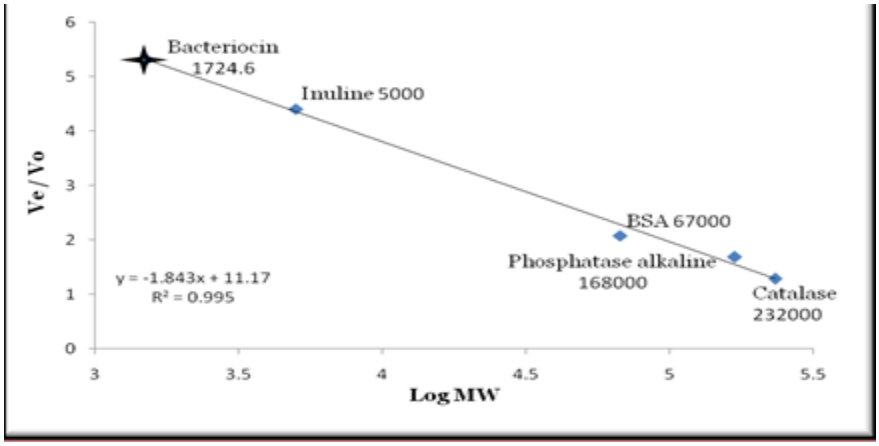
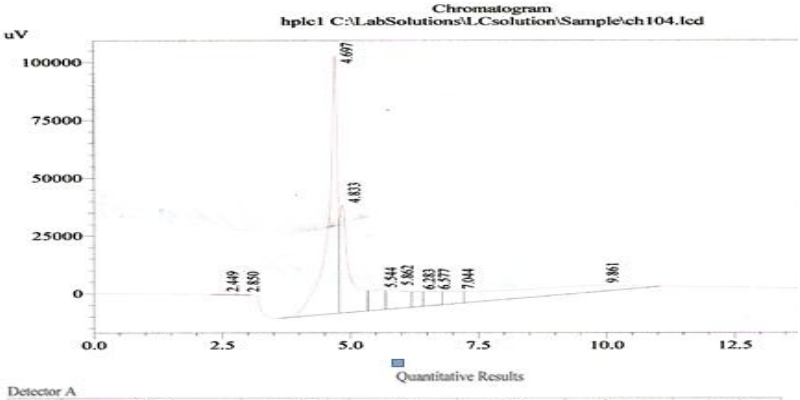


Figure 4: Determination of the molecular weight of purified bacteriocin of the local isolate Lc. mesenteroides NEF42 by Gel filtration chromatography using standard proteins of different molecular weights. Numbers in the figure indicate molecular weight of proteins in Daltons.

Guyonnet *et al*. [9] found that the molecular weight for mesenterocin Y105 was 3868.3Da and mesenterocin B105 was 3445.2Da using Mass spectrometry analysis which purified by cat-ion exchange chromatography. The molecular mass of the *Lb. acidophilus* 30SC bacteriocin was estimated at 3.5 kDa, whereas acidocin B migrated with a molecular mass of 2.4 kDa on SDS-PAGE, but themolecular mass was subsequently calculated to be 5.8 kDa from its DNA sequence [18, 19].

To characterize the structure and function of the bacteriocin produced by *Lc. mesenteroides* NEF42 further investigation is required, and is presently being performed.Bacteriocin NEF42 obtained by gel filtration was further purified by HPLC (reversed phase). After concentration (dialyzed against sucrose), the active fractions was applied directly to a C18 analytical reverse-phase (HPLC) column at a flow rate of 0.5 ml/min and bacteriocin NEF42 was eluted in the 80% acetonitrile fraction. The elution profile, recorded at 220 nm, is presented in figure 5 and the bacteriocin NEF42 appeared at the same retention time after 4.774 min.



Detector A

ID#	Name	Ret. Time	Area	Height	Conc.	Area %
1	RT4.774	4.774	1450705	136844	0.000	25.050
2	RT4.892	4.892	948678	85318	0.000	16.382
3	RT5.569	5.569	245656	10232	0.000	4.242
4	RT5.835	5.835	245987	10351	0.000	4.248
5	RT6.311	6.311	209352	7734	0.000	3.615
6	RT8.921	8.921	1243252	9059	0.000	21.468
7	RT9.325	9.325	1447509	8766	0.000	24.995

Figure 6: HPLC Technique for bacteriocin purification from the local isolate Lc. mesenteroidesNEF42 after Gel filtration chromatography

Conclusion

We can conclude that the possibility of obtaining of pure bacteriocin with two steps of purification methods includes cat-ion exchange chromatography and HPLC, and the pure productive bacteriocin of *Lc. mesenteroides* isolate (bacteriocin NEF42) has a molecular weight of 1724.6 Dalton

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