Partial purification and characterization of a bacteriocin produced by *Lactobacillus acidophilus*

التنقية الجزئية وتوصيف البكتريوسين المنتج من بكتريا *Lactobacillus acidophilus*

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Abstract

Bacteriocin produced by *Lactobacillus acidophilus* was partially purified by ammonium sulfate precipitation 60% saturation. The bacteriocin exhibited activity of 320AU/ml against *Serratia marcescens*. Characterization of bacteriocin showed that its active principle was proteinaceous in nature since it was inactivated by proteolytic enzymes but not by other enzymes. Treatment of bacteriocin with organic solvents 5% concentration did not affect the activity of it, but its activity was reduced to the half at 10% concentration of the solvents. Bacteriocin activity was stable at pH 4 -7, half of its activity was lost at pH8, and whole activity was lost at other pH values. Bacteriocin was stable at 40-100°C for 10min, but it retained only 50% of its activity at 40 and 60°C for 30min. whole activity was lost at 80 and 100°C for 30min and at 121°C for 15 min.

المستخلص

تمت تنقية البكتريوسين المنتج من بكتريا *Lactobacillus acidophilus* جزئيا بواسطة الترسيب باملاح كبريتات الامونيوم وبنسبة تشبع 60% . اظهر البكتريوسين فعالية بلغت 320 وحدة/مل ضد بكتريا *Serratia marcescens* . اظهر توصيف البكتريوسين انه ذو طبيعة بروتينية إذ فقد فعاليته بعد تعرضه للإنزيمات المحللة للبروتين ولكنه لم يتأثر بالإنزيمات الأخرى . لم تتأثر فعالية البكتريوسين بعد معاملته بتركيز 5% من المذيبات العضوية لكن فعاليته اختزلت إلى النصف بعد معاملته بتركيز 10% من هذه المذيبات . أظهرت فعالية البكتريوسين استقرارا عند الأرقام الهيدروجينية4 - 7 وفقدت نصف هذه الفعالية عند الرقم الهيدروجيني 8 وكامل الفعالية عند الأرقام الهيدروجينية الأخرى . اظهر البكتريوسين استقرارا في الفعالية بدرجة 40- 100^{O}م ولمدة 10 دقائق لكنه احتفظ بنصف هذه الفعالية عند تعرضه لدرجتي 40 و 60م ولمدة 30 دقيقة وفقد كامل فعاليته بدرجتي 80 و100م لمدة 30 دقيقة وكذلك عند تعرضه لدرجة 121م ولمدة 15 دقيقة .

Introduction

Since Metchnikoff proposed a role for lactobacilli in suppressing undesirable intestinal microflora, numerous researchers have investigated the antimicrobial activities of *Lactobacillus acidophilus* [1]. *L. acidophilus* strains have been widely utilized as a dairy starter culture for their therapeutic activities associated with an intestinal microbial balance, and has been used in fermented foods, and as a probiotic in dietary supplements [2].

Bacteriocins are proteinaceous antimicrobial compounds with activity against species that are usually closely related to the producer culture [3]. *L. acidophilus* has received more attention and has been the subject of much research because of its ability to produce bacteriocins against other bacteria [4].

Some bacteriocins that are produced by strains of *L. acidophilus* have been purified and characterized including acidocin 8912, lactacin B, acidophilin, acidolin, and lactocidine[5]. [6] first described a bacteriocin-type inhibitor produced in aged liver veal agar cultures of *L.acidophilus*. Crude "lactocidin" was nonvolatile, non-dializable, insensitive to catalyase, active at neutral pH and displayed inhibitory

activity against numerous genera, including *Proteus*, *Salmonella*, *Escherichia*, *Staphylococcus*, *Bacillus*, *Streptococcus*, and *L. actobacillus*. Lactacin F was the first biochemically and genetically characterized bacteriocin of a *L. acidophilus* strain. The majority of bacteriocins produced by the *L. acidophilus* group of lactic acid bacteria are heat-stable, low-molecular-mass, non-lantibiotic peptides which belong to class II [7].

Currently, there are no bacteriocins from *L.acidophilus* that are being applied commercially in food systems as food preservatives. But this is not only true for the bacteriocins from *L.acidophilus* but also true for many bacteriocins isolated from lactic acid bacteria (LAB). Many bacteriocins have been characterized biochemical and genetically but many aspects of these compounds are still unknown which explains why till date nisin, approved by the Food and Drug Administration, is the only purified bacteriocin widely used as a food preservative. Toxicity data exist for only a few bacteriocins, none of which are for bacteriocins from *L.acidophilus*, but research and their long time intentional use strongly suggest that bacteriocins can be safely used [8].

Because of bactriocins potential as antimicrobial agents for improving the safety of food products, therefore, this study was aimed to extraction, partially purification and characterization of abactriocin produced by *Lactobacillus acidophilus*.

Materials and Methods

Bacterial isolates and Culture media

The bacteriocin producer was *Lactobacillus acidophilus*, the indicator bacteria were *Staphylococcus aureus*, *Enterococcus feacalis*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Serratia marcescens* and *Lactobacillus acidophilus* (Department of biology/College of Science/Al-Mustansiriya University). *L. acidophilus* was grown in MRS broth (Hi-Media/ India) while other indicator bacteria were grown in nutrient broth (NB) (Difco/ USA).

Bacteriocin activity assay

This was carried out as described by [9] with some modification, *L. acidophilus* was cultivated overnight in MRS broth at 37°C. After centrifugation at 6000 rpm for 15min at 4°C, the cell-free supernatant was filtered through 0.22µm filters and neutralized to pH 6.5, to eliminate the inhibitory effect caused by the decrease of pH. Pour plates were prepared from nutrient agar seeded with10^6 cell/ml of indicator bacteria. Wells cut into the pour plates with 6mm sterile cork borer were filled with 100 µl of the cell-free supernatant. The plates were incubated at 37°C for 24 h. Inhibition was detected by a zone of clearing around the supernatant well. The highest dilution that produced a definite zone of growth inhibition of the indicator lawn was defined as 1 arbitary unit of bacteriocin activity per milliliter (AU/ml) [3]. AU was calculated as:

(1000 / 100) ×D, where 1000: constant, 100: volume of supernatant in a well (µl) and D: the dilution factor [10].

Partial purification of bacteriocin

Cell-free supernatant was obtained by centrifugation (6000 rpm for 15 min, 4°C) of MRS broth inoculated with *L. acidophilus* and incubated at 37°C for 24h. Ammonium

sulfate was added to cell-free supernatant at (40,50,60,70, 80) % saturation (w/v), the mixtures were stirred for 2h at 4°C and later centrifuged at 8000 rpm for 20 min at 4°C, the precipitates were resuspended in 50 mM potassium phosphate buffer (pH 6.5) and dialyzed against the same buffer for 24h at 4°C.

Characterization of bacteriocin

Effect of enzymes, solvents, pH and heat stability

Partially purified bacteriocin was treated with enzymes at a final concentration of 1mg/ml as the following: pepsin, lipase, α-amylase (Philip Harris Biological Ltd/UK), papain(BDH/England) and trypsin(Merck /Germany). All samples were adjusted to pH7 except that treated with pepsin, which was adjusted to pH3. Samples were filter-sterilized using filter membrane 0.22 µm and then incubated at 37°C for 2h. Residual enzyme activity was finally stopped by boiling for 5min [10]. The effect of 5 and 10 % of chloroform, ethanol, methanol and butanol was studied by adding the solutions of these materials, respectively to partially purified bacteriocin. All samples were incubated at 37°C for 2h.

For pH stability, partially purified bacteriocin samples were adjusted to pH range of 2- 9 (at increments of one pH unit), all samples were incubated at 37°C for 2h and then adjusted to pH 5.5.

For the study of thermal stability, partially purified bacteriocin samples were adjusted to a pH 5.5 and then heated to (40, 60, 80, 100) °C respectively. Bacteriocin activity was assayed after (10, 30) min at each of these temperatures. Activity also assayed after 15min at 121°C.

Results and Discussion

Bacteriocin activity assay

Serratia marcescens which exhibits the most sensitivity to bacteriocin was used as the indicator bacteria in all experiments in this study table (1). The sensitivity of gram negative bacteria to bacteriocins produced by LAB is not common. But some other researchers observed inhibition of pathogenic *Salmonella* and *Escherichia* by the bacteriocin of *L.acidophilus* [7, 11]. Previous finding of positive results for inhibitory action of bacteriocin produced by *Lactobacilli* on gram negative bacterium is consistent with the present observation [12].

Table (1): Antibacterial activity of the cell-free supernatant of *Lactobacillus acidophilus* against indicator bacteria by the agar well diffusion assay

indicator bacteria	Diameters of inhibition zones(mm)
Staphylococcus aureus	**0**
Enterococcus feacalis	**16**
Lactobacillus acidophilus	**0**
Pseudomonas fluorescens	**0**
Pseudomonas aeruginosa	**0**
Serratia marcescens	**18**

Extraction and Partial Purification of bacteriocin

Partial purification of bacteriocin was performed by precipitation with ammonium sulfate. Ammonium sulfate precipitation is the most commonly method used to purify protein from broths culture [13]. It is the best, first –choice salt because it is high

solubility and is relatively inexpensive [14]. The bacteriocin was recovered following the 60% ammonium sulfate saturation of the cell-free supernatant with an increase from activity unit of 160 AU/ml for cell-free supernatant 320 AU/ml for partially purified bacteriocin.The activity ratio between the various bacteriocins differed from species to species and even from strain to strain [15]. Even during controlled fermenter experiments, considerable differences in activity yields are obtained, and an influence of the environmental process conditions on the obtained bactriocin activity can be seen.

In addition, temperature, pH and nutrient availability seem to play a crucial role in bactreiocin production. In general, the cultivatin conditions directly affect bactriocin production as such (specific bactriocin production in particular) and, indirectly, through biomass production [16]. [13] showed that some bactriocin activity was lost in the supernatant, probably due to the saturation of adsorbing sites on the bacterial cells.

However, during the purification producers, each step resulted in a considerable loss of protein concentration while specific activity increases [17].

Characterization of bacteriocin

Bacteriocin was totally inactivated by treatment with trypsin, pepsin and papain, whereas treatment with lipase and α-amylase did not affect the activity of it table (2). The sensitivity of bacteriocin to the proteolytic enzymes indicate that it is proteinaceous in nature.

Table (2): Effect of enzymes on the partial purified bacteriocin activity produced by *actobacillus acidophilus*

Enzyme	Bacteriocin activity (AU/ml)
Trypsin	**0**
Pepsin	**0**
Papain	**0**
Lipase	**320**
α-amylase	**320**

The activity of bacteriocin was not affected after treatment with 5% concentration of all solvents that used in this study, but its activity was reduced to the half at 10% concentration of them table (3).

Similler results were reported for some other bactriocins [18]. Organic solvents alter the native structure of proteins by disrupting hydrophobic interactions between the non polar side chains of amino acids. Relatively high concentrations of these solvents are required to unfold the ordered structure of poly peptide chains. Some solvents showed strong stability effect, while others showed either no effect or a strong destabilizing effect, the stabilizing effect of these solvents gradually decreases as the concentration is increased and finally start to accelerate the denaturation of protein[19].

Table (3): Effect of solvents on the partial purified bacteriocin activity produced by *Lactobacillus acidophilus*

Solvent	Concentration (%)	Bacteriocin activity (AU/ml)
Chloroform	5	320
	10	160
Ethanol	5	320
	10	160
Methanol	5	320
	10	160
Butanol	5	320
	10	160

Full activity of bacteriocin was retained in samples at pH 4-7, at pH 8 activity was reduced to the half, and at the pH 2, 3, 9 no activity was observed table (4). LAB bacteriocins are generally highly stable under acidic conditions, but many of them, including nisin are easily inactivated under neutral and alkaline conditions [20]. At the low pH the solubility is often increased; less aggregation of hydrophobic peptides occurs and less bounding of bacteriocins to the cell surface takes place [21].The pH stability data suggests that this bacteriocin could prove to be useful in preservation of acidic foodstuffs, such as fermented products. [22] reported that piscicolin 126, a bacteriocin produced by *Carnobacterium piscicola* JG126 was stable at acidic pH values but it was inactivated at neutral or alkaline pH values.

However, different bacteriocins behave in different manner on exposure of different pH values. For example, bacteriocins of vaginal *Lactobacilli* strains are stable between pH range of 4.5-7.0 but sensitive to pH9. Similarly, bacterocins of *L. acidophillus* and *L. bulgaricus* are stable at pH range of 3-10. Conflictingly, bacteriocin produced by *L.helveticus* is stable at pH range of 3-9 but sensitive to pH 10[11, 23].

Table (4): Effect of pH treatment on the the partial purified bacteriocin activity produced by *Lactobacillus acidophilus*

pH	Bacteriocin activity (AU/ml)
2	0
3	0
4	320
5	320
6	320
7	320
8	160
9	0

The bacteriocin was resistant to treatments of (40,60,80,100)°C for 10 min, respectively. However, 50% activity was lost after 30min at 40 and 60°C.whole activity was lost at 80 and 100°C for 30 min and also after autoclaving (121°C /15min) Table (5).The thermostability feature might be related to the molecular structure of the bacteriocin.

Heat stability of bacteriocin could be due to the formation of small globular structures and the occurrence of strongly hydrophobic regions and stable cross-linkage [24].

Table (5): Effect of temperature treatment on the bacteriocin activity produced by *Lactobacillus acidophilus*

Temperature (°C)	Time (minutes)	Bacteriocin activity (AU/ml)
40	10	320
	30	160
60	10	320
	30	160
80	10	320
	30	0
100	10	320
	30	0
121	15	0

In conclusion, more characterization was needed for a bacteriocin produced by *Lactobacillus acidophilus* such as its molecular weight, effect of storage conditions on its activity and attempt to purify this bacteriocin to reach high purification to use it in food industry as biopreservative.

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