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Characterization, Production and Partial Purification of a Bacteriocin Produced by *Lactobacillus plantarum* LPS10 Isolated from Pickled Olives.

Mahmoud E F Abdel-Haliem*, Eman Tartour and Gamal Enan.

Department of Botany and Microbiology, Faculty of Science, Zagazig University, Zagazig, Egypt.

ABSTRACT

Lactobacillus (L) *plantarum* LPS10 isolated from pickled olives inhibited other lactic acid bacteria (LAB), some Gram positive and Gram negative pathogenic bacteria and the *Candida albicans* fungs. The inhibitory substance(s) was heat resistant protein and could be classified as a bacteriocin. This bacteriocin was active in the acidic pH range (pH 2-6-8), was free from lipid or carbohydrate moieties, was produced optimally in MRS broth adjusted initially at pH 6.5 and incubated at 35°C and was purified by two-step purification protocol including ammonium sulphate precipitation and gel filtration. Bacteriocin activity showed 7-fold increase; 520-fold increase in partially purified bacteriocin (PPB) obtained by ammonium sulphate precipitation; active fractions eluted from sephadex G200/50 column, partial purified extract (PPE) respectively. The PPE showed two active protein bands by SDS-PAGE of molecular sizes of about 15 kDa and 50 kDa. Amino acid analysis of PPE showed 15 amino acids in the bacteriocin molecule which designated plantaricin LPS10. This plantaricin LPS10 was non-lantibiotic and could be classified to belongs class IIb bacteriocins.

Keywords: *L. plantarum* LPS10, Pickles, Bacteriocin, Food-borne pathogens.

***Corresponding author**

INTRODUCTION

Lactic acid bacteria (LAB) are Gram-positive, catalase negative rods; are widely distributed and involved in dairy, meat and vegetable fermentation. The antimicrobial potential of LAB are due to lactic acid, acetaldehyde, diacetyl and bacteriocins which are antimicrobial protein [1]. Another property was studied and introduced nowadays in starter LAB which is the ability of starter LAB to produce enzymes to be used as probiotics; such enzymes can carry out necessary functions *in vivo* such as degradation of lipids, proteins, polysaccharides and cholesterol [2]. Hence there is a need to select starter, protective and probiotic LAB cultures.

The occurrence of pathogenic Gram positive and Gram negative bacteria in foods (3-5] attracts researchers to find out new biocontrol agents to control such food-borne pathogens and to extend the shelf life of foods. In this regard some *Lactobacillus* (*L.*) *plantarum* strains isolated from foods inhibited Gram positive and Gram negative pathogenic bacteria and fungi [6-8]. The inhibitory substances were characterized as bacteriocins which could be used as viable alternative to antibiotics since they can be produced in human or animal gut by probiotic bacteria [9]. This clearly showed that there is a need to continue research to find out starter, protective and probiotic LAB cultures.

Recently *L. plantarum* LPS10 isolated from pickled olvies showed fast growth and acidification of medium, produced enzymes and inhibited many food-borne pathogens [10]. Hence it could be used as starter, probiotic and protective cultures. The present work described the characterization, production and purification of a bacteriocin by *L. plantarum* LPS10 isolated from pickled olives.

MATERIALS AND METHODS

Bacterial strains and culture media:

L. plantarum LPS10 was isolated from pickled olives and was characterized peviously by both biochemical and molecular methods [10]. It was isolated and subcultured on MRS agar [11]. Indicator Gram positive and Gram negative bactertia were stored in brain heart infusion broth (BHI, Oxoid) plus 20% glycerol [12-14] and were subcultured in BHI broth. The *Candida albicans* LMZ 331 fungus used in this study was subcultured and propagated on Sabaroud medium (Oxoid).

Antibacterial spectrum and titration of the inhibitory substance(s):

Cell free supernatant fluids were collected by centrifuging (10000 rpm for 10 min) *L. plantarum* LPS10 cultures after their growth in MRS broth [11] for 16h; were redjusted to pH 6.8 to avoid inhibition due to organic acids produced by cultures; were sterilized by Millipore filters (0.45μm); this pH-adjusted (pH 6.8) and filter-sterilized cell free supernatants were designated CFS and were used in the experiments [15, 16]. The estimation of the antimicrobial titres of CFS was carried out by critical dilution assay as described previously [17]. One activity unit (AU) was defined as 5 μL of the highest dilution of filtrate yielding a definite zone of growth inhibition of the indicator organism. The highest dilution was multiplied by 200 (1 ml/5μL) to obtain the arbitrary units per milliliter (AU/ml).

Effect if different treatments on bacteriocin activity:

To test for enzyme sensitivity, heat sensitivity, pH-stability; organic solvents stability, aliquots of CFS (1 mL for each) each containing 1 mL CFS (1 mL for each) were treated with filter-sterilized enzymes tested (1 mg/mL final concentration in 10 mM potassium phosphate buffer, pH 6.5); heated at 100°C for 5 min and 10 min; mixed with 10% (v/v) of the organic solvents tested and incubated at 60°C overnight to evaporate organic solvents used; adjusted to different pH values tested for 24 h and then readjusted to pH 6.8 with 10 mM potassium phosphate buffer. Controls were either potassium phosphate buffer (pH 6.8) or cell-free supernatants (CFS) without treatments. Samples and controls were then assayed for residual antimicrobial activity (Au/ml) [16].

Production and purification of plantaricin LPS10:

A series of 500 ml Erlemeyer flasks, each containing 250 ml MRS broth were adjusted initially at different pH (3.0, 4.0, 6.5, 7.0, 8.0) and inoculated with (2 x 10 CFU/ml) of *L. plantarum* LPS10. The flasks were inoculated at 25°C, 30°C and 42°C for 96 h. After appropriate time intervals, samples were removed and examined for growth (CFU/ml) and bacteriocin activity (AU/ml) using *Listeria monocytogenes* LMG 10470 as the indicator organism [5, 18]

For purification of plantaricin LPS10, CFS of *L. plantarum* LPS10 was adjusted to pH 6.8 and treated with solid ammonium sulphate up to 50% saturation, incubated at 4°C overnight with sitting and then centrifuged at 2000 g for 1 h at 4°C. The precipitates (surface pellicles and pellets) were recovered in 10mM potassium phosphate buffer, pH 6.5 and dialysed against the same buffer for 24 h at 4°C in Visking dialysis tubing (Alex. Co., Egypt). This partially purified bacteriocin was sterilized by Millipore filtration (0.45 μm) (Amicon), adjusted at pH 6.5 and designated PPB and assayed against *Listeria moncytogenes* LMG 1047 as the indicator organism [19]. 100 ml of PPB were applied to 200 ml column (4-cm interior diameter) of sephadex G200/50 (Sigma) equilibrated with potassium phosphate buffer pH 6.5 at 4°C. Activity was eluted with the same buffer and the eluents were monitored for absorbance (OD 280 nm) and bacteriocin activity (Au/ml) [20]. The purified extract designated PPE and was used for further experiments.

SDS-PAGE and amino acid analysis of plantaricin LPS10:

Protein was analysed for SDS-PAGE using phast Gel High Density Strips [21]. Before application of PPE on the gel, 2-ml was boiled for 10 min in SDS buffer containing 2% 2-mercaptoethanol. Protein concentrations in the active fractions were determined as described previously [22]. Amino acids analysis of PPE was determined as described previously [23]. 200 μl of PPE were hydrolysed with 6N HCl in sealed tube, heated in an oven at 100°C for 24 h to evaporate HCl. The residue was then dissolved in diluting citrate buffer (pH 6.5).Chromatography was performed with an AAA 400 amino acid analyser (Ingos Ltd Czech Republic) equipped with an Ostion LG ANB ion exchange column. Free amino acids were separated by stepwise gradient elution using Na/K-citrate buffer system (Ingos Ltd., Czech Republic) Post-column derivatization with ninhydrin reagent and spectrophotometric measurement was used for determination of amino acids and biogenic amines.

RESULTS

Titration of CFS from *L. plantarum* LPS10 was studied against different organisms (Table 1). *Listeria monocytogenes* and *Bacillus* spp. tested were the more sensitive organisms as AU/ml values were 800-1000 Au/ml. *Pseudomonas aeruginosa* was not inhibited. Other organisms tested showed moderate response to CFS from *L. Plantarum* LPS10 and activity units were almost 200-600 AU/ml.

Table 1: Antibacterial activity of *L. plantarum* LPS10 isolated from pickles.

Sensitive organism and its source	AU/ml
Bacillus cereus ATCC 14579	800
Bacillus subtilis LMZ 12	800
Streptococcus pyogenes LMZ 107	600
Staphylococcus aureus DSM 1104	600
Listeria monocytogenes LMG 10470	1000
Salmonella typhi LMG 10395	200
Klebsiella oxytoca LMG 3055	200
Pseudomonas aeruginosa LMG 8029	0
Escherichia coli LMZ 8223	200
Candida albicans LMZ 331	400
Lactobacillus plantarum LMG 8155	100
Lactobacillus sake FRC 706	400
Lactococcus lactis ATCC 11454	400

ATCC: American Type Culture Collection. Rockville, Maryland, USA.

DSM: Deutsche Sammlung Von Mikroorganism and Zelkulturen, Gmbh, Braunschweig, Germany.
FRC: Federal Research Center for Nutrition, Institute for Hygiene and Toxicology, Karlsruhe, Germany.
LMG: Laboratory of Microbiology, Gent Culture Collection, Universiteit Gent, Belgium.
LMZ: Laboratory of Microbiology, Zagazig Culture Collection, Zagazig University, Zagazig, Egypt.

Characterization of the inhibitory substance in CFS collected from *L. plantarum* LPS10 was studied (Table 2). The inhibitory substance was heat resistant since residual activity did not affect by heating at 100°C for 15 min; was degraded by proteolytic enzymes; was stable in the acidic pH range (pH 2 - pH 6.5); was not affected by lipase, amylase or organic solvents.

Table 2: Effect of different treatment on antibacterial activity of CFS using *L, monocytogenes* LMG 1047 as the indicator organism.

Treatment	Residual antibacterial activity (AU/ml)
Heat treatment:	
100°C for 10 min	1000
1000°C for 15 min	1000
Enzyme treatments:	
Catalase	1000
Pepsin	0
α-chemotrypsin	0
Trypsin	0
Proteinase K	0
Pronase E	0
Lipase	1000
Amylase	1000
Organic solvent treatments:	
Chloroform	1000
Diethyl ether	1000
Ethyl alcohol	1000
Toluene	1000
pH-treatments:	
pH 2.0	1000
pH 4.0	1000
pH 6.0	1000
pH 6.5	1000
pH 7.0	0
pH 8.0	0

Production of *L. plaantarum* LPS10 bacteriocin was investigated in MRS broth adjusted initially at different pH values (Figure 1). Initial pH 6.5 was the optimum for obtaining maximum growth and bacteriocin production by *L. plantarum* LPS10. Bacteriocin production started in the early exponential growth phase and maximum values of growth and bacteriocin activity (1000 AU/ml) were obtained after 12h of growth. At this initial pH 6.5, bacteriocin production was detected in MRS broth incubated at 25°C, 35°C and 42°C (Figure 2). Bacteriocin production and correlated growth were the best at 35°C. At this incubation temperature, growth values; bacteriocin activity recorded 8.2 x 10^8 CFU/ml; 1200 AU/ml within 24h respectively. Considerable growth and bacteriocin activity were obtained at other two incubation temperatures reaching 1000 AU/ml; 800 AU/ml after 24h of incubation at 25°C, 42°C respectively; but these were still lower than that obtained in samples incubated at 35°C. Bacteriocin production was decreased by prolonged incubation (Figure 2).

L. plantarum LPS10 bacteriocin was purified using two-step purification protocol including ammonium sulphate precipitation and gel filtration across sephadex G200/50. A purification scheme is given in Table 3. PPB was obtained at pH 6.5 and 50% ammonium sulphate saturation. Bacteriocin activity increased from 1000 AU/ml in CFS to 7000 AU/ml in PPE and in this PPB almost 132-fold increase in specific activity was showed using *L. monocytogenes* LMG 10470 as an indicator organism. For further characterization of PPB; this PPB was divided into three aliquots. The first aliquot was heated for 15 min at 100°C; second aliquot was treated with pronase E and in another experiment proteinase K; third aliquot was used as control without treatments. No bacteriocin activity was obtained from protease treated PPB and bacteriocin activity of about 7000 AU/ml was

obtained in heat treated PPB and in control; indicating on proteinaeous nature and heat resistance ability of PPB.

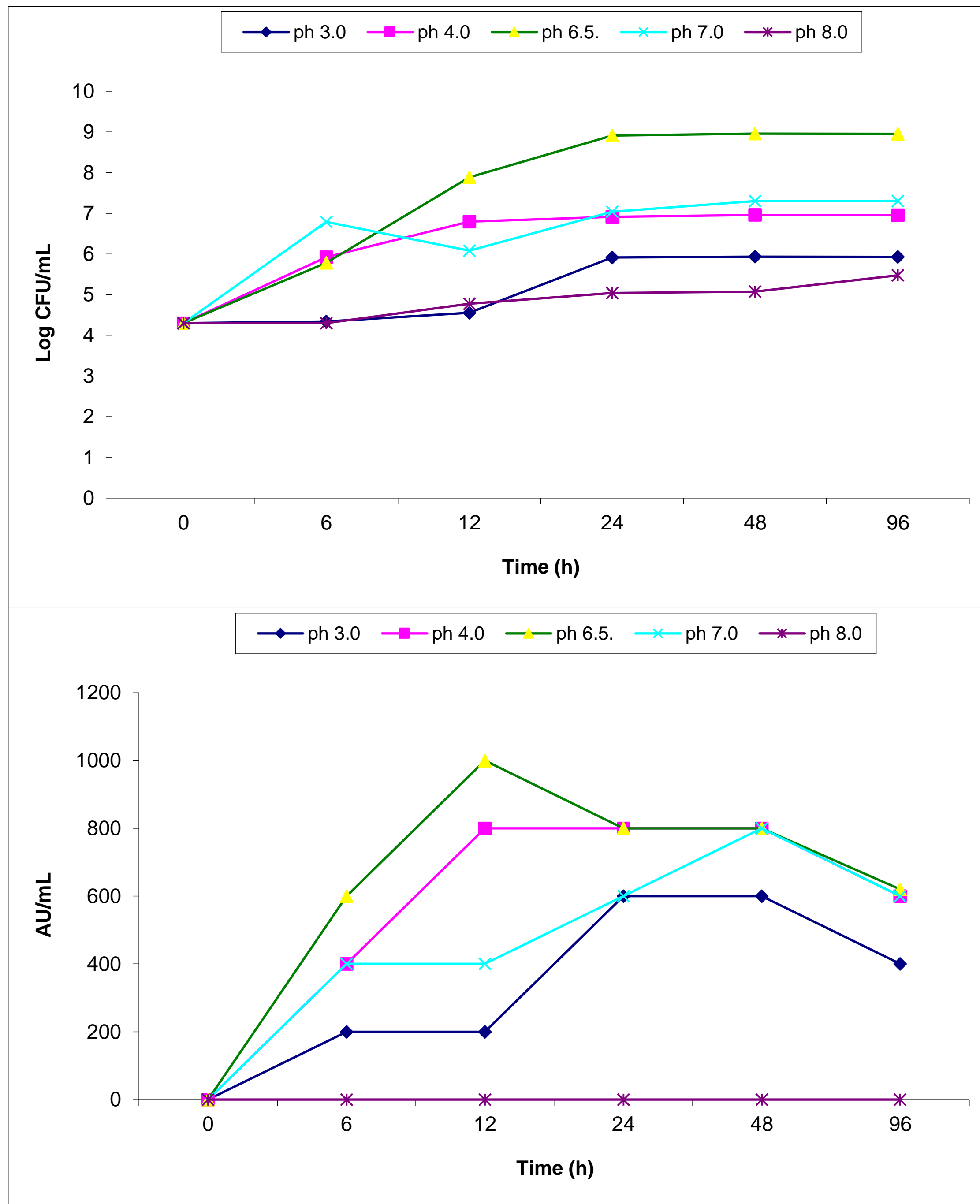

Figure (1): Effect of different pH values on *L. plantarum* LPS10 growth and bacteriocin production.

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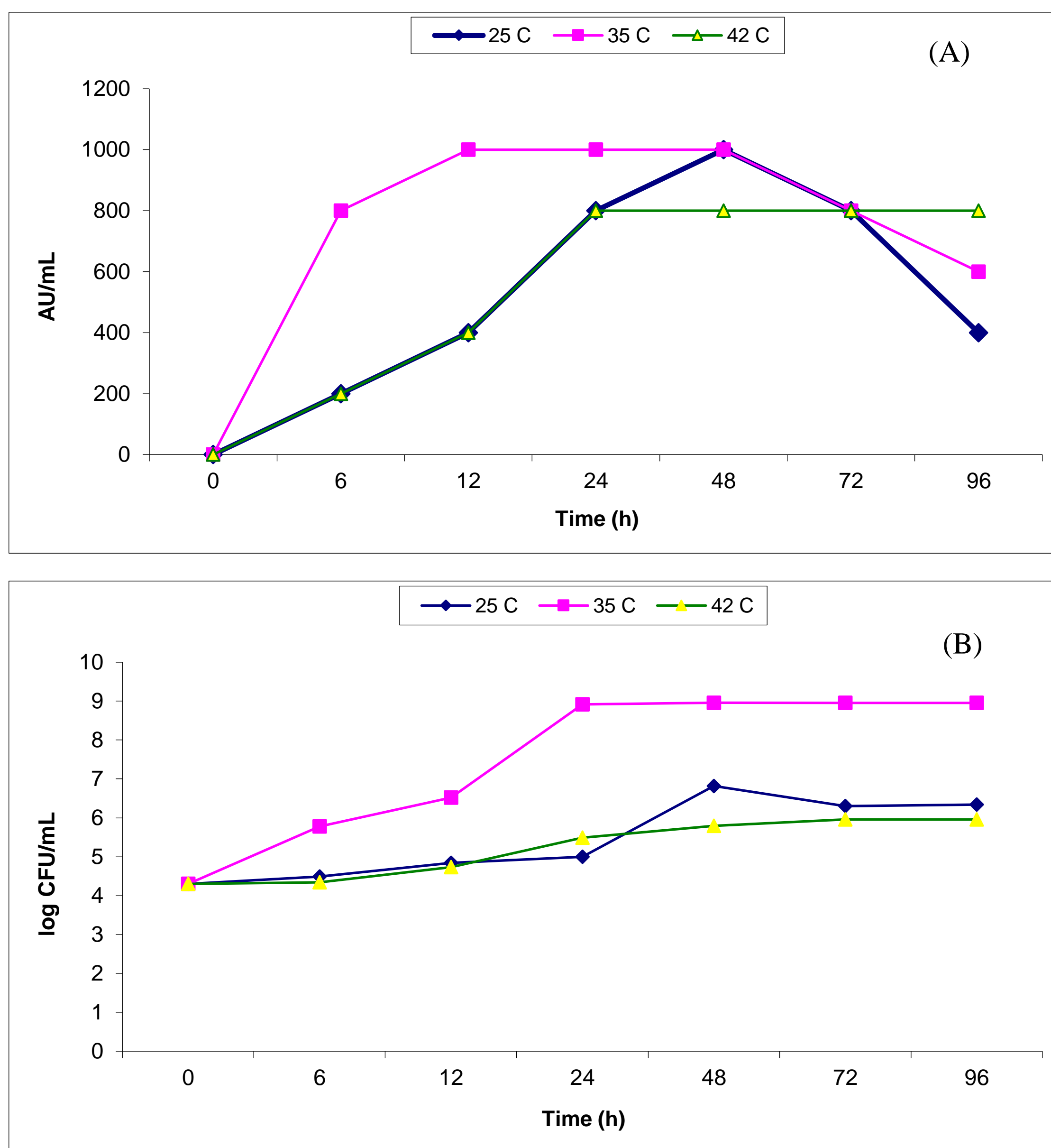

Figure (2): Effect of incubation temperatures on (A) bacteriocin production and (B) growth of *L. plantarum* LPS10. Symbols; A, incubation at 35◦C; 0 and incubation at 42◦C.

PPB was applied to sephadex G200/50 column. Elution was carried out by potassium phosphate buffer pH 6.5. Results are given in Figure 3. Two large peaks of bacteriocin activity were obtained in fractions 10, 15. They coupled with the highest absorbance (0.7 nm). PPE showed a distinctive increase in bacteriocin activity (45000 AU/ml) and a 520 fold-increase in specific activity was obtained (Table 3).

Table 3: Purification scheme of *L. plantrum* LPS10 bacteriocin.

Sample	Total Protein (µg/ml)	Bacteriocin activity (AU/ml)	Specific activity	Fold increase in specific activity
- CFS	0.083	1000	12.04	1
- PPB obtained by ammonium sulphate precipitation	4.42	7000	1590	132.5
- PPB obtained after elution on sephadex G200-50	7.2	45000	6250	520

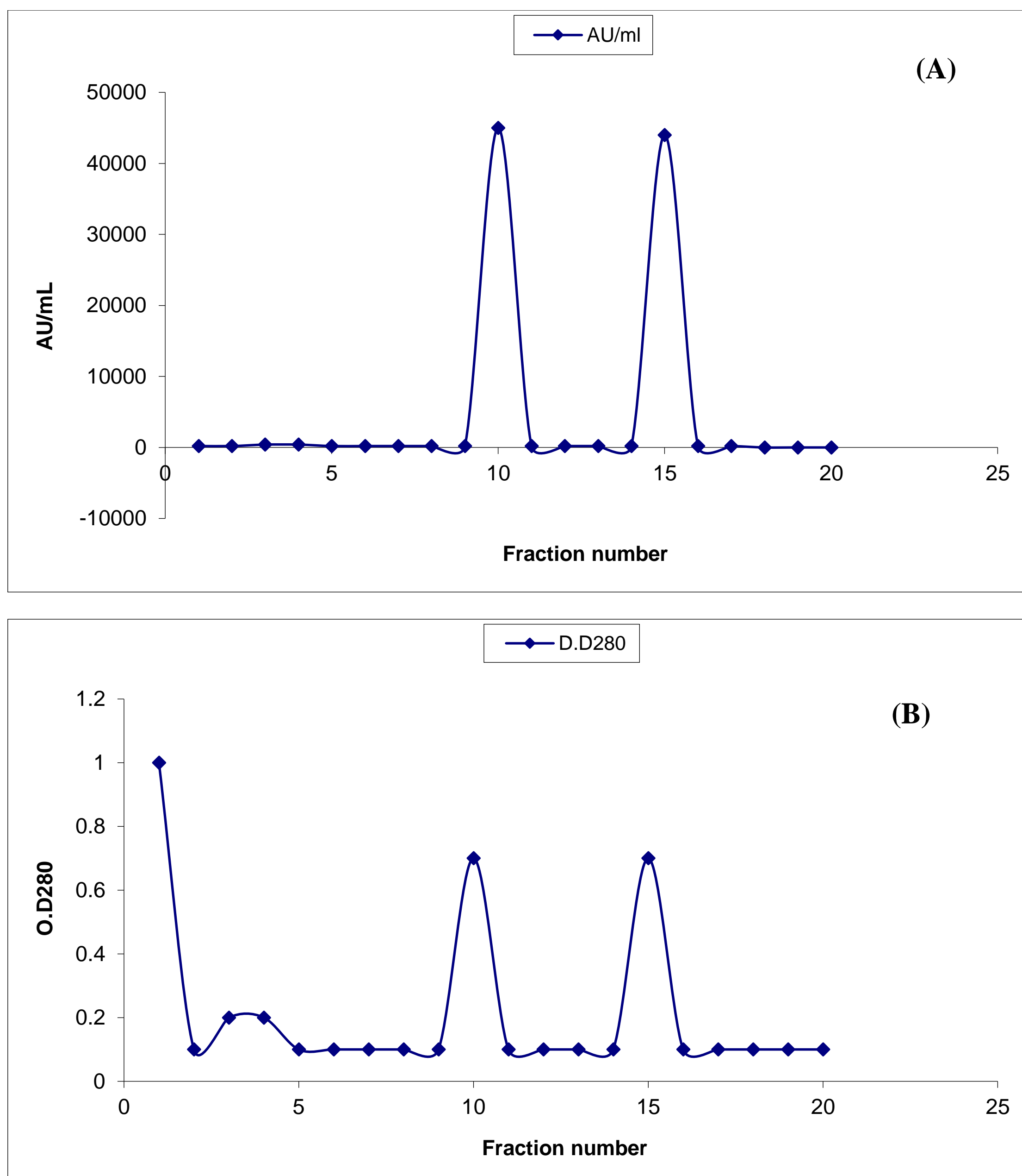

Figure (3): Elution profiles of PPB produced by *L. plantarum* LPS10; (A), bacteriocin activity (AU/mL); (B), absorbance (OD 280) obtained from the 20 fractions appeared.

To confirm the purity of PPE, SDS-PAGE was carried out. Two bands of pure protein were showed indicating on two polypeptides of molecular masses of about 15 kDa and 50 kDa (Figure 4). To ensure whether those two polypeptides were bacteriocin components or were other protein else, antibiogram was carried out using soft agar containing *L. monocytogenes* LMG 10470 as an indicator organism. This soft agar was seeded with the indicator strain and poured over the unstained gel, incubated at 30°C for 24 h. A large inhibition zones were showed in the indicator lawns on the protein bands; indicating a purity and activity of *L. plantarum* LPS10 bacteriocin components (Figure 4).

Table 4: Amino acid analysis of the bacteriocin plantaricin LPS10.

No of amino acid	Amino acid type	Concentration of amino acid (µg/ml)
1	Aspartic acid	721.76
2	Threonine	509.92
3	Serine	509.84

4	Glutamic acid	5677.92
5	Glycine	407.84
6	Alanine	1253.28
7	Valine	619.84
8	Methionine	312.16
9	Isoleucine	236.96
10	Leucine	949.2
11	Tyrosine	510.56
12	Phenyl alanine	1242.4
13	Histidine	208.96
14	Lysine	503.44
15	Cysteine	745.03

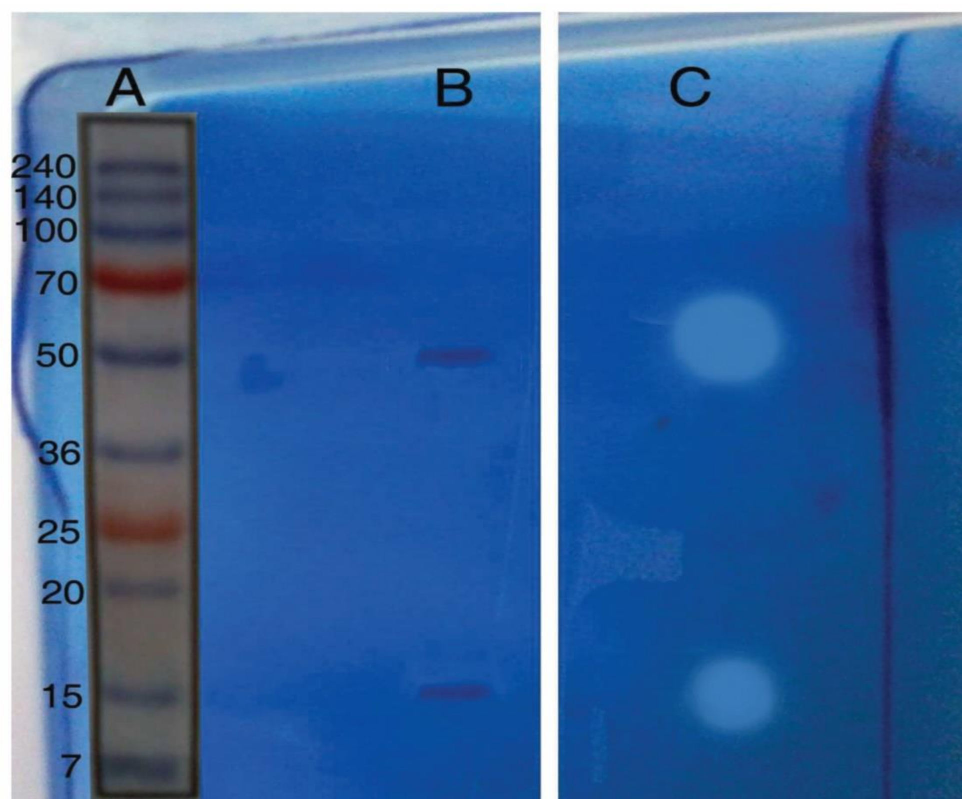

Figure (4): SDS-PAGE of PPB eluted from mix of fractions 10 and 15. (A) standard protein markers of known molecular size, (B) gel stained with Coomasie brilliant blue, (C) unstained gel showing the *in-situ* bacteriocin activity against *L. monocytogenes* LMG 10470 as an indicator organism.

Amino acid analysis of PPE was carried out (Table 4). PPE contained 15 amino acids and lanthionine did not appear; indicating that this bacteriocin was non-lantibiotic. It contained polar amino acids (serine, threonine, tyrosine and cysteine), non-polar amino acids (glycine, alanine, valine, leucine, isoleucine, phenyl alanine and methionine), amino acids with acidic side chains (aspartic acid and glutamic acid), and amino acids with basic side chains such as histidine and lysine. At this final step of experiments, it was proved that the PPE was bacteriocin and coupled with properties of class IIb bacteriocins which contain 2 polypeptides and are non-lantibiotic and, therefore, this bacteriocin designated plantaricin LPS10 and was classified as belonging to class IIb bacteriocins.

DISCUSSION

As *L. plantarum* LPS10 isolated from pickled olive showed interested properties such as fast growth, fast acidification of medium, production of enzymes and inhibition of many sensitive pathogenic bacteria which enable this LPS10 strains to be starter, protective and probiotic bacterium [10]; it was necessary to characterize and purify the antimicrobial substance produced by the LPS10 bacterium. The inhibitory

substance of either CFS or PPB was heat resistant protein and fits with bacteriocin definitions [24]; and classified as belonging to bacteriocin and designated plantaricin LPS10. This bacteriocin was active in the acidic pH levels (pH 2.0 – pH 6.8) but was not stable at neutral and alkaline pH levels (pH 7.5 – pH 14); this is similar to some *L. plantarum* bacteriocins [24, 25] but is different from some bacteriocins produced by *L. plantarum* [26]. Plantaricin LPS10 did not contain lipid or carbohydrate moieties in its active molecule and this corroborates latter findings in this respect [4, 10, 15, 20, 24, 27].

CFS of *L. plantarum* LPS10 containing crude plantaricin LPS10 inhibited other LAB tested, Gram positive and Gram negative bacterial pathogens with the exception of *Pseudomonas aeruginosa* and the *Candida albicans* LMZ331 fungus. It was known till the end of last century that bactertiocins are active against only Gram positive bacteria and/or closely related bacteria to the producer strains [1]. Recent studies described bacteriocin activity against both Gram positive and Gram negative bactreria and fungi [28]. Plantaricin A produced by *L. plantarum* C11 inhibited also Gram negative bacteria and fungi; it permeabilizes eukaryotic cell membranes by a mechanism dependent on negative surface charge linked to glycosylated membrane proteins [29]. Activity of bacteriocins from *L. plantarum* against Gram negative bacteria has been reported in other previous studies [6]. In general, different spectra of inhibitory action may be obtained deopending on the bacteriocin producing strain, and also the method used for bacteriocin detection [30]. The inhibition of other LAB by plantaricin LPS10 is of interest for starter cultures in controlled lactic acid fermentations of plant materials such as vegetables and silage which naturally contain competing LAB flora [2, 31].

Plantaricin LPS10 was produced in the mide to the late exponential phase of growth of *L. plantarum* LPS10 and optimum values were obtained in MRS broth adjusted initially at pH 6.5 and incubated at 35°C; similar results in bacteriocin production have been observed for other bacteriocins [16, 24].

Plantaricin LPS10 was purified successfully by two-step purification procedure including ammonium sulphate precipitation and gel-filtration. A notable increase in bacteriocin activity reaching 520-fold increase in specific activity of PPE; similar increase in bacteriocin activity was obtained upon purification of LAB bacteriocins [4, 6, 18, 20, 27]. The purity of planatricin LPS10 was judged by SDS-PAGE as two clear protein bands were observed denoting on molecular sizes of about 15 kDa, 50 kDa. This showed that plantaricin LPS10 contained two polypeptides and was, therefore, similar to class IIb bacterocins which contain two active polypeptides [27]. According to this author, bacteriocins are classified into separate groups such as the lanthionine containing bacteriocins (lantibiotics, class I); the non-lantibiotics (<10 KDa), heat stable posttranslationally proteins (Class II) which further subdivided to the pediocin-like and anti-*Listeria* bacteriocins (Subclass IIa); and the two-polypeptide bacteriocins (subclass IIb); and the large heat labile (>100 KDa) bacteriocins (Class III). The class IIb bacterocins include lactococcin G and plantaricins E, F, E, J, K. All of them showed narrow spectrum of activity against Gram positive bacteria and this did not apply on the broad spectrum plantaricin LPS10 described herein [25]. Many plantaricins were characterized as belonging to class IIa bacteriocins such as pediocin LB-B1 [7], bacteriocinST71KS produced by *L. plantarum* ST71KS [8], plantaricin C19 produced by *L. plantarum* C79 [32] and plantaricin Ip31 produced by *L. plantarum* [6]. Some biological and physiochemical properties of the above plantaricins fit with plantaricin LPS10 such as spectrum of activity and absence of lipid moieties in bacteriocin molecules but their distinctive properties are that all of them contained one polypeptide only and this is not the case in plantaricin LPS10 employed in this study which contained two active polypeptides as showed from the unstained gel, on which the *Listeria monocytogenes* indicator lawn was inhibited. In general comparison of different bacteriocins based upon spectra of activity or even stability in different conditions is elusive, as it is strongly dependent on the variability of strains used as indicators. Such comparison could only made at the molecular level.

Amino acid analysis of plantaricin LPS10 revealed 15 amino acids which did not include-lantionine; indicating that this bacteriocin is a non-lantibiotic. A non-polar hydrophobic amino acids such as glycine, alanine, valine, leucine, isoleucine, phenyl alanine and methionine are existed in plantaricin LPS10 molecule and these amino acids can be through of as lipid-like, a property that promotes hydrophobic interactions stabilizes protein amino acids such as serine, threonine, tyrosine and serine that can participate in hydrogen bond formation and the side chain of cysteine can become oxidized to form dimer of cystine which contains disulfide bond (-S-S-); giving greater antimicrobial activity of this bacteriocin. This is in agreement with previous studies in this respect [6, 24, 26]. The two acidic amino acids of plantaricin LPS10 such as glutamic and aspartic acids can be dissociated giving negatively charged side chains; the two basic amino acids of

plantaricin LPS10 such as histidine and lysine accept protons at physiologic pH and become fully ionized and positively charged and enable this bacteriocin to (i) attach positively charged phospholipids of cell membranes of sensitive prokaryotic or eukaryotic cells and to (ii) attach to positively charged groups of either cell wall or cell membranes of sensitive cells, making permeabilization of sensitive cells and then pore formation which causes cell death. This corroborates the findings of latter previous work in this respect [29].

Further work will be necessary to characterize plantaricin LPS10 at the molecular level regarding its amino acid sequence and its gene(s) encoding; in this case the actual classification of plantaricin LPS10 can be done,

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