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Partial Purification of two Novel Variants of Bacteriocins Produced by Lactic Acid Bacteria

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Abstract: The work employed described the partial purification and physicochemical characterization of two bacteriocins produced by two strains of lactic acid bacteria (LAB) namely: *Lactococcus lactis* subsp. *Lactis* Z_{11} (*L. lactis* Z_{11}) and *Lactobacillus delbrueckii* subsp. *Bulgaricus* Z_{55} (*Lb. bulgaricus* Z_{55}); both strains were isolated from Arabian yoghurt. The two bacteriocins were purified by two-stepsprotocol including ammonium sulphate precipitation and ion-exchange chromatography across sephadex G 200-50 column. The two bacteriocins were precipitated ideally at 50-60% ammonium sulphate saturation levels in the pH range 5.0-6.0. The specific activity of *L. lactis* Z_{11} bacteriocin and *Lb. bulgaricus* Z_{55} bacteriocin was increased by102 times; 12.7 times respectively. Bacteriocin activities showed further increase by ion-exchange chromatography asabout 383 fold-increase and 170 fold-increase in specific bacteriocin activity was reported for *L. lactis* Z_{11} bacteriocin and *Lb. bulgaricus* Z_{55} bacteriocins respectively. SDS-PAGE analysis revealed that *L. lactis* Z_{11} bacteriocin and *Lb. bulgaricus* Z_{55} bacteriocin had a molecular weight of 3.5 and 5.0kDa respectively. Amino acid analysis of two bacteriocins showed that *L. lactis* Z_{11} bacteriocin is alantibiotic bacteriocin and *Lb. bulgaricus* Z_{55} bacteriocin is a non-lantibiotic bacteriocin. Consequently the former designated lacticin Z_{11} andwas classified as a member within class I bacteriocins and the latter was designated *bulgaricin* Z_{55} and was classified as a novel variant within class IIa pediocin- like bacteriocins.

Key words: Lactic Acid Bacteria · Bacteriocins · Purification · Physicochemical Characterization

INTRODUCTION

Lactic acid bacteria (LAB) are widely distributed in nature. They are found on vegetables, grains, in milk, meat and their products [2-3]. LAB are the predominating microflora in food fermentations. Their presence in fermented food cause acidification resulting in inhibition of spoilage and pathogenic bacteria [1-4]. The antimicrobial activity of LAB is due to fermentation and their products such as organic acids, carbon dioxide, hydrogen peroxide, diacetyl; among others, as well as to the bacteriocins [5-7]. Bacteriocins are antimicrobial proteins active mainly in closely related bacteria [8]. Recently, bacteriocins of LAB inhibited many pathogenic Gram positive and Gram negative bacteria [9, 6].

Lactic acid bacteria are used as probiotics which confer a health benefit on the host and to prevent or limit the growth and colonization of potentially pathogenic bacteria [10]. LAB are the main microflora of raw milk, milk products and many fermented food [11]. The recent interest is focused to select and characterize LAB from foods to be used as starter, protective and probiotic cultures [12]. In this study, *Lactococcus lactis* subsp. *Lactis* Z_{11} (*L. lactis* Z_{11}) and *Lactobacillus delbrueckii* subsp. *bulgaricus* Z_{55} (*Lb. bulgaricus* Z_{55}) isolated from Arabian yoghurt (Zabady) inhibited many food borne pathogens and showed many probiotic capabilities such as proteolytic, lipolytic and ß-galactosidase activities. They also produced acetoin, organic acids which give yoghurt its tart flavor and aroma [3]. The inhibitory substances from both *L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55} were characterized as bacteriocins [4]. These two bacteriocins were heat resistant, stable in acidic and neutral pH levels and did not affect by either lipase or amylase indicating an absence of either lipidic or glucocidic moieties in their active molecules [3, 4, 7]. The present work was undertaken to purify, characterize and classify the bacteriocins produced by *L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55}. Molecular weight and amino acid analysis of bacteriocins were carried out.

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MATERIALS AND METHODS

Bacterial Strains and Culture Media: *L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55} were used as bacteriocin producers. They were isolated on M 17 agar (Oxoid) and De Man Rogosa and Sharpe agar (MRS agar) respectively and were sub-cultured on the same media [3]. Both Z_{11} and Z_{55} strains were characterized biochemically and biologically in a previous studies [3, 4].

Preparation of Cell-Free Supernatants (CFS): CFS were collected as described previously [13, 14, 15, 16, 17]. Briefly, 18 h. old cultures in broth media; M 17 brothfor *L. lactis* Z_{11} and MRS broth for *Lb. bulgaricus* Z_{55}; were centrifuged at 10.000 x g for 15 min at 4°C. CFS were neutralized to pH 7.0 by 1 M NaOH and treated with catalase (1mg/mL) to avoid inhibitory activity due to hydrogen peroxide. The CFS were then filter-sterilized through Milliporefilters (Amicon, 0.45 µm). This pH-adjusted, filter sterilized CFS were used immediately after preparation. One milliliter of either CFS or partially purified bacteriocin was assayed against *Listeria monocytogenes* LMG 10470 by critical dilution assay [18,19, 32,33].

Purification, Molecular Mass and Amino Acid Analysis of Bacteriocins: Bacteriocins were purified from the culture medium as the following steps.

Ammonium Sulphate precipitation of Bacteriocins: CFS from *L. lactis* Z_{11} and *Lb. bulgaricus*Z_{55} were adjusted at different pH values (pH 3, 4, 5, 6 and 7) and were treated with solid ammonium sulphate to 40, 50 and 60% saturation. The mixtures were stirred for 12 h at 4°C and centrifuged at 20.000 g for 1 h at 4°C. The precipitates (surface pellicles and pellets) were recovered in 10 mM potassium phosphate buffer, pH 6.5 and dialysed against the same buffer for 24 h at 4°C in Visking Dialysis Tubing (Pharmacia). This partially purified bacteriocin (PPB), was sterilized by filtration through cellulose membrane filters (Amicon 0.45µm, Milipores) and titrated against *L. mononcytogenes* [4, 7]. ThePPB was used for further purification experiments.

Elution of Bacteriocins and Chromatographic Analysis: 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 7.0 at room temperature. Activity was eluted with the same buffer and the eluent was monitored for A 280 (absorbance at 280 nm) and bactetriocin activity (AU/mL). Five mL fractions were collected. Time needed for collection of each fraction was varied from one to another one [4, 7].

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Fraction number 3 of *L. lactis* Z_{11} bacetriocin eluent and number 7 for *L. bulgaricus* Z_{55} bacteriocin eluent, eluted from the column contained the highest bacteriocin activity against *L. monocytogenes* were pooled and subjected to SDS-PAGE analysis. Protein were analysed for SDS-PAGE using Phast Gel High Density Strips,Phast gel SDS buffer strips [20] and Phast System (Pharmacia LKB Biotechnology). Before application on the gel, 2mL of each fraction were boiled for 10 min in SDS buffer containing 2% 2-mercaptoethanol.

Protein Determination: Concentration of proteins in the active fractions containing the highest bacteriocin activity were estimated by comparison with a Coomassie Blue binding assay [21] usingmixture of commercial standard protein, human albumin and globulin (5:3) as standard (Sigma).

Amino Acid Analysis of Bacteriocins: Aminoacidswere determined using the method described previously [22]. 200 µl of purified bacteriocin obtained after gel filtration were hydrolysed with 6 N 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- citric 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

Ammonium Sulphate Precipitation of Both *L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55} Bacteriocin: To precipitate bacteriocins with optimum antibacterial activity, the precipitated ammonium sulphate pellets were collected from CFS adjusted at pH value 3, 4, 5, 6, 7 by using 40, 50, 60 % ammonium sulphate saturation levels. Results are given in Table (1). The 50%; 60% saturation levels appeared to be the best saturation levels for *Lb. bulgaricus* bacteriocin; *L. lactis* bacteriocin respectively.

Table 1: Ammonium sulphate precipitation of bacteriocins from both *L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55}.

	Bacteriocin activity (AU/ mL) at different ammonium sulphate saturation levels					
	40%		50%		60%	
pH value of CFS	*L. lactis* Z_{11}bacteriocin	*Lb. bulgaricus* Z_{55}bacteriocin	*L. lactis* Z_{11}bacteriocin	*Lb. bulgaricus* Z_{55}bacteriocin	*L. lactis* Z_{11}bacteriocin	*Lb. bulgaricus* Z_{55}bacteriocin
3.0	12000	14000	16000	10000	32000	6000
4.0	12000	14800	16000	10000	38000	8000
5.0	46000	16000	64000	18000	82000	12000
6.0	32000	16000	64000	18000	88000	12000
7.0	18000	14800	48000	16000	44000	12600

Table 2: Amino acid composition of lacticin Z_{11} and bulgaricin Z_{55}

Amino acid	Lacticin Z_{11} (µg/ mL)	Bulgaricin Z_{55} (µg/ mL)
Aspartic acid	849.7	1227.6
Threonine	265.6	470.4
Serine	453.4	691.52
Lanthionine	268.0	0
Glutamic acid	2363.6	3304.48
Alanine	2362.2	2425.84
Valine	642.9	664.96
Methionine	1616.1	2456.72
Isoleucine	297.12	589.6
Leucine	748.9	612.8
Tyrosine	24.96	253.04
Phenyl alanine	9.2	771.44
Histidine	363.12	777.52
Lysine	396	725.04
Arginine	400.96	0

Table 3: Purification scheme oflacticin Z_{11} and bulgaricin Z_{55}.

	Lacticin Z_{11}				Bulgaricin Z_{55}			
Sample	AU/ mL	Total protein (mg/ mL)	Spacific activity AU/mg protein	Increase in sapcific activity	AU/mL	Total protein (mg/mL)	Spacific activity AU/mg protein	Increase in sapcific activity
CFS	1880	4.80	391.6	1	1860	4.2	443	1
Ammonium sulphatepreciptation	88000	2.20	40000	102	18000	3.2	5625	12.7
Purified fraction after ion-exchange chromatography	270000	1.80	150000	383	216000	2.86	75524	170

In the pH range 5.0-6.0 and ammonium sulphate saturation, the *Lb. bulgaricus* Z_{55} bacteriocin pellets had the maximal activity of about 18000 AU/mL. In the same pH range and 60% ammonium sulphate saturation level, *L. lactis* Z_{11} bacteriocin pellets had the maximal activity of about 82000-88000 AU/mL. Other pH values (pH 3, 4, 7) in the presence of 40% ammonium sulphate saturation, the recovered activities of the two precipitated bacteriocins pellets were comparatively low. It was necessary to remove salt from the precipitated pellets. Hence dialysis of bacteriocin pellets was conducted using Visking Dialysis Tubing (Pharmacia) against phosphate buffer pH 6.8. Dialysis was performed for 24 h at 4°C. Bacteriocin activity in each case was performed using *L. monocytogenes*as the indicator organism. After ammonium sulphate precipitation, the specific activity of *L. lactis* Z_{11} bacteriocin and *Lb. bulgaricus* Z_{55} bacteriocin was increased by 102 times; 12.7 times respectively (Table 3).

Ion-Exchange Chromatography: Elusion of both *L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55} bacteriocins was studied using ion-exchange chromatography on sephadex G200-50 column. Results are given in Figure (1). Large peaks of bacteriocin activities were obtained in fraction number 3 for *L. lactis* Z_{11} bacteriocin and fraction number 7 for

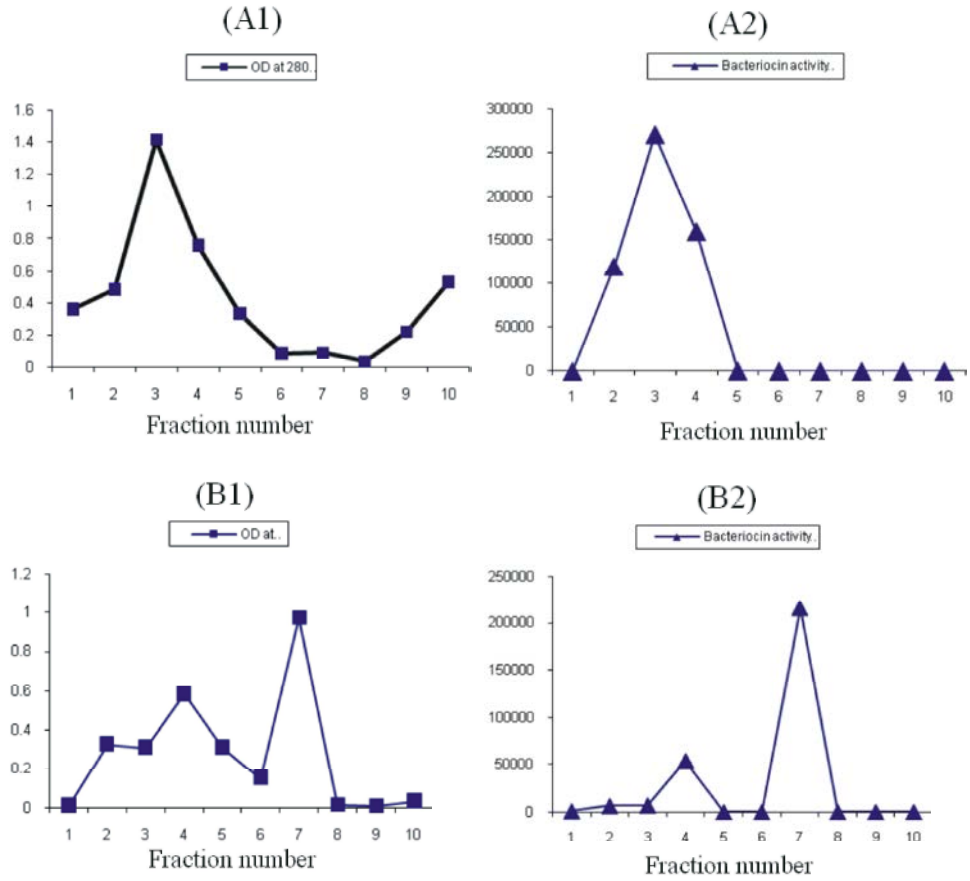

Fig. 1: Elution profiles of partially purified bacteriocins on Sephadex G200-50 of (A) *L. Lactis* Z_{11} and (B) *Lb. bulgaricus* Z_{55} symbols: ■, absorbance at 280nm; ▲ bacteriocin activity titre (AU/ml)

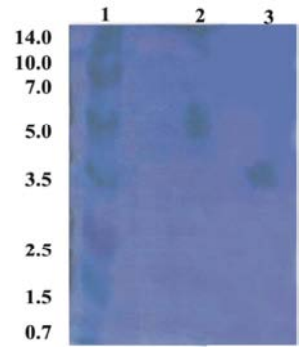

Fig. 2: SDS-PAGE of bacteriocins. Lane 1, protein standards; Lane 2, *Lb. bulgaricus* Z_{55} bacteiocins; Lane 3, *L. lactis* Z_{11} bacteriocin

Lb. bulgaricus Z_{55} bacteriocin. The bacteriocin activity was 270000 AU/mL for the former and was 216000 AU/mL for the latter. Peaks number 2, 4, 5, 6 showed lower activity of *L. lactis* Z_{11}bacteriocin and peaks 1-4 showed lower activity of *Lb. bulgaricus* Z_{55} bacteriocin. Other eluted peaks of the two tested bacteriocin fractions showed no activity using *L. monocytogenes*as the indicator organism. SDS-PAGE of both*L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55} bacteriocins

To determine the purity and molecular mass of the two experimental bacteriocins,the purified bacteriocins obtained in fractions number 3 of *L. lactis* Z_{11}bacteriocin and number 7 of *Lb. bulgaricus* Z_{55} bacteriocin were subjected to SDS-PAGE analysis. As shown in Figure (2), single bands of pure proteins were observed migrating at a position of about 3.5 kDa for *L. lactis* Z_{11} bacteriocin and of about 5.0kDa for *Lb. bulgaricus* Z_{55} bacteriocin (Fig. 2).

Amino Acid Composition of Both *L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55} Bacteriocins: It was necessary to prove that the bacteriocins employed herein consist of protein. Therefore, their amino acid composition was studied. Results are given in Table (2). *L. lactis* Z_{11}bacteriocin contained lanthionine amino acid indicating on lantibiotic produced by *L. lactis* Z_{11}. This lantibiotic produced by

L. lactis Z_{11} contained15 amino acids. Alanine and glutamic acid showed the higher content of almost 2362 µg/mL. Phenyl alanine showed the lower content, reaching 9.2 µg/mL. It could be designated lacticin Z_{11}.

The concentration of amino acids was more in *Lb. bulgaricus* Z_{55} bacteriocin than that found in Lacticin Z_{11}. Glutamic acid, methionine alanine and aspartic acid were the more concentrated amino acids in *Lb. bulgaricus* bacteriocin. Neither lanthionine nor arginine were found, while the average (253-777 µg/mL) of other amino acids listed in Table (2) were observed. Therefore, *Lb. bulgaricus* Z_{55}bacteriocin was protein and based on [8, 23]; it was designated bulgaricin Z_{55}.

Purification Scheme of Both Lacticin Z_{11} and bulgaricin Z_{55}: A purification schemes for both bacteriocins studied herein are shown in Table (3). Activity units per milliliter of lacticin Z_{11}; bulgaricin Z_{55} were increased from 1880 AU/mL; 1860 AU/mL in CFS to 270000 AU/mL; 216000 AU/mL in samples purified by ion exchange chromatography respectively. Z_{11} increased from 391.6 to150000 AU/mg protein with 383 fold-increases in bacteriocin activity. However, specific activity of bulgaricin Z_{55} increased from 443 AU/mg proteinin CFS to 75524 AU/mg protein with 170 fold-increase in bacteriocin activity. This indicated on the purity of both lacticin Z_{11} and bulgaricin Z_{55}.

DISCUSSION

The study employed herein was coincided by clear success since two strains ofLAB, isolated from Arabian yoghurt (Zabady),*L. lactis* Z_{11} and *Lb. bulgaricus* Z_{55} were showed to produce bacteriocins that inhibited many food borne pathogens [4,7]. Therefore, those two strains could be used as probiotics to prevent or limit the growth and colonization of potentially pathogenic bacteria and to improve many nutritional functions in humans. The number of probiotic dairy products has increased tremendously in the market during the last few years. Probiotic bacteria are mainly members of genera *Lactobacillus* and *Bifidobacterium* [31]. This clearly shows that there is a need to continue research to characterize a starter and protective LAB cultures; producers of bacteriocins.

Two- steps protocol have been developed for the purification of both *L. lactis* $Z_{11}$and *Lb. bulgaricus* Z_{55}bacteriocins. The protocol included ammonium sulphate precipitation and ion-exchange chromatography. The purification procedures resulted in a pure bacteriocin preparation as judged by SDS-PAGE analysis. The purification protocol adopted herein was also used successfully for purification of many bacteriocins of LAB [20,6].

Precipitation of the two studied bacteriocins with ammonium sulphate showed that when the pH of CFS was adjusted at 6.0 with 50%; 60% ammonium sulphate saturation, the bacteriocin activities of *L. lactis* Z_{11}; *Lb. bulgaricus* Z_{55} recovered in the pellets were higher than that present in initial CFS. Increased bacteriocin activity has been observed previously upon precipitation by ammonium sulphate[24].

A notable increase in bacteriocins activities reaching 383-fold increase in *L. lactis* Z_{11} bacteriocin and reaching 170-fold increase in *Lb. dulbrukii* Z_{55} bacteriocin was observed in the partially purified bacteriocin extracts. This suggested on purity of the two studied bacteriocins. Similar increase in bacteriocin activity was obtained after their purification by ion exchange chromatography [25].

The molecular masses of *L. lactis* Z_{11} bacteriocin; *Lb. bulgaricus* bacteriocin were 3.5kDa; 5kDa respectively. Amino acid composition of the two bacteriocinswas studied. *L. lactis* Z_{11} bacteriocin contained lanthionine amino acid this proved that *L. lactis* bacteriocin is a lantibiotic. This lantibiotic could be designated lacticin Z_{11} [8, 9, 23, 26]. According to Halami *et al*. [9] and Cotter *et al*. [27], bacteriocins are grouped into separate groups such as the lantibiotics (class I); the small (□10kDa) heat-stable non-lantibiotics (classII), further divided in the pediocin-like and anti-*Listeria* bacteriocins (subclass IIa), the two peptide bacteriocins (subclassIIb); and the large molecular weight (□30kDa) heat labile non lantibiotics (bacteriolysins) (classIII). Consequently, lacticin Z_{11} employed herein is similar to class I lantibiotic bacteriocins in molecular weight and existence of lanthionine in its active molecule, but differs in pH stability. Lacticin Z_{11} is stable at acidic and neutral pH levels, but lantibiotic bacteriocins are active at only acidic pH values [9]. Hence lacticin Z_{11} employed herein could be classified to class I bacteriocin and considered a novel one within them.

The molecular mass of *Lb. bulgaricus* bacteriocin was around 5kDa and this is similar to some bacteriocins produced by LAB [3, 4, 29]. Amino acid composition showed that the *Lb. bulgaricus* Z_{55} bacteriocin did not contain lanthioninein its active molecule. This indicated that the *Lb. bulgaricus* bacteriocin is protein and following definitions of bacteriocins [8, 23, 26], it could be

designated bulgaricin Z_{55}. The bacteriocin bulgaricin Z_{55} possessed molecular mass of about5kDa and did not contain lanthioninein its active molecule and was thermostable. Hence bulgaricin Z_{55} could be classified following class Iiabacteriocins [9, 26, 30]. However, class IIa anti-*Listeria* thermostable bacteriocins possessed molecular weight in the range 2.5-4.5kDa and mostly are active at either acidic or alkaline pH values [27, 28] and are active on closely related bacteria and *Listeria* spp. The bulgaricin Z_{55}bacteriocin showed dissimilar properties regarding molecular mass, spectrum of activity and stability. Consequently, bulgaricin Z_{55} could be considered othervariant within class IIapediocine like bacteriocins [6, 27, 28]. Further work regarding amino acid sequences will be necessary for both lacticin Z_{11} and bulgaricin Z_{55}. This will show whether those two bacteriocins are novel ones or variants of other described bacteriocins.

CONCLUSION

L. lactis Z_{11}bacteriocin; *Lb. bulgaricus* Z_{55}bacteriocin was purified. Their specific activity showed 383 fold-increase; 170 fold-increase and their molecular masses were 3.5 kDa; 5.0 kDarespectively. The amino acid analysis showed that their composition were pure protein. Consequently, *L. lactis* Z_{11}bacteriocin designated lacticin Z_{11} and was classified as another variant within class I lantibioticbacteriocin; *Lb. bulgaricus* Z_{55}bacteriocin designated bulgaricin Z_{55} and was classified as novel variant within class IIapediocin- like bacteriocin.

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