Purification and biochemical characterization of plantaricin UG1: a bacteriocin produced by *Lactobacillus plantarum* UG1 isolated from dry sausage

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Abstract: The bacteriocin plantericin UG1 produced by *Lactobacillus* (*Lb.*) *plantarum* UG1 was purified herein by three steps including ammonium sulphate precipitation, dissociation of bacteriocin activity and chromatographic separation. At pH 6.0 and 60% ammonium sulphate saturation, the precipitated pellets contained maximal plantaricin UG1 activity of about 22880 AU/ml. Some dissociating agents dissociated plantaricin UG1 activity, thereby releasing more active subunits. The bacteriocin plantaricin UG1 existed in ammonium sulphate pellets was dialysed against potassium phosphate buffer pH 6.5 and was easily purified using chromatographic separation. Purified plantaricin UG1 activity showed 54.69 fold -increase than that obtained in cell free supernatant (CFS). The purity of plantaricin UG1 was judged by SDS-PAGE which resolved a 3.7 KDa and 1.8 KDa protein bands; indicating that plantaricin UG1 is two polypeptides. Consequently it is another bacteriocin within class 11 b bacteriocins. Amino acid composition of plantaricin UG1 showed 29 to 31 amino acids. Amino acid sequence of plantaricin UG1 showed a double glycine leader and proved that it is a novel variant of plantaricins.
[Sahar M. Ouda, Johan Debevere and Gamal Enan. **Purification and biochemical characterization of Plantaricin UG1: a bacteriocin produced by *Lactobacillus plantarum* UG1 isolated from dry sausage.** *Life Sci J* 2014;11(8):271-279]. (ISSN:1097-8135). http://www.lifesciencesite.com. 37

Keywords: Purification; bacteriocin; plantaricin UG1; *Lb. plantarum*, UG1; amino acid sequence

1- Introduction

Bacteriocins are antimicrobial proteins produced by bacteria [1,2].Bacteriocins of lactic acid bacteria have attracted the attention of many investigators especially in the recent years because of their use as a natural food preservative with probiotic capability within human body after ingestion of food [3,4].Plantaricin UG1 was produced by *Lb. plantarum* UG1[5,6] It was found to inhibit many food-borne pathogens including *Bacillus cereus, Clostridium sporogenes, Clostridium perfringens and Listeria monocytogenes* [7]. Plantaricin UG1 was thermostable and active over wide pH range (pH 4-7.0). Its production was linked to gene located within chromosomal DNA but not in plasmid [8]. Plantaricin UG1 showed a bactericidal action against sensitive bacteria[9,10,11]. Plantaricin UG1 inhibited some food-born pathogens *in vitro* and in food [12,13,14] The bacteriocin producer strain: *Lb. plantarum* UG1 could be used in sausage making and with probiotic capabilities. Hence there is a need to select isolates to inhibit bacterial pathogens and with probiotic capabilities to the immune system of human body [15,16].

Plantaricin UG1 was produced in an extracellular form[7] and differed from the cell bound bacteriocin[17]. Hence, crude plantaricin UG1 preparations should be obtained by salt preparation[18]. Crude bacteriocins have a tendency to aggregate with other proteins. To precipitate them during dialysis, it must be solubilized by different buffers [11,19,20].

Due to the importance of plantaricin UG1 in control of food-borne pathogens during food industry, and owing to the promise and possible use of its producer: *Lb. plantarum* UG1 as probiotic culture, the present study was undertaken to (1) purify the bacteriocin plantaricin UG1, (2) elucidate its molecular mass and to (3) classify it and determine its amino acid composition and sequence.

2- Materials and methods

Bacterial cultures & media:

Lb. plantarum UG1, the plantaricin UG1 producer, was isolated from dry sausage and was characterized as described by Enan et al. [7]. It was maintained as frozen stocks at -20°C in De Man Rogosa and Sharpe broth (MRS)[21] plus 10% glycerol[22] and was subcultured in MRS broth.
Lactococcus lactis MG1614 was provided from Food Microbiology and Food Preservation Laboratory, University of Gent, Belgium. It was used as the indicator organism. It was maintained at -20°C in skim milk (Difco) and was subcultured every 15 days in brain heart infusion broth (Difco).

Preparation of cell free superuatants (CFS):

Lb. plantarum UG1 was grown for 16 h in casitone-glucose-yeast extract (CGY) medium which was optimized for bacteriocin production ability[23]. CFS was obtained by centrifuging the culture (10000 xg for 10 min. at 4°C). To exclude the inhibiting activity due to acids, the pH of the CFS was adjusted to pH 6.5 with 1M NaOH and filter-sterilized through cellulose membranes. This pH-adjusted, filter-sterilized CFS was used immediately after preparation. The quantitative estimation of the antibacterial titre(s) of CFS preparation was carried out by the critical dilution assay[24].

Purification of plantaricin UG1

1- Ammonium sulphate precipitation:

Plantaricin UG1 was precipitated by dry NH_4SO_4 according to Zaman and Verwiulghen [5]. CFS from *Lb. plantarum* UG1 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% saturations. The mixtures were stirred for 12 h at 4°C and centrifuged at 20000 x g for 1 hr at 4°C. The precipitates (surface pellicels 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. This partially purified plantaricin UG1 was sterilized by filtration through cellulose membrane filters (Amicon, 0.45 µm, milipore) and titrated against *L. lactis* MG1614. It was designated PPE (partially purified extract) and was used for further purification experiments. The AU/ml was determined according to Enan and Al-Amri [8].

2- Dissociation of plantaricin UG1 activity:

One ml aliquots of PPE, each containing 22880 AU/ml were treated with Tween 80 (polyoxyethelene 20 sorbitan monooleate), Triton X-100 (t-octylphenoxypolyethoxy ethanol), SDS (sodium dodecyl sulphate), EDTA (Ethylene diamine tetracetic acid), nonidet p-40 (actylphenolethylene oxide). B-mercaptoethanol, MOPS [3-(N-morpholino) propanesulphonic acid], HEPES (N-(2-hydroxyethyl) peperazine-N-2-ethane-sulphonic acid], and SPAN 85 (Sorbitan trioleate), all from Sigma at a final concentration of 1 %. Controls were PPE or 1% of each chemical agent dissolved in fresh CGY medium. They were also treated with 50% V/V of each of the following solutions which were adjusted with 1mM NaOH to pH 6.0 as an optimum pH value for plantaricin UG1 activity: 1mM dithiothreitol, 8 M urea, 1 M sodium acetate and 6 M guandine hydrochloride. Controls were 50% V/V of each solution with fresh CGY broth. PPE samples treated with chemical agents and controls were incubated at 30°C for 4 h, and were then assayed for antibacterial activity [4,7,15,16].

3- Chromatographic separation of plantaricin UG1

(a) Ion exchange chromatography:

100 ml of PPE were treated with 1% nonidet p-40 as a dissociating agent. They were also solubilized with 0.1% SDS and 0.02% sodium azide and were then applied to a 200-ml column (4-cm interior diameter) of Sephadex G 200-50 (Sigma) equilibrated with 8 M urea, pH 6, at room temperature. The column was connected with both peristatic pump and fraction collector (Pharmacia). Activity was eluted with the same buffer (8 M urea, pH 6) and the eluent was monitored for A280 (absorbance at 280 nm) and plantaricin UG1 activity. The fraction collector was adjusted to collect 5 ml fractions at variable times. 10 ml of fraction number 12, containing the highest plantaricin UG1 activity were pooled and used for the next step.

(b) Reverse-phase chromatography:

Fifty ml of fraction number 12 containing the highest plantaricin UG1 activity were pooled from the ion exchange column. They were subjected to reverse-phase chromatography. A C_{12}-C_{18} reverse phase column (Pep RPC HR 5/5) equilibrated with 8 M urea, pH 6, was used. The plantaricin UG1 activity was eluted with the same buffer at a flow rate 1ml/min [22]

SDS-PAGE:

Fraction number 8 eluted from reverse phase chromatography contained the highest plantaricin UG1 activity and was collected. Proteins were analysed for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Phast Gel High Density Strips; Phast gel SDS buffer strips [26], and the Phast System (Pharmacia-LKB Biotechnology. Before application on the gel, 2 ml of fraction number 8 (see above) were boiled for 10 min in SDS buffer containing 2% 2-mercaptoethanol [26].

Protein determination:

Concentrations of protein in the active fractions containing the highest plantaricin UG1 activity were estimated by comparison with a Coomassie Blue binding assay[25] using a mixture of commercial standard protein, human albumin and globulin (5:3) as a standard (Sigma).

Amino acid composition and sequence:

Amino acids were determined using the method described previously[27]. 200 µl of purified bacteriocin obtained after gel filtration was hydrolyzed 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 analyzer (Ingos Ltd., Czech Republic) equipped with an Ostain 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 components. The half – cystine content was determined as cysteic acid by the method of Stoffels et al.[26], Hirs [28].

N - terminal amino acid sequencing and mass spectrometry analysis was achieved according to Atrih et al. [29].The amino – terminal sequence analysis of plantarocin UG1 was performed by Edman degradation using Applied Biosystems (A.B.I) 476A gas phase sequencer. Phenylthiohydantion amino acids were identified on line by reverse phase HPLC with Brownlee C18 column (A.B.I.). The sample was sequenced with polybrene – coated glass fiber as a support.

3- Results

The procedures carried out to purify plantaricin UG1 from the supernatants of CGY medium were as the following:

A- Ammonium sulphate precipitation of plantaricin UG1:

To precipitate plantaricin UG1 with optimum antibacterial activity, the precipitated ammonium sulphate pellets were collected from CFS adjusted at pH values 3,4,5,6 and 7 by using 40, 50 and 60% ammonium sulphate saturation (Table 1). The 60% saturation level showed the best precipitation of plantaricin UG1 pellets with the highest values of antibacterial titres. In the pH range 4.0 -6.0 at 60% ammonium sulphate saturation the pellets had the maximal values of plantaricin UG1 activity (22880 AU/ml) (Table 1). However, at pH values 3 and 7 in the presence of 40% to 60% ammonium sulphate saturation, the recovered activity of the precipitated plantaricin UG1 pellets was comparatively low (16500-18668 AU/ml).

B- Dissociation of plantaricin UG1 activity:

To determine if plantaricin UG1 was present in an aggregated form, PPE obtained by ammonium sulphate precipitation were treated with the dissociating agents listed in Table 2. The plantaricin UG1 activity increased by 33.48%, 29.38% and 20.55% upon treating with SDS, nonidet p-40 and EDTA, respectively. In contrast, it decreased by 73.69% and 32.147% upon treatment with 6M guanidine hydrochloride and β-mercaptoethanol, respectively. The other chemical agents tested had no effect. Most of the chemical agents controls had no effect on the indicator strain: *L. lactis* MG1614, except for 6M guanidine hydrochloride, hexadecyltrimethylammonium bromide and B-mercaptoethanol which were strong inhibitors (Table 2). SDS and EDTA had a slight cytotoxic effect on the indicator organism. Since nonidet p-40 was not inhibitory to the indicator strain tested and dissociated the complex molecule of plantaricin UG1, resulting in increase in its activity, it was used as a dissociating agent for subsequent purification experiments. About 4.47 fold – increase in plantaricin UG1 activity was obtained in PPE treated with 1% nonidet p-40.

C - Chromatographic separation:

1- Ion- exchange chromatography:

100 ml of PPE were solubilized and dissociated as described above. They were then subjected to ion-exchange chromatography using sephadex G 200-50 column. The elution profile is shown in Figure1. Plantaricin UGl activity was detected in the first eleven peaks and showed its maximal value in fraction No. 12 and decreased in latter fractions. The plantaricin UGl activity was parallel to the absorbance results at 280 nm. About 23.57 fold – increase in bacteriocin activity was obtained in purified material after this step (Table 3). This showed an increase in specific activity of plantaricin UG1 by 269.8 times.

2- Reverse phase chromatography and amino acid analysis:

The ion exchange chromatography doubled the plantaricin UGl activity. Therefore, 10 ml from fraction No. 12 eluted using sephadex G 200-50 column were subsequently subjected to reverse phase chromatography using C12-C18 column. As shown in the elution profiles (Fig. 2), *a* large peak of activity corresponding to the largest absorbance peak was obtained. As given in Table 3, a vigorous increase in plantaricin UGl activity was obtained after reverse phase chromatography. The amount of protein in plantaricin UGl samples decreased with increasing the purification procedures (Table 3). About 54.69 fold – increase in plantaricin UG1 activity was obtained in purified bacteriocin after this step (Table 3). Also 1453 fold- increase in specific activity of plantaricin UG1 was showed (Table 3).

The purity of plantaricin UG1 was judged by SDS-PAGE. The purified plantaricin UG1 obtained from reversed phase HPLC (C12 – C18 column) was prepared and was electrophoresed via polyacrylamide gel. Two protein bands of about 3.70 K Da and 1.80 K Da were obtained. They showed an antibacterial activity against the indicator bacterium used; indicating on purity of the bacteriocin plantaricin UG1 obtained (Figure 3).

The amino acid composition of the purified plantaricin UGl which was pooled from reverse phase chromatography is shown in Table 4. In the amino acid chromatogram; three residues of alanine, aspartic acid, serine and valine; two residues of glycine, glutamic acid, proline, phenylalanine and ornithine; one residue of arginine, isoleucine; leucine, lysine, tryptophane and histidine were observed. Also, amino acid analysis revealed one to three residues of cysteic acid per plantaricin UGl molecule. The total number

of residues would then amounted to about 31 [28 + 1-3 cysteic acid] (Table 4).

Plantaricin UG1 contain double glycine leader (GG). Amino acids with non-polar side chains viz. alanine, phenylalanine, leucine, isoleucine, tryptophane and valine were obtained. Also, amino acids with uncharged polar side chains viz. serine, cysteine and glutamic acid were also showed. In addition two basic amino acids (lysine, eristidine) were detected. The three residues of cysteine did not appear thoroughly; one was appeared in each trial; became this amino acid was unstable under acid hydrolysis.

The sequence of amino acids obtained was as follows:
AHADDOESOSDLEKISGGARPFPWVCVFV plus unstable 2 C. This sequence was compared to other *Lb. plantarum* bacteriocins (plantaricins) which were discussed by Diep et al. (2009). Plantaricin UG1 showed to be different from them. The short consensus LEKISGG including the GG leader is similar to plantaricin NC8.

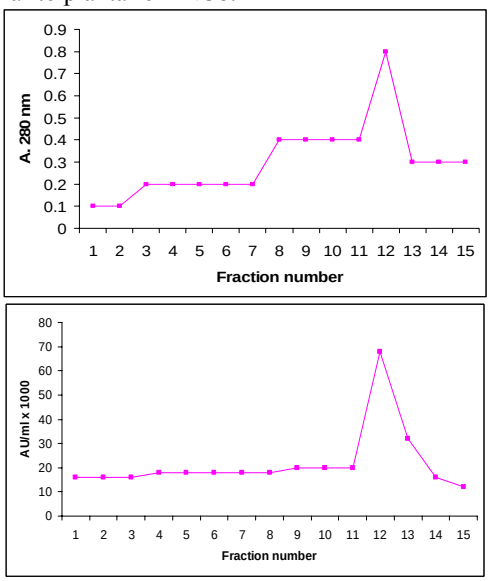

Figure 1: Elution profile of the dissociated partially purified plantaricin UG1 on sephadex G200-50 column. A. 280, absorbance at 280nm; AU/ml, plantaricin UG1 activity.

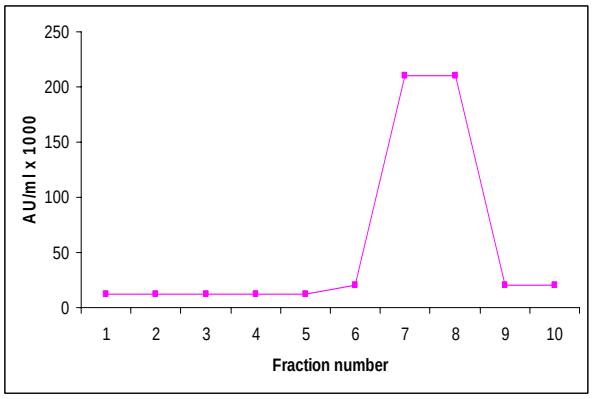

Figure 2: Reversed phase high performance liquid chromatography of partially purified plantaricin UG1, which eluted on column of sephadex G200-50-, using C12-C18 column, plantaricin UG1 titres (AU/ml).

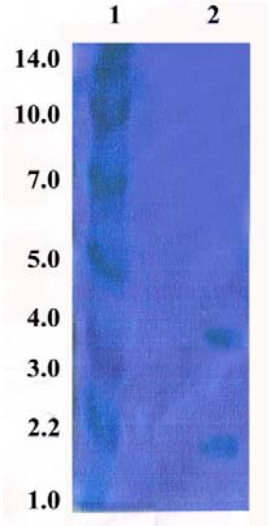

Figure 3: SDS – PAGE of purified plantaricin UG1, Lane 1, protein marker of known molecular mass (KDa); Lane 2, plantaricin UG1 which showed 2 bands of about 3.7 KDa and 1.8 KDa.

Table (1): Ammonium sulphate precipitation of plantaricin UGl.

pH value of CFS	Plantaricin UGl activity (AU/ml) at different ammonium sulphate saturation levels		
	40%	50%	60%
3.0	16500	16500	16500
4.0	16800	17200	20880
5.0	18000	20000	20880
6.0	18000	20000	22880
7.0	18620	18600	18660

Table (2): Effect of dissociating agents on plantaricin UG1 activity using *L. lactis* MG1614 as the indicator organism.

Dissociating agents	Plantaricin UG1 activity (AU/ml)[a]	
	Plantaricin UG1 (22880 AU/ml) + chemical agent	Chemical agent control
None	22880	
Tween 20	22880	None
Tween 80	22880	None
Triton X-100	22880	None
SDS[a]	34400	200
EDTA	28800	200
Nonidet p-40	32400	None
1m M dithiothreitol (pH 6.0)	22880	None
P-mercaptoethanol	16000	1080
MOPS	22880	None
HEPES	22880	None
SPAN 85	22800	None
8M Urea (pH 6.0)	22880	None
1M sodium acetate buffer (pH 6.0)	22800	None
6M Guandine hydrochloride (pH 6.0)	8060	2040

[a] To determine the antimicrobial titre of the dissociated bacteriocin, the antibacterial activity of the chemical agent control was substracted from total activity of the treated bacteriocin samples.

Table (3): Purification scheme for plantaricin UGl.

Purification step	Volume (ml)	Plantaricin UGl activity (AU/ml)	Total plantaricin UGl activity (AU/ml)	Amount of protein (mg/ml)	Specific plantaricin UG1 activity (AU/mg protein)	Increase in specific activity	Purification fold
Cell-free supernatant (CFS)	2000	6880	13760000	1.03	6679.6	1	1
Dissociated PPE by nonidet p-40	200	32440	648800	0.31	104645	15.6	4.74
Cation-exchange chromatography	100	162200	1622000	0.09	1802222	269.8	23.57
Reverse phase chromatography	15	376300	564450	0.04	9707500	1453	54.69

Table (4): Amino acid composition of plantaricin UGl.

Amino acid	Standard and International abbreviations	Number of amino acid
Alanine	A	3
Arginine	R	1
Aspartic acid	D	3
Glycine	G	2
Glutamate	E	2
Isoleucine	I	1
Leucine	L	1
Lysine	K	1
Proline	P	2
Phenylalanine	F	2
Tryptophane	W	1
Serine	S	3
Valine	V	3
Ornithine	O	2
Histidine	H	1
Total number of residues		28
Cysteic acid	C	1-3

4- Discussion

A three-step protocol has been developed for the purification of plantaricin UGI. The protocol included; ammonium sulphate precipitation, dissociation of bacteriocin activity and chromatography. The purification procedures resulted in a pure plantaricin UGI preparation as judged by SDS-PAGE analysis and reverse-phase high performance liquid chromatography. The purification protocol employed herein was also used successfully for the purification of different bacteriocins[26].

Precipitation of plantaricin UGI with ammonium sulphate showed that, when the pH of CFS was adjusted at pH 6.0 and with 60% ammonium sulphate saturation, the activity recovered in the pellets was higher than that present in the initial CFS. Increased activity has been observed previously upon purification of bacteriocins of seemingly high molecular weights; dissociation of the complexes appears to be responsible for increased activity [22,30,31]. Multimolecular dissociation of plantaricin UGI was judged by SDS-PAGE analysis, as two protein bands of molecular mass of about 3.70 K Da, 1.80 K Da were obtained. This is parallel to latter findings in this respect [1,32,33].

A notable increase in planlaricin UGI activity was detected after treatment with SDS, nonidet p-40 and EDTA; suggesting that the detergents may dissociate the bacteriocin, thereby releasing more active subunits. Such phenomenon is in conform with[34]. The possible effect of chemical agents as dissociating agents was also reported for staphylococcin 462[35] and mutacin[36]. The activity of planlaricin UGI was reduced after its treatment with 6M guanidine hydrochloride and β-mercaptoethanol. This may be due to the possible partial denaturation of this bacteriocin; resulting in an adverse effect on activity. This is in agreement with previous work [37].

The ion exchange chromatography using sephadex G200-50 column was better for elution of planlaricin UGI. This may be due to the affinity of plantaricin UGI to sephadex G22-50 and C12-C18 reverse-phase chromatography. The plantaricin UGI seemed, therefore, to be a hydrophobic peptide. This is similar to carnocin U149[26] and different from pediocins[1].

The amino acid composition suggested that plantaricin UGI consists of approximately 31 amino acid residues. These numbers of residues are almost similar to the number of amino acids obtained for carnocins and nisin[26].

Plantaricin UG1 was proved to consist of two polypeptides and hence it was classified as belonging to class IIb bacteriocins[38,39,40]. According to Badarinath and Halami [41], bacteriocins are classified into separate groups such as the lantibiotics (class I) ; the small (<10 KDa) heat stable posttranslationally unmodified non – lantibiotics (class II) which further subdivided to the pediocin – like and anti - *Listeria* bacteriocins (subclass IIa), the two – polypeptide bacteriocins (subclass IIb); and the second dependent bacteriocins (subclass IIc) and the large (> 30 KDa) heat – labile non lantibiotics (class III).

Similar to most bacteriocins produced by lactic acid bacteria[2] and [41] and like all bacteriocins produced by *Lb. plantarum*[42], plantaricinUG1 contained an N – terminal double glycine leader peptide, which is cleaved off concomitantly with externalization of biologically active bacteriocins by a dedicated ABC – transporter and its necessary protein. Plantaricin UG1 contained almost in 50% of its amino acid residues a non – polar hydrophobic side chains which can through of as " lipid – like " a property that promotes hydrophobic interactions giving a stable protein structure and increasing antibacterial activity[42,43]. The existence of three cysteic acid residues in plantaricin UG1 molecule and other acidic uncharged polar amino acids such as serine, glutamate and aspartate enable the compound to form cross – linkages such as disulfide bridges with other components of bacterial cell surface or other cross reactions, increasing the antibacterial activity of this bacteriocin. This is in conform with latter work in this respect [1,44]. Two residues of the imino acid proline were appeared in the amino acid sequence of plantaricin UG1. This indicated that this bacteriocin is an unusual one and ring side imino chains are existed[43].

The obtained amino acid sequence of plantaricinUG1 was compared with other amino acid sequences of bacteriocins of lactic acid bacteria published by Cintas et al. [2], Badarinath and Halami [41] and also was compared to amino acid sequences of other plantaricins in this respect [29,42]. It was shown that the amino acid sequence of plantaricin UG1 employed herein differed from the amino acid sequences of plantaricin EF and JK[45] and of plantaricin A[45] and [46] and of plantaricins NC8 and J51[47] and [48]. The short consensus LEKISGG including the double glycine leader existed in plantaricin UG1 was also existed in the amino acid sequence of plantaricin NC8[47]. Plantaricin NC8 contained also 2 polypeptides like plantaricin UG1. Consequently, plantaricin NC8 is the nearest one to plantaricin UG1. With the exception of LEKISGG consensus, plantaricin UG1 sequence differed in the rest of its sequence from plantaricin NC8[47]. Hydrophobic non – polar amino acids, imino acids and sulphure amino acids were more in plantaricin UG1 than other plantaricins published previously[42].

Due to its novel amino acid sequence, its

hydrophobicity, its structure of imino acids, plantarocin UG1 is a novel varient of plantaricins within class IIb bacteriocins.

Conclusion

Plantaricin UG1 produced by *Lb. plantarum* UG1 isolated from dry sausage was purified in this study. After its purification, about 1453 fold- increase in its specific activity was obtained. Plantaricin UG1 was two polypeptids, of molecular weights of about 3.7 KDa, 1.8 KDa was classified as novel varient within class IIb bacteriocins. Plantaricin UG1 contained the double GG leader and is different from other amino acid sequences of other bacteriocins.

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5/2/2014