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Purification and Characterization of Plantaricinvgw8, A Bacteriocin Produced by Lactobacillus Plantarum VGW8

Wala'a Shawkat Ali[1*] Rashid M. Musleh[2]
1.Department of Biology , College of Science , Al-Mustansiriya University, P.O.box 14022 ,Baghdad, Iraq
2.Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq
*E. mail of the corresponding author: microrose.2788@yahoo.com

Abstract
PlantaricinVGW8, a bacteriocin produced by *Lactobacillus plantarum* VGW8 was purified by two steps method including extraction with n-butanol followed by gel filtration chromatography on Sepharose 6B column, the results showed that the specific activity was 10666.67AU/mg protein with 13.067purification folds and 12% recovery yield. Plantaricin VGW8 was characterized and the results showed that the molecular weight of it was 14400 Dalton by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Plantaricin VGW8 activity was stable at pH values (3-9) but 50% of its activity was lost at (2 and 10) pH values, and whole activity was lost at extreme alkali pH values(11 and 12).Also, plantaricin VGW8 showed high thermostability at different temperatures (20- 100)°C for (10,30 and 60)min, it remained active after being treated with (20- 80)°C for the periods above, but it retained only 50% of its activity after treatment at 100°C for one hour and autoclaving treatment (121°C/ 15 min).Also, the results showed that the activity of plantaricin VGW8 disappeared when it treated with proteolytic enzymes (pepsin, trypsin and papain), whereas it retained whole activity when treated with lipase, lysozyme and α-amylase, indicating pure proteinaceous nature of purified plantaricin. As well as, treatment of plantaricin VGW8 with organic solvents (chloroform, acetonitrile, diethyl ether, ethanol, methanol, isopropanol, toluene and ethyl acetate), surfactants (tween 80, tween 20, triton X-100, SDS, EDTA and urea) and the metal ions: Na, K, Ca, Ba, Zn and Fe, showed no effect of these treatments on the activity of plantaricin VGW8, while treatment with Mn and Mg reduced its activity to the half. In conclusion, the characteristics of plantaricin VGW8 are promising for application of it in the food production processes as food preservative instead of chemicals food preservatives.
Keywords: purification of plantaricin, *Lactobacillus plantarum*, plantaricin activity, characterization of plantaricin.

1. Introduction

Bacteriocins are ribosomally synthesized, biologically active proteins or protein complexes that display antimicrobial action towards usually closely related species (Sánchez *et al*.,2008). Depending on their chemical structure, molecular weight, sensitivity to enzymes, contents of modified amino acids and activity mechanism, the bacteriocins were divided into four classes (Bodaszewska-Lubas *et al*.,2012),but the classes I and II are the main of them due to their abundance and potential use in commercial applications(Fan and Song,2013).
Because of their natural ability to preserve food, bacteriocins of lactic acid bacteria (LAB) have particular interest to researchers in the food industry (Hu *et al*.,2010). Nisin, the lantibiotic produced by *Lactococcus lactis* strains, is the most well-known, studied and characterized bacteriocin and the only one with widespread commercial use in most major food-producer countries. The success of nisin has led many research groups to search for novel bacteriocin – producer strains and bacteriocins over the last years (Papagianni and Anastasiadou, 2009). This has resulted in a growing range of potential biopreservatives, with most promising the plantaricins, these are bacteriocins produced by *Lactobacillus plantarum.* Production of an antimicrobial compound (lactolin) which resembled a bacteriocin from *L.plantarum* was firstly reported by Kodama in 1952.Later many other bacteriocins produced by different strains of *L.plantarum* were identified and characterized (Abo-Amer, 2007).Several bacteriocin producer strains of *L.plantarum* may be successfully applied in the food fermentation processes to increase the shelf-life and safety of the final products (Todorov, 2009).

Because of the biotechnological importance of bacteriocins produced by LAB and relatively few local studies about them in Iraq, particularly, those produced by *L.plantarum*. Therefore, this study was aimed to extraction, purification and characterization of plantaricin VGW8 produced by *Lactobacillus plantarum* VGW8.

2. Materials and Methods

2.1 Bacterial isolates and Culture media

The bacteriocin producer was *Lactobacillus plantarum* VGW8 (that isolated previously from fermented vegetables) , the indicator organism was *Pseudomonas aeruginosa* (Department of Biology , College of Science , Al-Mustansiriya University,Baghdad, Iraq)and the culture medium which used for production of plantaricinVGW8 was production liquid medium (PLM) (Ali,2010).

2.2 Plantaricin activity assay

Measurement of plantaricin VGW8 activity was carried out by serial two-fold dilutions of cell-free supernatant (crude plantaricin)(Pilasombut *et al*.,2005). These dilutions were used to detect the antibacterial activity of plantaricin VGW8 against indicator bacteria by agar well diffusion assay (Lewus and Montville,1991).The arbitrary unit (AU) was defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the test isolate.AU was calculated as: (1000 / 100) × D, where 1000:constant,100:volume of supernatant in a well(µl) and D : the dilution factor(Pilasombut *et al*.,2005).

2.3 Plantaricin concentration

It was determined according to Bradford(1976) . The concentration and antibacterial activity of plantaricin VGW8 were determined in all experiments of this study.

2.4 Extraction and Purification of Plantaricin

PLM was inoculated with 2% of 1×10^9cell/ml of *L.plantarum* VGW8 and incubated at 30°C for 12 hours under anaerobic conditions(Ali,2010). Cells were harvested by centrifugation at 6000 rpm for 15minutes , the cell-free supernatant was referred as crude plantaricin extract (CPE).CPE was heated at 80°C for 10 minutes, then cooled and centrifuged at 6000 rpm for 15minutes (Powell *et al.,*2007).The supernatnat was mixed thoroughly with n-butanol at a ratio 1:1.The mixture was centrifuged at 4000 rpm for10 minutes to achieve phase separation .The organic phase was evaporated at 80°C by rotary evaporator, then the sediment was re-suspended in 20mM sodium citrate buffer (pH 5) and referred to as partial purified plantaricin (PPP) (Abo-Amer, 2007).

Gel filtration chromatography by sepharose 6B gel was used as a second step for plantaricin purification . This gel was prepared according to Pharmacia catalogue(Pharmacia Fine Chemical/ Sweden). PPP was loaded slowly over the sepharose 6B gel.The plantaricin was eluted from the column((1.5x64) cm dimensions) by 20mM sodium citrate buffer (pH 5)and flow rate was adjusted to give 40 ml per hour. 5 ml for each fraction was collected. Activity and absorbance at 280nm of each fraction were determined,the active fractions were mixed .Activity and protein concentration were determined, then loaded again on the same column at the same conditions above.

2.5 Characterization of Plantaricin

2.5.1 Determination of the Molecular Weight

The purity of the plantaricin and its molecular weight were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Garfin,1990).

2.5.2 Effect of pH

Plantaricin solution was mixed with 10mM potassium phosphate buffer of pH values ranging from 2 to 12 (at increments of one pH unit), After 30 minutes of incubation at 30°C, the samples were re- adjusted to pH 5 with 1N NaOH or 1N HCl and tested for remaining activity. It was calculated as follows:

$$\text{Residual activity (\%)} = \frac{\text{Remaining units}}{\text{Original units}} \times 100$$

2.5.3 Effect of Temperature

It was assayed by treating plantaricin solution with (20, 25,30,35,40, 60, 80, and 100) °C respectively. Plantaricin activity was assayed after (10, 30 and 60) minutes at each of these temperatures. Activity also assayed after 15 minutes at 121°C.

2.5.4 Effect of Enzymes

Plantaricin solution was incubated for 1 hour at 30°C in the presence of the enzymes(pepsin , lipase, α-amylase(Philip Harris Biological Ltd/UK),papain(BDH/England), trypsin(Merck /Germany) and lysozyme(Sigma /USA)) at a final concentration of 1mg/ml. After incubation, the enzymes were heat-inactivated (3 minutes at 100°C) and tested for remaining activity.

2.5.5 Effect of Surfactants, Solvents and Metal Ion Salts

The effect of 1 ,2.5 and 5 % of tween 80, tween 20 and triton X-100, 2.5 and 5 mM of SDS, EDTA and urea, 5 and 10 % of solvents(chloroform, acetonitrile, diethyl ether, ethanol, methanol, isopropanol , toluene and ethyl acetate) and 5 and 10 mM of metal ion salts (NaCl, KCl, $MgCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, $FeSO_4$, Na_2SO_4, $Ca(NO_3)_2$ and $Mg(NO_3)_2$)was studied by adding the solutions of these materials, respectively to plantaricin solution. All samples, were incubated at 30°C for 1 hour and then tested for remaining activity.

3. Results and Discussion

CPE was heated to denaturant any proteases and heat-sensitive proteins. Butanol extraction exhibited complete recovery of plantaricin activity, suggesting that at least part of the plantaricin molecule has a hydrophobic character and shares this property with other bacteriocins (Enan,2006; Noonpakdee *et al.,*2009).The specific

activity of plantaricin recorded 2285.71 AU/mg protein with 2.799 purification folds and 40% yield (table 1). In the gel filtration chromatography, the maximum activity of plantaricin was observed in the fractions (30-32).The specific activity for these fractions was 5517.24 AU/mg protein with 6.759 purification folds and 12% yield (figure 1/stepI and table 1). The three active fractions of this step were collected and applied once again on the same column; this step gave a single active peak that was identical with the peak of plantaricin activity (figure 1/stepII), the specific activity was 10666.67 AU/mg protein with 13.067 purification folds (table 1).The purified plantaricin was called plantaricin VGW8 according to the producer isolate *L.plantarum* VGW8.

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 (Stellwagen,1990).

The molecular weight of plantaricin VGW8 was determined as 14400 Dalton which is larger than most bacteriocins thus far described for *L. plantarum*, however, molecular weight of 14KDa was reported for some bacteriocins produced by *L. plantarum*(Todorov and Dicks, 2005; Todorov *et al*., 2006). Appearance only one band of protein reflects the purity of plantaricin VGW8 by SDS-PAGE (figure 2).

Purified plantaricinVGW8 was stable at pH values ranging from 3 to 9.At these values plantaricinVGW8 remained active while at pH values 2 and 10, it lost 50% of its activity. Whole activity of plantaricin was lost at the pH values 11 and 12. These results agreed with the findings of other researchers (Messi *et al*., 2001;Lim and Im,2007).Many bacteriocins display greater antibacterial activity at lower pH values than at physiological pH, because a higher amount of bacteriocin molecules are available at lower pH values. At these values, the solubility is often increased; less aggregation of hydrophobic peptides occurs and less bounding of bacteriocins to the cell surface takes place (Messens and DeVuyst,2002).Activity at neutral pH constitutes an advantage over other bacteriocins used as food preservatives and particularly over nisin, which maximal solubility and stability are at pH 2.

The plantaricin VGW8 was resistant to treatments of (20, 25, 30,35,40,60 and 80)°C for (10,30 and 60) min, respectively. At 100°C for (10 and 30) min, respectively, plantaricin VGW8 was also appeared thermostability. However, 50% activity was lost after 60min at 100°C.Also, 50% activity was lost after autoclaving(121°C /15min).The thermostability feature might be related to the molecular structure of the plantaricin VGW8.However,thermostability is a general characteristic of plantaricins (Messi *et al*., 2001; Noonpakdee *et al.,*2009).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 (Navarro *et al*.,2000;Lim and Im,2007). Most processing of food involve a heating step so, thermostability is very important feature if the bacteriocin will be used as a food preservative.

Complete inactivation of antibacterial activity was observed after treatment plantaricin VGW8 with trypsin, pepsin and papain, confirming its proteinaceous nature. Whereas treatment with lipase, α-amylase and lysozyme did not affect the activity of it, suggesting that structure of plantaricin VGW8 did not contain a lipid or carbohydrate moity. This high sensitivity of LAB bacteriocins to metabolic proteolytic enzymes is very interesting with respect to food safety, since it means that the ingestion of bacteriocins will not alter digestive tract ecology and seem to be nontoxic (Lim and Im,2007).

The activity of plantaricin VGW8 was not affected after treatment with all solvents that used in this study, which suggests the absence of lipophilic structures in the plantaricin molecules. Stability under treatment with organic solvents is a typical behaviour for plantaricins, thus, these organic compounds can be used as solvents for the extraction of plantaricins from the culture medium or as eluents in further purification steps (Hernández *et al*.,2005).

The effect of some surfactants on plantaricin VGW8 activity was studied, the surface-active agents SDS, EDTA and urea at a final concentration of (2.5 and 5) mM as well as tween 80, tween 20 and triton X-100 at concentrations of (1, 2.5 and 5) % did not affect the activity of plantaricin VGW8.

Nonionic detergents such as tween 80 and tween 20 are known to increase the supernatant activity relative to total activity ,probably by desorption and disaggregation of the bacteriocin(Keren *et al*.,2004). Chelating agents such as EDTA permeate the outer membrane of Gram negative bacteria by extracting Ca and Mg cations that stabilize lipopolysaccharide of this structure ,allowing bacteriocins to reach the cytoplasmic membrane (Gálvez *et al*.,2007). The synergistic effect of tween 80 and other surfactants with plantaricin VGW8 may be useful in different applications.

Results showed that incubation of plantaricin VGW8 with (5 or 10) mM of NaCl, KCl, $CaCl_2$, $BaCl_2$, $Ca(NO_3)_2$, $ZnSO_4$, FeSO4 and $Na2SO_4$ did not reduce the activity of it, indicating that activity of plantaricin VGW8 did not affected by presence of these salts in the same environment but incubation of plantaricin VGW8 with (5 or 10)mM concentrations of $MgSO_4$, $MgCl_2$, $Mg(NO_3)_2$ and $MnSO_4$ resulted in loss of 50% of the activity. Decreasing of plantaricin VGW8 activity in the presence of some of these salts could be due to

denaturing of the peptide or changes in its confirmation. Also, it could be explained by the binding of Mg or Mn to anionic phsopholipids resulting in an enhanced rigidity of the cytoplasmic membrane and a reduced affinity of bacteriocin to the cytoplasmic membrane(Ganzle *et al*.,1999).

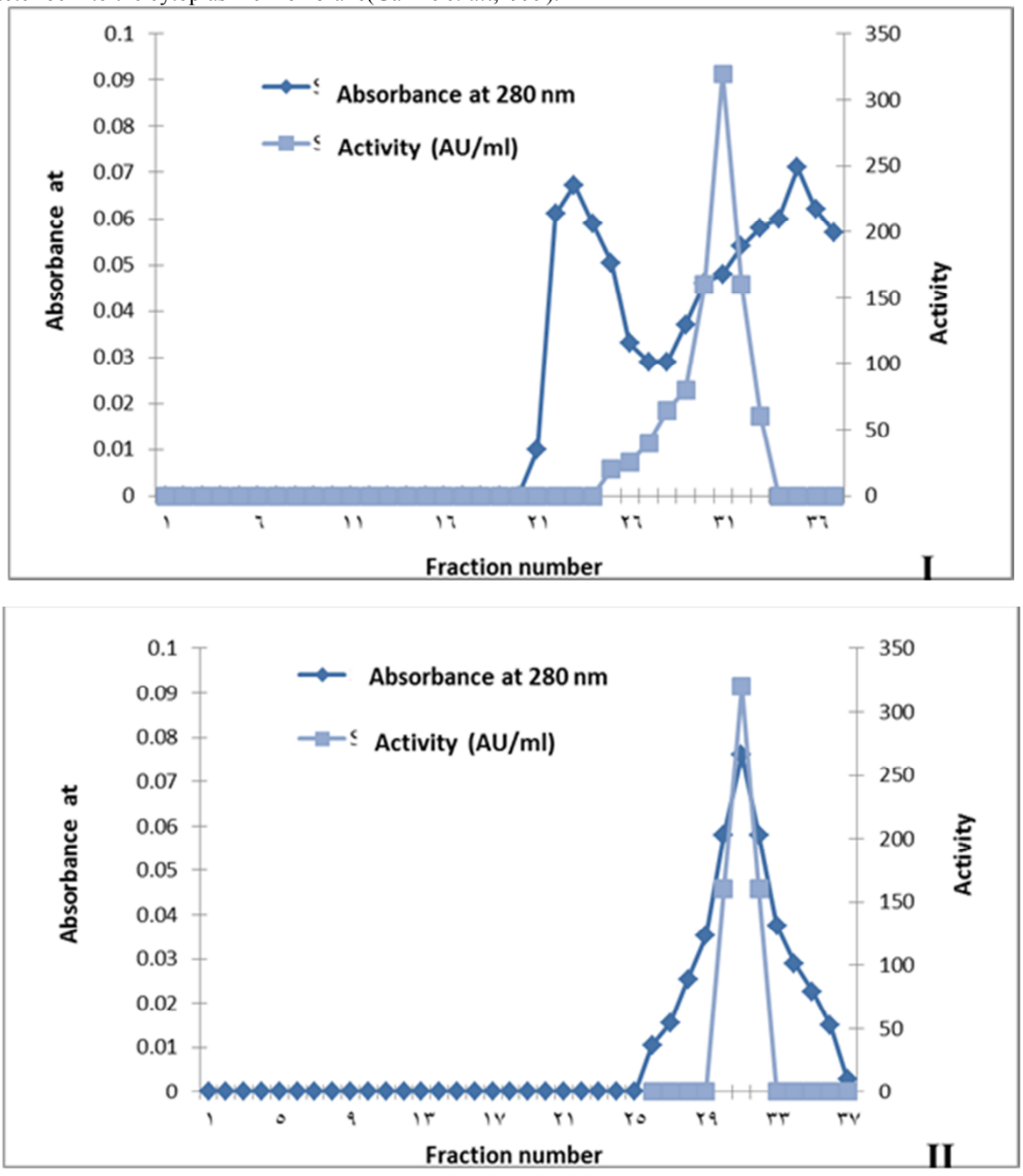

Figure 1: Purification of plantaricin VGW8 by gel filtration chromatography (Steps I and II), using Sepharose 6B column with dimensions (1.5x64) cm, that equilibrated and eluted by 20mM sodium citrate buffer(pH 5), flow rate was 40ml/hour, with 5ml for each fraction.

Table 1: Steps of purification of plantaricin VGW8 produced by *Lactobacillus plantarum* VGW8

Purification steps		Volume (ml)	Activity (AU/ml)	Protein concentration (mg/ml)	[a]Specific activity (AU/mg)	[b]Total activity (AU)	[c]Purification fold	[d]Yield (%)
Crude plantaricin extract(CPE)		250	160	0.196	816.33	40000	1	100
After heating (80°C/10min)		250	160	0.160	1000	40000	1.225	100
Extraction with butanol (1:1)		25	640	0.280	2285.71	16000	2.799	40
Gel filtration (Sepharose 6B)	First step	15	320	0.058	5517.24	4800	6.759	12
	Second step	15	320	0.030	10666.67	4800	13.067	12

[a]Specific activity (AU/mg): represents plantaricin VGW8 activity divided by protein concentration.
[b]Total activity (AU): represents Activity (AU/ml) × Volume (ml).
[c]Purification fold: represents specific activity of purified fraction divided by specific activity of crude extract.
[d]Yield (%): represents (total activity of purified fraction divided by total activity of crude extract) × 100.

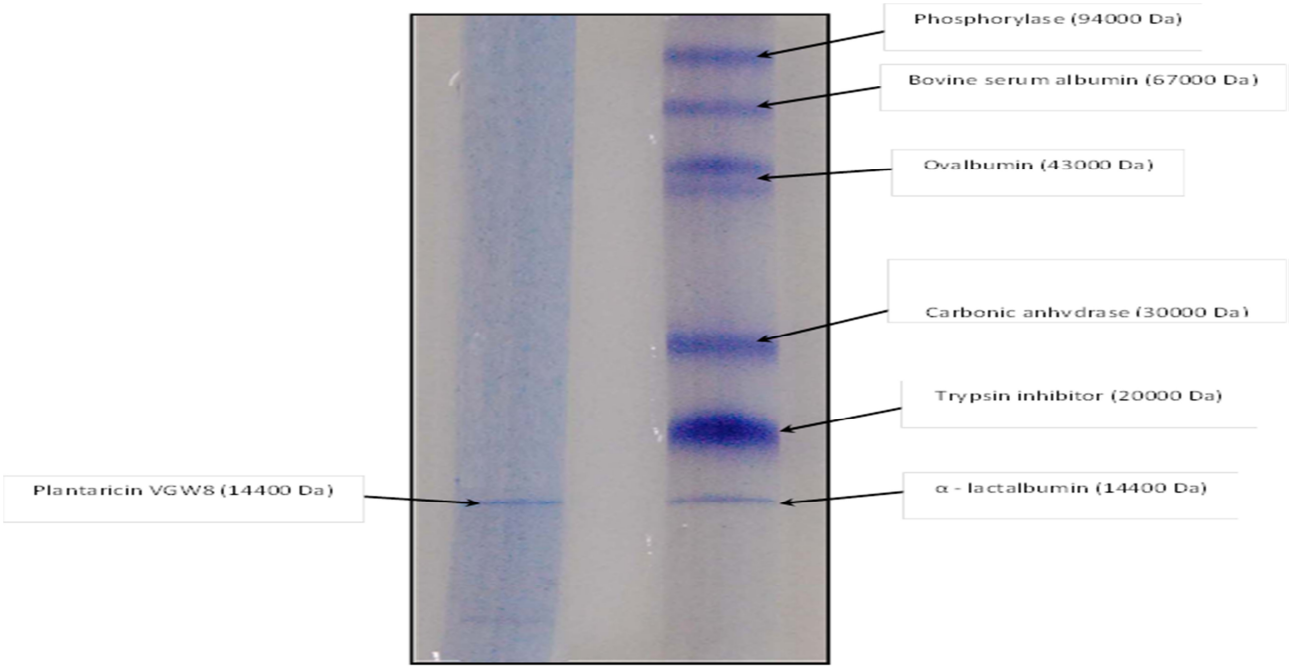

Figure 2: Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE)for the purified plantaricin VGW8.

4. Conclusion

Taking into account its proteinaceous nature, heat resistance, and molecular weight, plantaricin VGW8 can be classified as a small, heat stable peptide presumably belonging to class II according to the classification of Klaenhammer. The characteristics of plantaricin VGW8 are promising for application of it in the food production processes as food preservative instead of chemicals food preservatives.

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