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Full Length Research Paper

Purification and characterization of bacteriocin isolated from *Streptococcus thermophilus*

Aslam M.[1]*, Shahid M.[1], Rehman F. U.[2], Naveed N. H.[3], Batool A. I.[3], Sharif S.[1] and Asia A.[1],

[1]Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan.
[2]Department of Chemistry, University of Sargodha, Sargodha, Pakistan.
[3]Department of Biological Sciences, University of Sargodha, Sargodha, Pakistan.

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Streptococcus thermophilus **is used primarily as starter cultures to counter the harmful bacteria grown in cheese and yogurt making/preservation processes. These bacteria produce some exogenous toxins called bacteriocins having the antimicrobial activities against both Gram positive and Gram negative bacteria. In our study *S. thermophilus* growth was obtained at pH 5.5 and temperature 40°C. Bacteriocin activities were checked after their treatment with different enzymes, organic solvents, sodium chloride (NaCl) and detergents as well as their heat stability and effect of pH was studied. Bacteriocin activity was found heat stable at 100°C for 30 min and was found stable in the 3-10 pH range but lost the activity after the treatment with proteinase-K and protease enzymes. Activity was lost in treatment with lipase and amylase which shows the presence of lipo-glycolated peptide. Bacteriocin activity was lost on the presence of Dithiothreitol (DTT) and β-mercaptoethanol which showed the presence of disulphide bond present in bacteriocin and essential for its activity. Urea and ethylene diamine tetraacetic acid (EDTA) also affected the bacteriocin activity but found stable to survive in the presence of 6% NaCl. Antibacterial assay showed the strong growth inhibition of test bacteria. Bactericidal activity was further purified to homogeneity by ammonium sulphate precipitation and different chromatographic techniques. Molecular weight was calculated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as 2.7 kDa.**

Key words: *Streptococcus thermophilus*, bacteriocin, antimicrobial peptide, yogurt culture bacteriocin, disc diffusion test.

INTRODUCTION

Microbial defense system carries extraordinary armament in form of bacteriocins. These peptides are now well recognized for their property to inhibit the wide spectrum of Gram negative and Gram positive bacterial growth (Abdelkader et al., 2009; Mezaini et al., 2009; Tuncer and Ozden, 2010). Bacteriocins are produced by almost every bacterium and within a species tens or even hundreds of different kinds of bacteriocins are produced (Line et al.,2008). Bacteriocins are produced in both Gram-negative and Gram-negative bacteria and can be divided into different subgroups on the basis of structural and amino acid sequence similarities. Colicins (Parret et al., 2003) and the microcins (Kemperman et al., 2003) are major subgroups of Gram negative bacteriocins. Gram-positive
bacteria are sub-grouped in five major classes on the basis of their internal cross linking, post translational structures and primary structures (Nes et al., 1996; Nissen-Meyer and Nes, 1997; Kemperman et al., 2003). Subclasses include class one lantibiotics or lanthionine containing bacteriocins (McAuliffe et al., 2001; Guder et al., 2000), class two, small (<10 kDa) heat-stable, non-lanthionine-containing peptides bacteriocins (Van-Kraaij et al., 1999; Yi et al., 2010; Netz et al., 2001), Class three**,** large (>30 kDa) heat-labile proteins that are of lesser interest to food scientists (De-Jong et al., 2010; Chen et al., 2004), class four, complex proteins requiring

*Corresponding author. E-mail: mehwish.aslam9@gmail.com. Tel: +923004474407.

the association of carbohydrate or lipid moieties (Alpay et al., 2003) and Class five, circular, head-to-tail ligated bacteriocins (McAuliffe et al., 2001; Kemperman et al., 2003).

Bacteriocins from Gram-positive organisms, such as lactic acid bacteria (LAB), have attracted much attention and have been the subject of intensive investigation due to their extensive incorporation as bio-preservatives ingredients into model foods particularly in the dairy industry (Diop et al., 2007) and also in human therapeutics (Martin-Visscher et al., 2008). Normally the cells producing the bacteriocins are immune to its antagonistic action and therefore might enjoy a competitive advantage over sensitive bacteria inhibiting the same ecological niche (Joerger, 2003).

Yogurt starter cultures commonly contains *Streptococcus thermophilus* which have been well studied for the production of the bacteriocins, is a heterogeneous group of peptide which have wide spectrum of activity against bacteria (Ivanovaa et al., 1998; Kabuki et al., 2007; Iyera et al., 2010). *S. thermophilus* bacteriocins have been reported to have a broad inhibitory spectrum against several bacteria *Listeria monocytogenes*, *Salmonella typhimurium*, *Escherichia coli*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Ivanovaa et al., 1998), *Clostridium tyrobutyricum* (Mathot et al., 2003).

These bacteriocins are mostly heat (Kabuki et al., 2007) and pH stable (Vinderola et al., 2002; Mezaini et al., 2009). As the LAB have now achieved the status of generally recognized as safe (GRAS), these bacteria or their bacteriocin can be used as probiotic or food preservative in food to keep the food product safe from spoiling bacteria (Buriti et al., 2007). In our study, we have reported isolation and purification of heat and pH stable bacteriocin activity from *S. thermophilus* isolated from yogurt. This activity may be further used as a preservative of food products as it showed strong growth inhibition of both Gram-negative and Gram-positive bacteria.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used during the research were molecular biology grade and were purchased from Oxoid, Riedel Dehaen, Merck, ICN, BBI, BDH Chemicals, Panerac, Schwan, Sigma and Fermentas Different culture media like Nutrient Agar/broth, M 17 agar/broth and Muller Hinton Broth media were used. *E. coli* and *Staphylococcus aureus* were used as test bacteria. *S. thermophilus* isolated from yogurt and maintained in protein molecular biology labortory (PMBL), Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad was used as starter culture.

Screening of bacteriocin activity

Inoculum with $1x10^6$ – $2x10^8$ CFU/mL culture in selective broth (M17) was incubated at 37°C for 24 h and then centrifuged at 10,000 rpm (4°C) for 20 min. The residue was discarded and cell free extract was used to screen the bacteriocin activity against *Bacillus substilis* and *S.aureus* by using disc diffusion method (CLSI, The Clinical and Laboratory Standards Institute, 2007). Nutrient agar (Oxoid, UK) was prepared and test bacteria was added to the medium and poured in sterilized Petri plates. Small filter paper discs were laid flat on growth medium containing 100 µL of cell free supernatant. The Petri plates were then incubated at 37°C for 24 h, for the growth of bacteria. Antibacterial activity was measured by clear zones formation.

Production of bacteriocin

Broth culture (500 mL) with inocula was incubated to obtain a large repertoire of bacteriocin at 37°C and pH 5.0 for 18 h. After the incubation cell free supernatant was obtained by centrifugation of media at 12,000 x*g* for 30 min, the cell free supernatant was used further for ammonium sulphate precipitation.

Bacteriocin characterization

Sensitivity of bacteriocin to pH, temperature and enzymes

To determine the pH stability of bacteriocin, pH values were adjusted within the range of 3 to 10 by hydrochloric acid (HCl) or sodium hydroxide (NaOH), and each sample was held for 1 h at 37°C. Sensitivity of bacteriocin to heat was checked by heating the culture supernatant for 15 min at 65°C, 80°C, 100°C and 121°C. Sensitivity to proteolytic enzymes was checked when bacteriocin sample was treated with Proteinase-K (1 mg/mL), Trypsin (0.5 mg), Protease (1 mg/mL) and Lipases (1 mg/mL). 1 mL of crude bacteriocin was added to 1 mL of enzyme and incubated at 37°C for 2 h and then boiled at 100°C for 5 min. The disc diffusion assay was performed after treatment to detect activity against test organism peptide (Mathot et al., 2003).

Effect of organic chemicals and detergents

The effect of organic solvents on bacteriocin was tested with organic solvents including dithiothreitol (DTT) (5%), β-mercaptoethanol (50 mM), ethylene diamine tetraacetic acid (EDTA) (10 mM), and organic solvents; acetone, chloroform, ethanol and methanol at final concentration of 5.0% (Todorov et al. 2006). The surfactants tested were sodium dodecyl sulphate (SDS), Tween 20, Tween 80, Triton X-100 and urea at final concentrations 1%. Controls consisted of either active precipitates or detergents used. All samples and controls were incubated at 37°C for 5 h and tested for activity.

Effect of sodium chloride (NaCl)

The effect of NaCl on bacteriocins activity was checked by growing the bacteriocin producing strain in agar medium with 2, 4 and 6% NaCl and assaying the activity by disc diffusion method (Larsen et al., 1993).

Bacteriocin purification

The partial purification of bacteriocin was conducted by 80% ammonium sulphate precipitation and centrifuged at 10,000 x*g* and 4°C for 10 min to separate residues and supernatants from crude precipitated extracts; the supernatants were stored at 4°C in 100 mL sterilized bottles (pH; 8.0). Dialysis was followed in a tubular

cellulose membrane (1000 cut off) against 2 L distilled water for 24 h, from which the bacteriocin titer was performed. Protein concentration of the bacteriocin in supernatant was determined by the Bradford method of protein estimation (Bradford, 1976), using bovine serum albumin as the standard.

Gel filtration and ion exchange chromatography

The dialyzed samples were purified by gel filtration using Sephadex G-200. The elution of sample was performed by 10 mM Tris-HCl buffer (pH 8.0). The fractions with maximum protein contents were pooled out and applied for antimicrobial assay. The active factions obtained from the gel filtration were further purified by ion exchange chromatography. DEAE-Sephadex was used as resin in ion exchange chromatography column equilibrated with the 10 mM phosphate buffer (pH, 7.0). Elution was done by the gradient of 10 mM phosphate buffer and 0.1-1 M NaCl buffer (pH, 7.0). All samples of crude extract and partially purified extracts were then subjected to antibacterial assay.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The characterization of the bacteriocin (peptides/proteins) was performed by SDS-PAGE (Rehman et al., 2009). The SDS-PAGE was carried out for the crude and purified protein samples possessing antimicrobial activity. The experiment was carried out on Mini-PROTEIN, mini vertical electrophoresis apparatus (BioRad, UK) using the 12% gel. The samples were prepared by mixing the protein 2:1 ratio with SDS-sample buffer of Sigma. The gel was stained for protein with coomassie brilliant blue, along with the marker proteins (Lewus and Montville, 1991). The apparent molecular mass of the sample was calculated by comparison with the mobility of the standard markers.

RESULTS AND DISCUSSION

Screening and production of bacteriocin activity

The zones of inhibition showed the presence of bactericidal activity against *B. Substilis* and *S. aureus* in initial screening. Bacteriocin production was strongly dependent on pH, time and temperature as claimed by Todorov and Dicks (2004). Cell free supernatant showed the bacteriocin activity as 2600 AU/mL obtained after centrifugation.

Characterization of bacteriocin

Sensitivity of bacteriocin to pH, temperature and enzymes

Bacteriocin activity was remained stable at the 100°C for 30 min but it lost its activity after its incubation at 121°C (Table 1). These findings are similar to the Thermophilin 110 which remained stable when held at 100°C for 60 min but lost its activity against *Pediococcus acidilactici* after 90 min or longer exposure. Contradictory observations were made for *S. thermophilus* 580 bacteriocin, where its heat instability was due to a heat labile peptide (Mathot et al., 2003). Bacteriocin activity was also found stable at wide range of pH 3-10. Khali (2009) reported the *S.thermophilus* bacteriocin which was pH resistant over the wide range of 3-9 (Table 2). Proteinase-K and pepsin were strongly inhibited the bacteriocin activity showing the bacteriocin protein in nature. This bacteriocin activity was similar to Thermophilin 110 Gilbreth and Somkuti (2005), Thermophilin A (Ward and Somkuti, 1995) Thermophilin T (Aktypis et al., 1998) and the bacteriocin of *S. thermophilus* 580 (Mathot et al., 2003) in this manner. The inactivation of the bacteriocin by treatments with lipase enzymes (Table 3) suggests that activity was dependent on the presence of either a carbohydrate or lipid moiety as described by Maurad and Meriem (2008).

Table 1. Temperature sensitivity of bacteriocin activity.

Temperature	Bacteriocin activity
Control	2600 (AU/mL)
04°C (30 min)	-
65°C (30 min)	+
80°C (30 min)	+
100°C (30 min)	+
121°C (15 min)	-

Table 2. pH stability of bacteriocin activity.

pH	Bacteriocin activity
Control	2600 (AU/mL)
2.0	-
3.0	+
4.0	+
5.0	+
6.0	+
7.0	+
8.0	+
9.0	+
10.0	+

Effect of organic solvents, sodium chloride and detergents

Bacteriocin activity was lost after treatment with DTT and β-mercaptoethanol. This may be due to the reduction of disulphide bonds (Table 4). Our findings were in agreement with Todorov et al. (2006) and Khalil et al. (2009). Bacteriocin activity remained unaffected in the presence of all detergents (Table 5). Similar results were observed by Ivanova et al. (2000) and Ogunbanwo et al. (2003). Bacteriocin activity was found stable in the presence of 2, 4 and 6% NaCl (Table 6).

Table 3. Effect of enzymes on bacteriocin activity.

Enzyme	Bacteriocin activity
Control	2600 (AU/mL)
Proteinase-K	-
Trypsin	+
Protease	-
Lipases	-
Amylase	+

Table 4. Effect of organic solvents on bacteriocin activity.

Organic solvent	Bacteriocin activity
Control	2600 (AU/mL)
Dithiothreitol (DTT)	-
β-mercaptoethanol	-
Acetone	+
Chloroform	+
Ethanol	+
Methanol	+

Table 5. Effect of detergents on bacteriocin activity.

Treatment	Bacteriocin activity
Control	2600 (AU/mL)
Sodium Dodecyl Sulphate (SDS)	+
Tween 20	+
Tween 80	+
Triton X-100	+
Urea	-
EDTA	-

Table 6. Effect of NaCl on bacteriocin activity.

Treatment	Bacteriocin activity
Control	2600 (AU/mL)
2% NaCl	+
4% NaCl	+
6% NaCl	+

Purification of bacteriocin

Crude of the recovered proteins were applied to ammonium precipitation and dialysis. Protein recover was 26.0 fold after ammonium sulphate precipitation. Dialysis removed the salt and concentrated the protein with 15 mL volume. Dialyzed protein sample was then fractionated by gel filtration and ion-exchange chromatography, using DEAE-Sephadex. Gel filtration using Sephadex G-200 active fractions (Figure 1) were pooled out and applied to ion exchange chromatography (Figure 2). Bacteriocin activity was purified up to 597 folds chromatographic techniques (Table 7). The overall yield and activity are summarized in Table 7.

Antibacterial assay

Bacteriocin activity was found active against Gram-positive bacteria; *S. aureus*, *B. subtilis* and Gram-

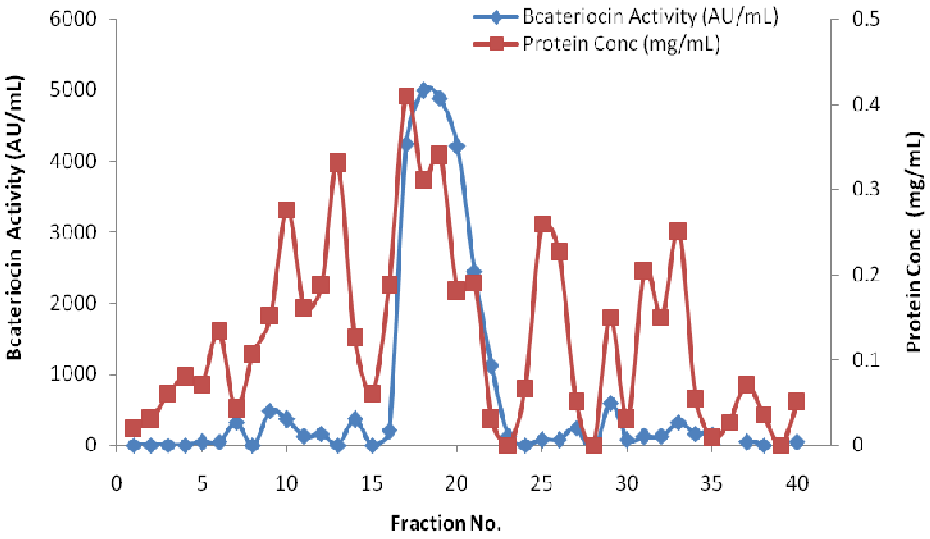

Figure 1. Gel fitration of bacteriocin activity showing the protein concentration and the bacteriocin activity in the active fractions.

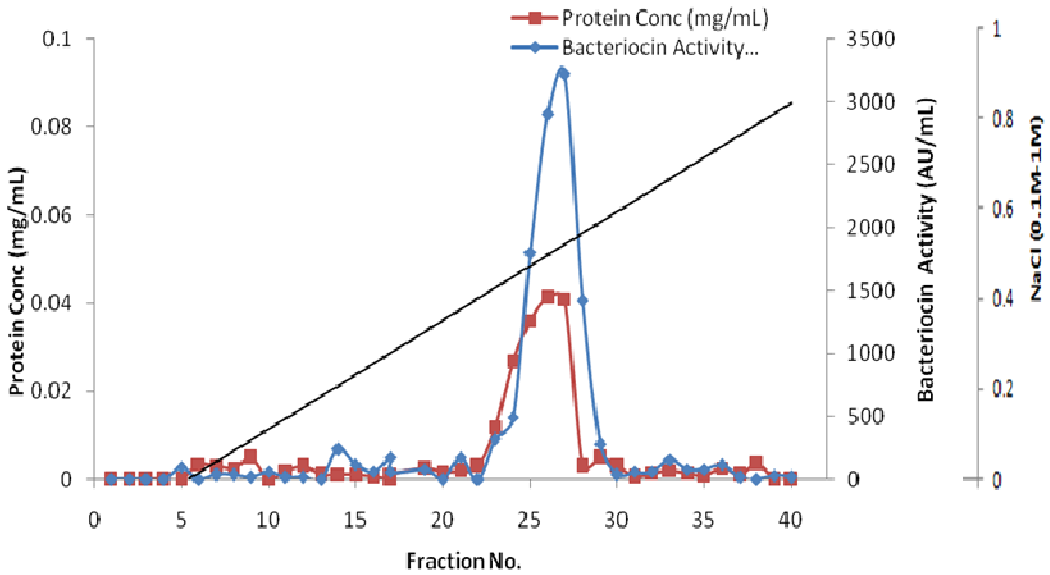

Figure 2. Ion exchange chromatography of bacteriocin activity.

Table 7. Purification fold bacteriocin activity purification steps.

Purification Level	Volume (ml)	Activity (AU/ml)	Total act. (AU)	Protein (mg/ml)	Specific activity	Recovery	Purification fold
Culture supernatant	500	2600	1300000	13.5	192.6	100.0	1.0
Ammonium sulphate precipitation	20	10500	210000	2.1	5000.0	16.2	26.0
Dialysis	15	10200	153000	1.9	5368.4	11.8	27.9
Gel filtration chromatography	4	4600	18400	0.4	11500.0	1.4	59.7
Ion exchange chromatography	2	3450	6900	0.03	115000.0	0.5	597.1

Activity unit (AU/mL), Reciprocal of the highest dilution x1000/volume of bacteriocin added; Specific activity (AU/mg), total activity of the subsequent purification step/total protein of the same step; Recovery (%), total activity of subsequent step x 100/total activity of crude preparation; Purification fold, Specific activity of subsequent step/ Specific activity of crude preparation.

Table 8. Antimicrobial activity of bacteriocin against Gram negative and Gram positive bacteria.

	S. aureus	*B. substilis*	*E. coli*	*P. multocida*
Bacteriocin activity (mm inhibition zone)	24.5 ± 05	9.5 ± 03	10.7± 05	8.7± 03
Rifampin	26.25 ± 08	12.5 ± 03	10.77 ± 01	10.26 ± 07

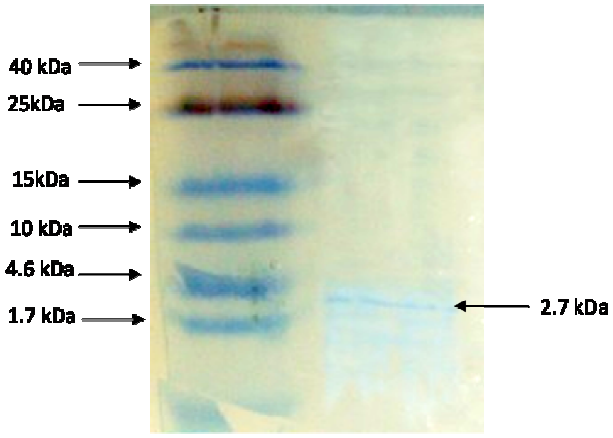

Figure 3. SDS-PAGE showing the molecular weight of the bacteriocin activity as 2.7kD.

negative bacteria; *E. coli*, *Pasteurella multocida*. Results are shown in Table 8.

Molecular weight determination

Molecular weight of the bacteriocin was determined by SDS-PAGE (Figure 3). The molecular weight of the purified bacteriocin activity was 2.7 kDa. Aktypis et al. (1998) reported that SDS-gel electrophoresis of partially purified thermophilin T showed that bacteriocin activity was associated with a protein band of approximately 2.7 kDa molecular mass (Figure 3).

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