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Full Length Research Paper

Isolation and characterization of toebicin 218, a bacteriocin, produced by *Geobacillus toebii* HBB-218

Gamze Başbülbül Özdemir* and Haci Halil Biyik

Biology Department, Faculty of Arts and Science, Adnan Menderes University, 09010 Aydin, Turkey.

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A novel bacteriocin Toebicin 218 was isolated from *Geobacillus toebii* HBB-218, a soil inhabiting Gram positive bacterium. The cell free culture supernatants of *G. toebii* HBB-218 showed antibacterial activity against many Gram positive bacteria including thermophilic strains. Purification of the bacteriocin was achieved after ammonium sulphate precipitation, gel filtration and ion exchange chromatography. Tricine-SDS-PAGE yielded a single protein band observed with a molecular mass of 5.5 kDa. The antibacterial compound was heat stable and sensitive to proteolytic enzymes. Bacteriocin production started at the early logarithmic phase and maximum production was observed at the end of the stationary phase. Bacteriocin was found to be effective especially against thermophilic bacteria and it may have a potential for use as a biopreservative in canned foods. This study provides the first data on bacteriocin produced by a strain of *G. toebii*.

Key words: bacteriocin, *Geobacillus toebii,* toebicin 218, thermophilic, characterization.

INTRODUCTİON

Bacteriocins are a heterogeneous group of ribosomally synthesized antibacterial peptides that inhibit strains and species that are usually, but not always, closely related to producing bacteria (Tagg et al., 1976). Both Gram positive and Gram negative bacteria produce small, heat-stable bacteriocins. Bacteriocins produced by Gram positive bacteria are often membrane permeabilizing cationic peptides with fewer than 60 amino acid residues (Jack et al., 1995). The bacteriocins produced by lactic acid bacteria offer several desirable properties that make them suitable for food preservation: (1) Are generally recognised as safe substances, (2) Are not active and nontoxic on eukaryotic cells, (3) Become inactivated by digestive proteases, having little influence on the gut microbiota, (4) Are usually pH and heat-tolerant, (5) Have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (6) Show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: No cross resistance with antibiotics, and (7) Their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Galvez et al., 2007). According to the Klaenhammer classification scheme, bacteriocins produced by lactic acid bacteria are grouped into four main classes: Class I is the modified bacteriocins which are known as lantibiotics. Class II is the heat-stable, minimally modified bacteriocins. Class III is the larger heat labile bacteriocins and Class IV is complex bacteriocins, these are carrying lipid or carbohydrate moieties (Klaenhammer, 1993). Although, reported bacteriocins are generally isolated from food-grade, mesophilic microorganisms, there are a few examples of bacteriocins produced by thermophilic bacteria and archea (Shafia, 1966; Sharp et al., 1979; Becker et al., 1986; Novotny and Perry, 1992; Prangishvilli et al., 2000; Martirani et al., 2002; Pokusaeva et al., 2009). Thermophilic bacteria belonging to *Bacillus* genetic group 5 have been reclassified as members of *Geobacillus* gen. nov., with *G. stearothermophilus* as the type species (Nazina et al., 2001). *Geobacillus* species, literally named as earth or soil *Bacillus,* are widely distributed and readily isolated from natural or man made thermophilic biotopes (McMullan et al., 2004). *Geobacillus toebii* was firstly identified as a thermophilic, aerobic, spore forming Gram positive bacteria by Sung et al. (2002). The aim of this

*Corresponding author. E-mail: gbasbulbul@adu.edu.tr Tel: 0090 256 218 2000-1937. Fax: 0090 256 213 53 79.

study was to evaluate the antibacterial activity of a bacteriocin produced by *Geobacillus toebii* HBB-218 isolated from soil collected from a the thermal spring in Denizli, Turkey. The antibacterial spectrum, characterization and purification of the bacteriocin are described in this report.

MATERİALS AND METHODS

Bacteria and media

Strain HBB-218 was isolated from soil that was collected around thermal spring in Denizli,Turkey. Soil samples were inoculated into *Thermus* broth and then incubated aerobically at 65°C for 48 h. One mililiter of culture was mixed with Caso agar cooled at 45°C, and then poured onto the plates. Colonies grown after 24 h were purified two times and pure culture of isolate was obtained. Indicator strains used in this study were obtained from different sources and were maintaned in skim milk 20% (v/v) at -20°C. Thermophilic isolates used as indicator strain from our culture collection were identified by partial 16S rDNA analysis, enterococci were identified by API 20E (Biomerieux, France). All the indicator strains were propagated in appropriate media and at temperatures before conducting the experiments (Table 1).

Identification of strain HBB-218

Strain HBB-218 was identified with biochemical tests and 16S rDNA analysis. API 50 CH (BioMerieux, France) kit was used to determine the carbohydrate utilization pattern of the strain. Genomic DNA of strain was isolated as described by Ronimus et al. (1997), 16S rRNA gene was amplified via PCR and then amplicon was sequenced. The primers used for the amplification were: 20F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1390R (5'- GAC GGG CGG TGT GTA CAA-3') (Orphan et al., 2000). The partial 16S rRNA gene sequence of strain HBB-218 was submitted to GenBank Nucleotide Sequence Database and accession number GQ255948 has been assigned.

Antibacterial activity assays

The antibacterial activity of cell free culture supernatants (CFS) were obtained from 24, 48, 72 and 96 h cultures of HBB-218 were detected by modified agar-well diffusion method (Hechard et al., 1992). For this purpose, HBB-218 was grown in BHI broth, and samples were centirifuged at 8 000 × g for 10 min. The supernatant was then sterilized through a milipore filter (Sartorius) with 0.45 µm pore size. 350 µl bacterial suspension (0.5 McFarland) was mixed with appropriate 35 ml agar media for each strain and then poured into plates. Wells with 6 mm diameter were cut into agar media by using cork borer and then 50 µl bacteriocin were placed into each well. The plates were kept at 4°C for 2 h for bacteriocin diffusion then incubated at the given temperatures for each bacterial strain and examined for zones of inhibition.

Effects of heat, enzymes, pH and chemicals on bacteriocin activity

In order to obtain crude bacteriocin sample, HBB-218 was grown in BHI broth at 60°C which is the optimum temperature detected for bacteriocin production (data not shown) for 24 h and then cell-free culture supernatants were prepared by centrifuge at 8 000 × g for 10 min. Both CFSs and purified samples were used for characterization of antibacterial substance. To determine the thermal stability, bacteriocin samples were heated for 30 min and 2 h at 30, 45, 60 and 95°C, also autoclaved for 20 min at 121°C, cooled and assayed for activity. Aliquots were also maintained for one month at 4°C, and then activity was tested. Untreated samples served as control (Powell et al., 2007). The effects of various enzymes on bacteriocin activity were also tested. Aliquots were treated with pronase E, proteinase K, papain, RNase, Lipase, B-glucoronidase, lactase at final concentration of 1 and 10 mg/ml, catalase at final concentration of 300 IU/ml for 1 and 24 h. An untreated bacteriocin and the enzyme in the buffer were both served as controls (Riosen et al., 2005). In a separate experiment, the effect of the pH on bacteriocin activity was tested by adjusting each of the aliquots to between 3.0 and 11.0 with sterile 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl. After 24 h of incubation at 4°C, the treated samples were adjusted to pH 7.0 with sterile 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl and tested for activity (Deraz et al., 2005). Acetone, chloroform, dimethyl sulfoxide (DMSO), ethanol, methanol and xylol were added to 24 h-old cell-free culture supernatants at final concentration of 10% (v/v) and then incubated for 1 h at 30°C. Trichloroacetic acid (TCA) was added at a final concentration of 100 mg/ml, incubated for 1 h at 30°C. After treatment with TCA, samples were centrifugated at 10 000 × g for 5 min and the supernatants were neutralized to pH 7.0 before testing for antibacterial activity. Tween 80 and urea were used at final concentration of 1 mg/ml, and ethylenediaminetetraacetic acid (EDTA) was used at 0.1 mmol l^{-1}. Chemicals and bacteriocin aliquots diluted with sterile distilled water at same final concentration were used as control (Todorov and Dicks, 2005).

After each treatment, the samples were tested for antibacterial activity against *G. stearothemophilus* DSMZ 22 using well diffusion method.

Growth and bacteriocin production

A 24 h-old culture of strain HBB-218 was inoculated (2%, v/v, $OD_{600\ nm}$ = 0.1) into BHI broth and incubated at 65°C without agitation. Samples were taken at 3 h intervals and absorbencies were recorded. To determine the bacteriocin titers, samples were centrifugated at 8 000 × g for 10 min and then put into wells. The titer was defined as the reciprocal of the highest dilution that exhibit inhibition of the sensitive strain and was expressed in arbitrary units (AU) per mililiter.

Purification of bacteriocin

Purification of bacteriocin was achieved by ammonium sulphate precipitation, gel-filtration chromatography and ion-exchange chromatography, sequentially. All the purification steps were performed at 4°C. Strain HBB-218 was grown in 500 ml modified media (soja peptone 3%, galactose 0.1%, NaCl 5 g l^{-1}; di-sodium phosphate 2.5 g l^{-1}) at 60°C for 72 h, then cells were harvested by centrifugation at 8 000 × g for 10 min. 450 ml culture supernatant were autoclaved at 121°C for 20 min, precipitants were removed again via centrifugation at 8 000 × g for 10 min. Ammonium sulphate was added to the resulting culture supernatant at 80% saturation. The pelleted amonnium sulphate precipitate was dissolved in 20 mmol l^{-1} sodium phosphate buffer (pH 7.0), and dialyzed against the same buffer by using a 1000 Da dialysis membrane (ZelluTrans-Roth E887.1). The dialyzed sample was stirred for 24 h in 20 mmol l^{-1} sodium phosphate buffer (pH 7.0), then concentrated by using 5 000 MWCO ultrafiltration membrane (Sartorius). 3 ml concentrated sample was applied to a gel filtration column (100 cm length and 1-cm internal diameter), Sephadex G-50, previously equilibrated with 20 mmol l^{-1} sodium phosphate buffer (pH 7.0) and eluted at a flow rate of 0.35 ml min^{-1} with the

Table 1. Indicator strains used in the study

Strain	Strain number	Media	Temperature (ºC)
Geobacillus stearothermophilus	DSMZ 22	Nutrient Agar	55
Bacillus sphaericus	DSM 396	Nutrient Agar	30
Bacillus sp.	Soil isolate	Nutrient Agar	30
B. mycoides	DSM 299	Nutrient Agar	30
B. thrungiensis	Soil isolate	Nutrient Agar	30
B. cereus	ATCC 11778	Nutrient Agar	30
B. subtilis	ATCC 6633	Nutrient Agar	30
Serratia marcescens	Soil isolate	Nutrient Agar	37
Pseudomonas fluorescens	DSM 50090	Nutrient Agar	30
Proteus sp.	Clinical isolate	Nutrient Agar	37
Pectobacterium carotovorum	DSM 30168	Nutrient Agar	26
Enterococcus faecalis	ATCC 51299	BHI Agar	37
Listeria sp.	Food isolate	BHI Agar	37
Staphylococcus aureus	ATCC 25923	BHI Agar	37
Micrococcus luteus	ATCC 9341	BHI Agar	37
Streptococcus vestibularis	DSM 5636	BHI Agar	37
Escherichia coli	ATCC 35218	BHI Agar	37
Listeria innocua	DSM 20649	BHI Agar	37
Lactobacillus plantarum	DSM 20174	MRS Agar	37
L. acidophilus	DSM 20079	MRS Agar	37
L. sakei	DSM 6333	MRS Agar	30
Leuconostoc mesenteroides	DSM 20343	MRS Agar	30
Brochothrix thermosphacta	DSM 20171	Corynebacterium agar	30
Cellulomonas fimi	DSM 20114	Trypticase soy yeast Agar	30
Clostridium pasteurianum	DSM 525	Glucose yeast extract Agar	37
Geobacillus sp.	HBB-103	Caso Agar	65
Anoxybacillus sp.	HBB-134	Caso Agar	65
Anoxybacillus sp.	HBB-225	Caso Agar	65
Anoxybacillus sp.	HBB-226	Caso Agar	65
Anoxybacillus sp.	HBB-229	Caso Agar	65
Geobacillus sp.	HBB-269	Caso Agar	65
Geobacillus sp.	HBB-270	Caso Agar	65
Geobacillus sp.	HBB-301	Caso Agar	65
Geobacillus sp.	HBB-247	Caso Agar	65
Geobacillus sp.	HBB-234	Caso Agar	65
E. faecium	HBB-M-1	BHI Agar	37
E. faecium	HBB-MS-1	BHI Agar	37
E. faecium	HBB-KT-2	BHI Agar	37
Enterococcus gallinarium	HBB-MÇ-3	BHI Agar	37
E. gallinarium	HBB-LC-M1	BHI Agar	37
E. faecalis	HBB-NÇ	BHI Agar	37
E.faecalis	HBB-AS-1	BHI Agar	37
Enterococcus avium	HBB-AS-3	BHI Agar	37
Enterococcus durans	HBB-KT-1	BHI Agar	37
E. durans	HBB-K-1	BHI Agar	37

same buffer containing 0.15 mol l^{-1} NaCl. Fractions were pooled and their absorbancies at 280 nm were measured using spectrophotometer (Shimadzu UV-1601) and bacteriocin activities were detected. One of the fractions obtained by gel chromatography, fraction B, was concentrated and then applied to DEAE-sepharose column (Sigma, C-3794), eluted with 20 mmol l^{-1} sodium phosphate buffer (pH 7.0) followed by a gradient from 0 to 1 mol l^{-1} NaCl at a flow rate of 0.6 ml min^{-1} .

Protein concentrations were determined by Bradford method using bovine serum albumin as the standart (Bradford, 1967).

Bacteriocin activities were tested against *G. stearothermophilus* DSMZ 22 as indicator strain.

SDS-PAGE

The Tricine-SDS-PAGE method (4 and 16% acrylamide for the stacking and separating gel, respectively) was performed to control the purification steps and to determine the molecular weight of the bacteriocin (Schagger, 2006). Molecular weight standarts were from Sigma (M3546). One half of the gel was stained with Coomasie blue, and the other half was used for direct detection of bacteriocin activity. The gel, assayed for antibacterial activity was overlaid with soft (0.7%) BHI agar, inoculated with the indicator strain *G. sterathermophilus* DSMZ 22 (1 % v/v). Plate was incubated at 55°C for 14 h, and observed for the formation of inhibition zone.

RESULTS

Identification of the strain HBB-218

The strain HBB-218 is a Gram-positive, aerobic, endo-spore forming thermophilic bacterium. According to the biochemical test results, HBB-218 was found to be catalase and oxidase positive, and produced acid from glucose, ribose and glycerol. The 16S rDNA sequence of the strain HBB-218 showed highest similarity (99.6%) with *G. toebii* according to BLAST results.

Antibacterial spectrum of bacteriocin

The bacteriocin produced by *G. toebii* HBB-218 showed inhibition of a wide range of Gram positive bacteria whereas none of the tested Gram negative strains were inhibited. Bacteriocin inhibits some clinically important bacteria like *Enterococcus faecalis* and *Micrococcus luteus* and it is more active against thermophilic bacterial isolates. The cell-free supernatans obtained from 72 and 96 h-old culture were effective especially against enterococcal species. The antibacterial spectrum of bacteriocin produced by strain HBB-218 is compiled in Table 2.

Effects of heat, pH, enzymes and chemicals on bacteriocin activity

As a result of enzyme treatment, inhibitory agent was found to be sensitive to pronase E and proteinase K; the crude bacteriocin was found to be relatively resistant to proteolytic enzymes. Also, the activity was partially or completely lost by lipase and B-glucoronidase enzymes depending on the enzyme concentration and incubation time (Table 3). Inhibitory agent was found to be a very heat stable, a loss of activity (31%) of the purified bacteriocin was determined after testing at 121°C for 20 min. Storage of either crude or purified bacteriocins at 4°C did not affect the activity after one month. Crude bacteriocin activity was stable after incubation for 24 h with pH values ranging from 3 to 11, while partial inactivation was observed for purified bacteriocin at pH 3, 4 and 5. Among the tested chemicals, only TCA was able to fully inactivate both of the crude and pure bacteriocin. Tween 80 and chloroform treatment resulted with decrease in activity (20 and 13%, respectively) (Table 4).

Bacteriocin production

The production of bacteriocin was found to be starting at early logarithmic phase of growth and it reached to the maximum level (320 AU/ml) after 72 h of cultivation. Figure 1 represents the correlation between bacteriocin activity and growth of *G. toebii* HBB-218.

Purification of the bacteriocin

The bacteriocin was purified from cell free culture supernatants by a combination of ammonium sulphate precipitation, gel filtration and ion exchange chromato-graphy. As a result of gel filtration chromatography, active fractions were collected at four different parts. Fraction B which is the mostly active one and has lower protein content applied to anion exchange column. On the anion exchange column, fractions with inhibitor activity were detected at initial samples without using NaCl gradient. The data for recovery and degree of purification are summarized in Table 5.

Molecular weight determination

According to Tricine-SDS-PAGE analysis, the purified bacteriocin provided only one band which corresponds to a molecular mass of approximately 5.5 kDa. On the other, for half of the identical gel, this band was active against the indicator strain *G. stearothermophilus* DSMZ 22 (Figure 2).

DİSCUSSİON

In this study, a bacteriocin tentatively named as Toebicin 218 produced by *G. toebii* HBB-218 was isolated, characterized and purified. HBB-218 isolated from soil collected around the thermal spring (Denizli, Turkey) was identified as *G. toebii* according to 16S rDNA results. To our knowledge, this is the first description of a bacteriocin produced by a *G. toebii* strain. Bacteriocin produced by strain HBB-218 was active against many of the tested Gram positive bacteria, especially the thermophilic species. One of the most sensitive strains, *G. stearothermophilus* is a canned food spoilage bacterium and spores of this bacterium are extremely resistant to

Table 2. Antibacterial spectrum of toebicin 218.

Indicator strain	Zone of inhibition (mm)			
	24 h CFS	48 h CFS	72 h CFS	96 h CFS
G. stearothermophilus DSMZ 22	20	20	20	20
B. sphaericus DSM 396	9	9	9	9
M. luteus ATCC 934	9	9	9	9
Lact. plantarum DSM 20174	10	10	10	10
Listeria sp.	-	9	9	9
E. faecalis ATCC 51299	11	11	11	11
E. gallinarium MÇ-3	-	-	12	13
E. gallinarium LC-M1	-	-	14	14
E.faecium MS-1	-	-	10	10
E. faecium KT-2	-	-	13	13
E. durans K-1	9	10	12	13
E.durans KT-1	-	-	14	14
E. avium AS-3	-	-	14	13
Brochothrix thermosphacta DSM 20171	-	-	10	8
Cellulomonas fimi DSM 20114	16	20	22	24
Clostridium pasteurianum DSM 525	20	20	24	25
Anoxybacillus sp. HBB-134	10	16	18	18
Geobacillus sp. HBB-234	8	11	11	11
Geobacillus sp.HBB-247	18	18	21	21
Geobacillus sp. HBB-269	-	-	12	12
Geobacillus sp. HBB-270	8	8	10	15
Anoxybacillus sp.HBB-229	15	16	18	20
Geobacillus sp. HBB-301	15	18	18	18
Bacillus sp. (soil isolate)	-	-	-	-
B. mycoides DSM 299	-	-	-	-
B.cereus ATCC 11778	-	-	-	-
B. subtilis ATCC 6633	-	-	-	-
S. marcescens (soil isolate)	-	-	-	-
Pseudomonoas fluorescence DSM 50090	-	-	-	-
Pectobacterium carotovorum DSM 30168	-	-	-	-
Staphylococcus aureus ATCC 25923	-	-	-	-
Streptococcus vestibularis DSM 5636	-	-	-	-
E.coli ATCC 35218	-	-	-	-
Listeria innocua DSM 20649	-	-	-	-
Lact. acidophilus DSM 20079	-	-	-	-
Lact. sakei DSM 6333	-	-	-	-
Leuconostoc mesenteroides DSM 20343	-	-	-	-
Geobacillus sp. HBB-103	-	-	-	-
Anoxybacillus sp. HBB-225	-	-	-	-
Anoxybacillus sp. HBB-226	-	-	-	-

heat treatment. Low-acid foods such as meat and marine products, milk, vegetables, meat and vegetable mixtures (such as soups) can be spoiled by *G. stearothermophilus* under improper storage conditions (Ayres et al., 1980). Toebicin 218 is also heat stable and active over a wide range of pH values. Because of these properties, it can be very useful in canned food industry against some thermophilic, endospore former spoilage-causing bacteria.

The results obtained from the bacteriocin treatment with different enzymes revealed the proteinaceous nature of the analyzed antimicrobial substances. Inhibitory agent is sensitive to pronase E and proteinase K, which indicates that a bacteriocin is indeed responsible for the antibacterial activity observed. Both purified and crude bacteriocins were also inactivated by TCA treatment. Among tested enzymes, lipase and B-glucuronidase also

Table 3. Effects of different enzymes on activity.

Enzyme	Residual activity (%)[a]							
	1 h incubation				24 h incubation			
	CFS	Pure	CFS	Pure	CFS	Pure	CFS	Pure
	1 mg/ml		10 mg/ml		1 mg/ml		10 mg/ml	
Proteinase K	100	0	100	0	100	0	0	0
Pronase E	100	80	100	69	100	0	75	0
Papain	100	100	87,5	0	100	100	87,5	0
Lipase	62,5	80	62,5	0	62,5	0	0	0
β-Glucuronidase	100	100	62,5	100	100	100	0	50
Lactase	100	100	100	100	100	100	100	100
RNAse	100	100	100	100	100	100	100	100
Catalase (300IU/mL)	100	100	100	100	100	100	100	100

[a] Residual activity was estimated according to formula "zone diameter of treated bacteriocin x100 / zone diameter of control"

Table 4. Effects of heat, pH and chemicals on toebicin 218 activity.

Treatment	Residual activity[a]	
	Crude (%)	Pure (%)
Organic solvent (10%)		
Acetone, xylol, DMSO, methanol, ethanol,	100	100
Chloroform	100	87
TCA (100 mg/ml)	0	0
Detergent		
EDTA (0.1 mM), urea (1 mg/ml)	100	100
Tween 80 (1 mg/ml)	100	80
pH		
3-5	100	86
6-11	100	100
Temperature (°C)		
30, 45, 60, 95 for 30 min	100	100
30, 45, 60, 95 for 2 h	100	100
121 for 20 min	100	69
+4 for 1 month	100	100

[a]Residual activity was estimated according to formula "zone diameter of treated bacteriocin x100 / zone diameter of control

affected the antibacterial activity which may be indicating that carbohydrate and lipid moieties are essential for bacteriocin activity. Reduction in the activity can be related to longer incubation times or bacteriocin instability. Similar results on bacteriocin activity and carbohydrate or lipid related enyzmes were reported for other bacteriocins produced by *Lactobacillus curvatus* L422, *Streptococcus thermophilus* ST110, *L. paracasei* and *L. rhamnosus, G. stearothermophilus* 17 (Xiraphi et al., 2005; Gilbert and Somkuti, 2005; Gulahmadov et al., 2006; Pokusaeva et al., 2009).

Production of bacteriocin starts at logarithmic growth phase and it reaches maximum level at the end of the stationary phase. Our results are similar to those described for bacteriocins produced by *Lactobacillus salivarius*, *Lactobacillus plantarum* TF711, *S. auerus* AB188, *E. faecium* MMT21 (Ocana et al., 1999; Hernandez et al., 2005; Saeed et al., 2006; Ghrairi et al., 2007). The reduction observed at the end of the stationary phase may be due to the proteolytic degradation or other environmental factors such as pH level or adsorption to the producer cell. To understand the

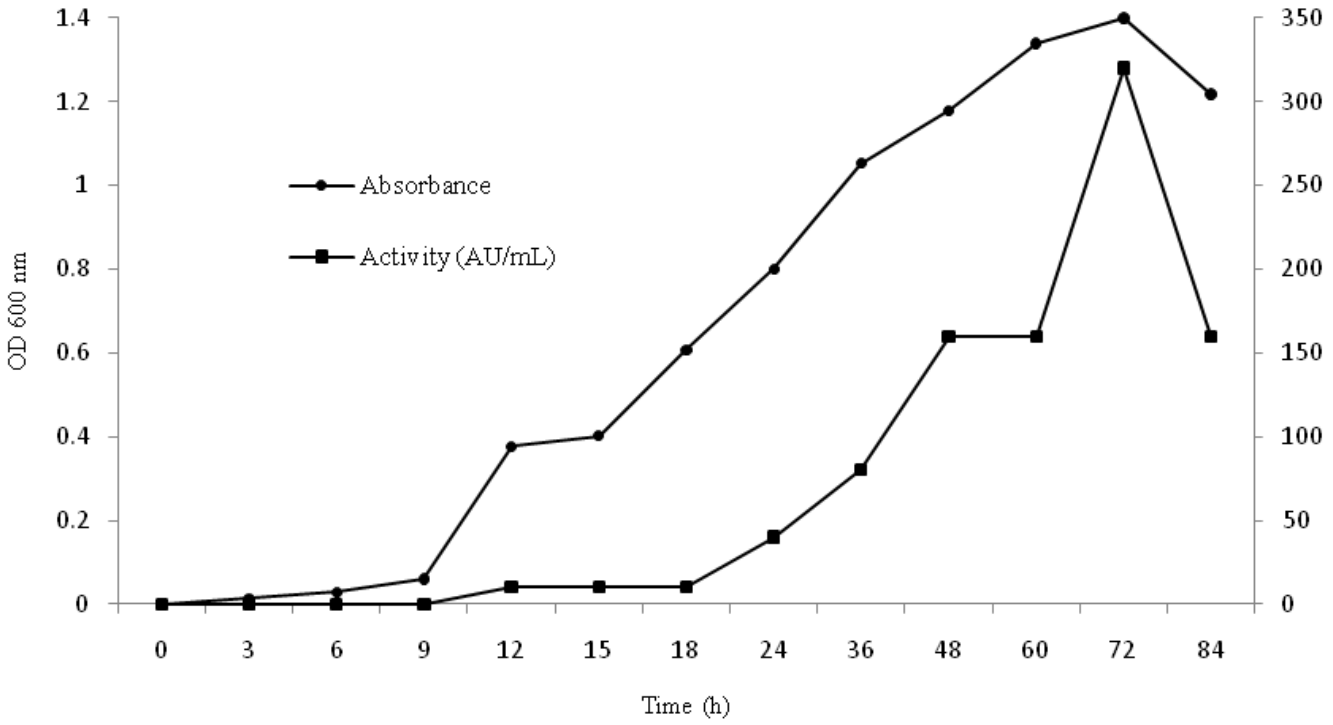

Figure 1. Kinetics of growth and bacteriocin production by *G. toebii* HBB-218 in BHI broth; (■) $OD_{600,}$ (●) bacteriocin activity (AU/mL) against *G. stearothermophilus* DSM22.

Table 5. Purification of the bacteriocin produced by *G. toebii* HBB-218.

Fraction	Volume (ml)	Activity (AU/ml)	Protein (mg/ml)	Total activity (AU)	Total protein (mg/ml)	Spesific activity (AU/mg)	Purification fold	Recovery (%)
CFS	450	160	0.234	72000	105.3	6 84	1	100
Autoclaved CFS	400	160	0.154	64000	61.6	1039	1.52	89
Dyalisate	21	2560	2.03	53760	42.63	1261	1.84	75
Concentrated dyalisate	3	10240	11.35	30720	34.05	902	1.32	43
Fraction B (gel chromatography)	12.5	640	0.240	8000	3.00	2667	3.90	11.1
Fraction 1 (anion exchange)	3	320	0.029	960	0.087	11035	16	1.33

correlation between lyses of cells and increasing bacteriocin activity, we also investigated the intracellular bacteriocin activity (data not shown). As a result of sonication of 24 h-old culture of strain, bacteriocin activities were detected in all three fractions including extracellular (80 AU/ml), intracellular (<40 AU/ml) and membrane-bound (<40 AU/ml) aliquots. These results are similar with some bacteriocins produced by *B. linens* ATCC 9171, *Sulfolobus islandicus* HEN2/2, and *L. curvatus* (Kato et al., 1991; Prangishvilli et al., 2000; Chung and Yousef, 2005).

Toebicin 218 was purified by sequential precipitation, gel filtration, and ion exchange chromotography process with a purification fold of approximately 16. A single band of about 5.5 kDa was observed after purification corresponded to the activity detected after incubation of the indicator strain with a gel slice containing bacteriocin. During anion exchange chromatography experiments, aliquots with antibacterial activity were obtained at first fractions. Bacteriocins produced by Gram positive bacteria are known as cationic molecules and our results are in agreement with this nature of bacteriocins. Pokusaeva et al. (2009) estimated the molecular weights of bacteriocins produced by some strains of *G. stearothermophilus* about 6.8, 5.6, 7.1 and 7.2 kDa (strain 17, 30, 31 and 32A, respectively) and indicated that these values differ from all bacteriocins produced by endospore-forming strains described previously. Toebicin 218 has some similarities to that produced by *G. stearothermophilus* strain 30 in terms of its molecular

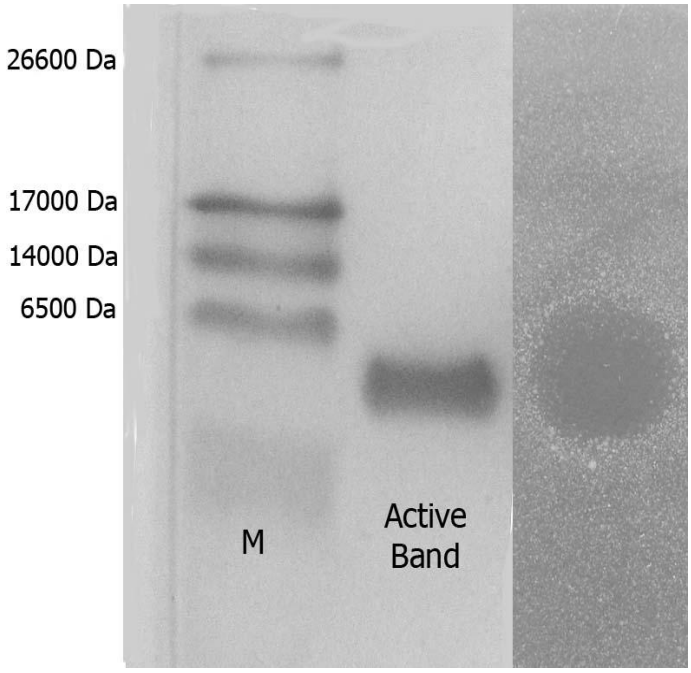

Figure 2. Tricine SDS-PAGE visualization of purified bacteriocin. M, Marker. Right line corresponds to zone of inhibition on the portion of the gel overlaid with the *G. stearothermophilus* DSM22.

wiehgt and antibacterial spectrum (Pokusaeva et al., 2009). Bacillocin 490 produced by a thermophilic strain of *Bacillus licheniformis* is also a small (2 kDa) and heat stable bacteriocin (Martirani et al., 2002). On the other hand, some examples of high molecular weight bacteriocins produced by thermophiles, such as thermoleovorine-S2 (42 kDa) and thermoleovorine-N9 (36 kDa), isolated from *B. thermoleovorans* and sulfolobicin purified from *S. islandicus* HEN2/2 had a molecular mass of about 20 kDa (Novotny and Perry, 1992; Prangishvilli et al., 2000).

The bacteriocin, designated toebicin 218, is inhibitory to several food spoilage and pathogenic bacteria and shows a remarkable stability to heat treatment.

In conclusion, toebicin 218 appears to be a potential biopreservative for food products subjected to pasteurization, sterilization and other heat processing treatments. Our study increases recent knowledge about bacteriocins produced by thermophilic bacteria, which were not previously investigated in depth. Further studies regarding the genetic determinants of bacteriocin production and its usefulnes in food systems are required to accumulate knowledge on bacteriocins produced by thermophilic bacteria.

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