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Detection and Identification of Bacteriocins Produced by Propionibacteria Isolated from Commercial Swiss Cheese Products

Ji-Woon Hur, Na-Kyoung Lee, Haa-Yung Lee* and Hyun-Dong Paik†

Dept. of Food Engineering, Kyungnam University, Masan 631-701, Korea
**Microbiology, Immunology and Preventive Medicine(MIPM) Program, Iowa State University, Ames, IA 50011, USA*

Abstract

Wild propionibacteria isolated from different commercial swiss cheese samples were tested for antimicrobial activities. In initial screening, six of these *Propionibacterium* isolates showed antagonistic activity against 10 selected indicator organisms by the deferred method. In next, only two *Propionibacterium* strains JW6 and JW14 showed antibacterial activity in the cell-free supernatants by the modified well diffusion method. *Propionibacterium* strains JW6 and JW14 were finally identified as bacteriocin producers which exhibited a bactericidal effect against closely related species. The antimicrobial substances were proteins, since their activities were completely destroyed following several degradative enzyme treatments. The bacteriocins showed a narrow inhibitory spectrum of activity against two propionibacteria and two bacilli of strains tested in this study.

Key words: bacteriocin, propionibacteria, identification, swiss cheese

INTRODUCTION

Bacteriocins are defined as bactericidal proteins with a narrow spectrum of activity targeted species related to the producer culture(1). Because bacteriocins are natural products of many microorganisms associated with foods, there is currently much interest in their use as natural food preservatives.

Numerous bacteriocins from gram-positive bacteria have been identified(2), but only a few bacteriocins have been found in propionibacteria. Among the dairy propionibacteria, 2 bacteriocins have been reported: propionicin PLG-1 from *P. thoenii* P127(3-9) and jenseniin G from *P. jensenii* P126(10)

Propionicin PLG-1 is active against a variety of microorganisms(3,5), and was purified to homogeneity by ammonium sulfate precipitation followed by ion exchange column chromatography and reverse-phase high performance liquid chromatography(6). It has been shown to have a calculated molecular weight of 9,328 and amino acid residues of 99, of which 42% are hydrophobic according to amino acid composition analysis, and was identified a 10-amino acid sequence from the N-terminal end, and was determined as a new bacteriocin when compared to other bacteriocins in SWISS-PROT database(6). Jenseniin G is a bacteriocin which shows proteinaceous nature and bactericidal activity against *L. delbrueckii* subsp. *lactis* ATCC 4797(10). Jenseniin G shows the narrow spectrum of activity and heat stability(100°C, 15min) when compared to propionicin PLG-1. The other comparison of two *Propionibacterium* bacteriocins is well documented(11,12).

The propionibacteria have the significant industrial importance on the production of antimicrobial substances, such as propionic and acetic acids, on the production of bacteriocins for foods and feeds, and on the development of probiotic ingredients in animal feeds or as dietary adjuncts to human foods(13).

Although propionibacteria are considered as GRAS(Generally Recognized As Safe), purified bacteriocins produced by the same microorganisms are not automatically regarded as GRAS item. The designation of purified bacteriocins as 'food additives' would require extensive testing to obtain approval for use in foods. However, the public already are consuming a wide variety of bacteriocin producing propionibacteria and/or their bacteriocins because propionibacteria are readily found on many commercial food products(14). Use of bacteriocin producer in starter culture of various foods will be practical and thus, the detection of bacteriocin (s) of starter culture isolated from commercial swiss cheese samples was performed in this study.

†Corresponding author

Propionibacteria, bacteriocin producers, yield their bacteriocins in liquid culture at relatively low concentrations and show slow production rate compared to that of other bacteriocins produced by lactic acid bacteria(7). Thus, more sensitive detection method(the modified well diffusion assay) and production method(fed-batch fermentation) should be used in order to identify bacteriocin(s) produced by propionibacteria(6,7).

The goal of this study was the detection of antimicrobial activity of bacteriocin(s) produced by propionibacteria isolated from commercial swiss cheese samples, and identification of the *Propionibacterium* bacteriocin(s).

MATERIALS AND METHODS

Bacterial cultures and media

Propionibacterium strains were isolated from commercial swiss cheeses and maintained as described previously by Lyon and Glatz(3). Working cultures were propagated in sodium lactate broth(NLB) without shaking at 32°C. *Propionibacterium acidipropionici* P9 and other *Propionibacterium* indicator cultures were obtained from Dr. B. A. Glatz(Iowa State University, Ames). Stock cultures were maintained at -70°C in NLB containing 20% glycerol. Working cultures were prepared from stock cultures and grown in NLB without shaking at 32°C. The strains used as indicator organisms were obtained from different culture collections and indicator strains were grown in appropriate medium as indicated in Table.

Detection of antimicrobial activity

In the initial screening of isolates for antagonistic activity, the deferred method was performed under anaerobic conditions on sodium lactate agar plate as follows. After spotting 3μl of a 18-h isolate culture, propionibacteria were grown on NLA at 32°C for 2 days. 5ml soft NLA(0.7% agar), containing about 10^7 cells of the indicator strain per overlay, was overlaid on 1.5% NLA plates and after 2 day of incubation at 32°C, an inhibition halo was clearly visible.

Bacteriocin assay

Bacteriocin activity was assayed by the modified well diffusion method described by Paik and Glatz(6). The basal layer of NLA contained 2.5% agar and 0.1% Tween 80 and was 5mm deep. After pouring the agar layer, plates were incubated for 24h at room temperature before wells were cut. After 7-mm diameter wells were cut, plates were incubated at 37°C for 2h or at room temperature for 2 days to dry the plates and to facilitate sample diffusion into the agar. The indicator strain was *P. acidipropionici* P9, which was added to 5-ml soft agar(0.7% agar) overlays of NLB medium at about 10^7 cells per overlay. Cell-free supernatants were prepared by centrifugation(8,000×g for 15min at 4°C) of the culture grown on NLB and by filtration through a 0.45μm-pore size disposable filter. Cell- free supernatants(200μl) were added to the wells, allowed to diffuse at 4°C, and the base agar was flipped into the petri dish lid before the overlay was applied. Plates were incubated for 2 days at 32°C before diameters of zones of inhibition were measured. Inhibition zone diameter(mm) was calculated by subtracting well diameter from total inhibition diameter. All assays were performed in duplicate, and the results presented are the means of duplicate trials.

Bacteriocin production

Batch and fed-batch fermentations were performed in the pH controlled bottle reactors(500ml working volume). Fermentation medium in batch fermentations was NLB and the fermentation was started with a 1%(v/v) inoculum of an 18-h culture in NLB, and was incubated at 32°C. The pH was controlled at 7.0±0.1 by the addition of 3M HCl and 3M NaOH. Fed-batch fermentations were slightly modified compared to previous study(8). Fermentation medium was NLB with 1.2% sodium lactate rather than 0.6% sodium lactate as a substrate. Fed-batch fermentations were started as batch fermentations and were incubated for up to 14 day. Sodium lactate was first fed at about 48h of incubation and was added every 24h to give a final concentration in the medium of 0.6%. In addition, a 20X-concentrated preparation of NLB(without lactate) was fed every 7 days to replenish about 15% of the other nutrients in the medium at each feeding.

Sensitivity to enzymes

For enzyme stability, cell-free supernatants were treated for 1h with various enzymes at a final concentration of 1mg/ml. All enzymes(protease XIV, protease IX, pepsin, α-chymotrypsin, and proteinase K) were dissolved in buffers as recommended by the supplier(Sigma, St. Louis, MI). Untreated bacteriocin plus buffers, buffers alone, and enzyme solutions served as controls. The residual bacteriocin activity was determined by the modified

well diffuison method. All data were the average of duplicate trials.

Inhibitory spectrum of activity

The deferred and the modified well diffusion methods were used to assess the antimicrobial activity of isolated propionibacteria towards several Gram-positive and Gram-negative bacteria, a yeast and a mold. All strains were previously subcultured in appropriate growth agar medium and were propagated in liquid medium, then inoculated into the soft-agar medium(0.7% agar) of the same composition.

Mode of inhibition

An exponentially growing culture of the indicator strain *P. acidipropionici* P9 was suspended in 0.1M phosphate buffer(pH 7.0) and exposed for 60min to cell-free culture supernatants. The number of surviving bacteria(CFU per milliliter) was determined by plate counting.

RESULTS AND DISCUSSION

Screening of *Propionibacterium* isolates for antibacterial activity

Seventeen propionibacteria isolated from commercial

Table 1. Initial screening of bacteriocin producer(s) by deferred method

(Inhibition zone diameter: mm)

Selected indicator	*Propionibacterium* isolates(JW)																
	1	2	3	4	5	6	7	8	9	10	11	13	14	15	16	17	18
Propionibacterium acidipropionici P5	3.0	-	3.0	3.0	-	3.0	-	-	-	1.5	-	-	2.5	-	-	-	-
Propionibacterium acidipropionici P9	2.0	-	2.0	2.0	-	2.0	-	-	-	1.5	-	-	2.5	-	-	-	-
Propionibacterium thoenii P127	1.0	-	1.0	2.0	-	2.0	-	-	-	2.5	-	-	2.0	-	-	-	-
Propionibacterium freudenreichii KCTC 1063	3.0	-	4.0	-	-	4.0	-	-	-	6.0	-	-	4.0	-	-	-	-
Propionibacterium acidipropionici P200910	2.0	-	2.0	2.0	-	2.0	-	-	-	2.0	-	-	2.0	-	-	-	-
Lactobacillus delbrueckii subsp. *lactis* ATCC 4797	5.0	-	1.0	1.5	-	1.5	-	-	-	10.0	-	-	2.5	-	-	-	-
Lactococcus lactis KCCM 40104	6.0	-	3.0	3.0	-	3.0	-	-	-	4.0	-	-	3.0	-	-	-	-
Leuconostoc mesenteroides KCCM 11324	3.0	-	3.0	2.0	-	2.5	-	-	-	2.0	-	-	2.0	-	-	-	-
Pediococcus acidilactici KCTC 1626	4.0	-	1.0	2.0	-	2.0	-	-	-	3.0	-	-	0.5	-	-	-	-
Lactococcus lactis BH5	2.5	-	2.5	2.8	-	4.0	-	-	-	2.0	-	-	2.0	-	-	-	-

Table 2. Screening of bacteriocin producer(s) by the modified well diffusion method

(Inhibition zone diameter: mm)

Selected indicator	*Propionibacterium* isolates(JW)					
	1	3	4	6	10	14
Propionibacterium acidipropionici P5	-	-	-	-	-	-
Propionibacterium acidipropionici P9	-	-	-	12.0	-	9.0
Propionibacterium thoenii P127	-	-	-	-	-	-
Propionibacterium freudenreichii KCTC 1063	-	-	-	-	-	-
Propionibacterium acidipropionici P200910	-	-	-	9.0	-	12.0
Lactobacillus delbrueckii subsp *lactis* ATCC 4797	-	-	-	-	-	-
Lactococcus lactis KCCM 40104	-	-	-	-	-	-
Leuconostoc mesenteroides KCCM 11324	-	-	-	-	-	-
Pediococcus acidilactici KCTC 1626	-	-	-	-	-	-
Lactococcus lactis BH5	-	-	-	-	-	-

Table 3. Effect of various enzymes on bacteriocins
(Inhibition zone diameter: mm)

Treatment	Bacteriocins	
	JW6	JW14
Control	8.0	10.5
Protease XIV	0	0
Protease IX	8.0	11.0
Pepsin	0	10.0
α-Chymotrypsin	10.0	0
Proteinase K	8.0	8.5

swiss cheese products were screened for their capacity to produce bacteriocin-like activity against 10 selected Gram-positive bacteria including 5 propionibacteria by the deferred method(Table 1). Six of these *Propionibacterium* isolates tested in this study showed antagonistic activities which inhibited all of the indicator strains used. From these data, the 6 propionibacteria were tested for their antimicrobial activity against same 10 indicator strains by the modified well diffusion method. At first, the antimicrobial activities of cell-free supernatants of 6 propionibacteria produced in pH-controlled batch fermentations was not found. Thus, we tried pH-controlled fed-batch fermentations for enhanced bacteriocin production of isolated propionibacteria because this fermentation tool was very efficient for propionicin PLG-1 production(9). Only two strains, *Propionibacterium* strains JW6 and JW14 still showed antibacterial activity against a few strains of 10 selected indicators for the screening in the modified well diffusion assay(Table 2). Strain JW6 had been isolated from a commercial swiss cheese(Craft; Aged). Strain

Table 4. Antimicrobial spectrum of activity on bacteriocins by deferred method
(Inhibition zone diameter: mm)[1]

Indicator	Growth medium[2]	Temp. (°C)	Producer	
			JW6	JW14
Gram-positive bacteria				
Propionibacterium acidipropionici P5	NLB	32	3.0	2.5
Propionibacterium acidipropionici P9	NLB	32	2.0	2.5
Propionibacterium thoenii P127	NLB	32	2.0	2.0
Propionibacterium freudenreichii KCTC 1063	NLB	32	4.0	4.0
Propionibacterium acidipropionici P200910	NLB	32	2.0	2.0
Lactobacillus delbrueckii subsp. *lactis* ATCC 4797	MRS	37	1.5	2.5
Pediococcus acidilactici KCTC 1626	MRS	37	2.0	0.5
Lactococcus lactis BH5	MRS	30	4.0	2.0
Lactococcus lactis KCCM 40104	MRS	30	3.0	3.0
Leuconostoc mesenteroides KCCM 11324	MRS	30	2.5	2.0
Leuconostoc curvatus CA170-12	MRS	30	4.0	5.0
Bacillus cereus	NB	30	-[3]	-
Bacillus pumilis	NB	30	2.0	2.0
Bacillus subtilis IFO 12113	NB	37	-	-
Staphylococcus aureus KCCM 32359	NB	37	0.5	1.0
Gram-negative bacteria				
E. coli KCCM 32396	LB	37	-	1.2
E. coli JM109	LB	37	2.0	-
Aeromonas hydrophila	NB	30	1.5	2.0
Pseudomonas cepacia(SBA9611)	NB	30	3.0	4.0
Pseudomonas cepacia(SBA9613)	NB	30	1.5	2.0
Pseudomonas fluorescence	NB	30	1.0	1.0
Pseudomonas putida	NB	30	1.5	1.5
Xanthomonas maltophila	NB	30	0.7	0.7
Chryseomonas luteola	NB	30	6.0	1.0
Zymomonas mobilis KCTC 1535	YPD	30	-	-
Yeast and Mold				
Aspergillus oryzae KCCM 11371	PDB	28	-	-
Penicillium chrysogenum KCTC 6933	PDB	28	-	-

[1]These data were the average of duplicate trials
[2]NLB, sodium lactate broth; MRS, lactobacilli MRS; NB, nutrient broth; LB, Luria broth; YPD, yeast extract peptone dextrose; PDB, potato dextrose broth
[3]Not inhibited

JW14 was isolated from a commercial swiss cheese(Crystal Farm; Aged over 60 days). This phenomenon is well known in the bacteriocin screening works because inhibition due to propionic and acetic acids was excluded by neutralizing the supernatants before testing in the modified well diffusion method compared to deferred method. In addition, solid environment seems to be favorable to produce *Propionibacterium* bacteriocin(s) in comparison with liquid culture(3,10). From these data, strains JW6 and JW14 were selected as good candidates for bacteriocin production. Upon the dilution of culture supernatants, the zones of inhibition on lawns of the indicator strain diminished in size without the appearance of plaques, suggesting the inhibition was not caused by replication of bacteriophage.

Effect of various enzymes

The effect of various enzymes on cell-free supernatants were investigated(Table 3). As shown in Table 3, all the inhibitory substance(s) of strain JW6 were completely inactivated by treatment with protease XIV or pepsin, while those of strain JW14 were completely inactivated by treatment with protease XIV or α-chymotrypsin. No modification of activity was observed when cell-free supernatants were treated with other enzymes tested. Buffers and enzyme solutions alone had no effect on the indicator strain(data not shown).

Inhibitory spectrum of activity

For the antimicrobial spectrum of activity, cell-free

Table 5. Antimicrobial spectrum of activity on bacteriocins by a modified well diffusion method

(Inhibition zone diameter: mm)[1)]

Indicator	Growth medium[2)]	Temp. (°C)	Producer	
			JW6	JW14
Gram-positive bacteria				
Propionibacterium acidipropionici P5	NLB	32	-[3)]	-
Propionibacterium acidipropionici P9	NLB	32	12.0	9.0
Propionibacterium thoenii P127	NLB	32	-	-
Propionibacterium freudenreichii KCTC 1063	NLB	32	-	-
Propionibacterium acidipropuionici P200910	NLB	32	9.0	12.0
Lactobacillus delbrueckii subsp. *lactis* ATCC 4797	MRS	37	-	-
Pediococcus acidilactici KCTC 1626	MRS	37	-	-
Lactococcus lactis BH5	MRS	30	-	-
Lactococcus lactis KCCM 40104	MRS	30	-	-
Leuconostoc mesenteroides KCCM 11324	MRS	30	-	-
Leuconostoc curvatus CA170-12	MRS	30	-	-
Bacillus cereus	NB	30	6.0	3.0
Bacillus pumilis	NB	30	2.0	4.0
Bacillus subtilis IFO 12113	NB	30	-	-
Staphylococcus aureus KCCM 32359	NB	30	-	-
Gram-negative bacteria				
E. coli KCCM 32396	LB	37	-	-
E. coli JM109	LB	37	-	-
Aeromonas hydrophila	NB	30	-	-
Pseudomonas cepacia(SBA9613)	NB	30	-	-
Pseudomonas cepacia(SBA9611)	NB	30	-	-
Pseudomonas fluorescence	NB	30	-	-
Pseudomonas putida	NB	30	-	-
Xanthomonas maltophila	NB	30	-	-
Chryseomonas luteola	NB	30	-	-
Zymomonas mobilis KCTC 1535	YPD	30	-	-
Yeast and Mold				
Aspergillus oryzae KCCM 11371	PDB	28	-	-
Penicillium chrysogenum KCTC 6933	PDB	28	-	-

[1)]These data were the average of duplicate trials

[2)]NLB, sodium lactate broth; MRS, lactobacilli MRS; NB, nutrient broth; LB, Luria broth; YPD, yeast extract peptone dextrose; PDB, potato dextrose broth

[3)]Not inhibited

supernatant was tested against various Gram-positive and Gram-negative bacteria, a yeast and a mold by the deferred and the modified well diffusion method. Table 4 indicates that strains JW6 and JW14 showed wide spectrum of activity against all propionibacteria, lactic acid bacteria tested and most of Gram-negative bacteria, but *Aspergillus oryzae* and *Penicillium chrysogenum* were not inhibited. Table 5 shows that the two strains showed a similar spectrum of activity which had a narrow antimicrobial spectrum of activity against two propionibacteria (*P. acidipropionici* P9 and its mutant *P. acidipropionici* P200910) and two bacilli(*B. cereus* and *B. pumilis*). No inhibition was observed against lactic acid bacteria, *Escherichia coli*, Gram-negative plant pathogens, a yeast and a mold tested in this study(Table 5).

Mode of inhibition

To determine whether the antimicrobial substance had a bactericidal or a bacteriostatic effect, cell-free supernatant was added to the indicator cells suspended in phosphate buffer(pH 7.0). The antimicrobial substances of isolates JW6 and JW14 showed a bactericidal mode of action. A decrease in CFU per milliliter was observed after the exposure of the indicator cells to the cell-free supernatants(data not shown).

The bacteriocins produced by two *Propionibacterium* strains were sensitive to proteolytic enzymes, and showed bactericidal activity against the sensitive indicator, *P. acidipropionici* P9. Thus, they could be classified as bacteriocins. Further partial purification and characterization of the bacteriocins are in progress.

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