J. Microbiol. Biotechnol. (2000), **10**(2), 195–200

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

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Identification and Partial Characterization of Cerein BS229, a Bacteriocin Produced by *Bacillus cereus* BS229

PAIK, HYUN-DONG*, NA-KYOUNG LEE, KWANG-HO LEE, YONG-IL HWANG, AND JAE-GU PAN[1]

Division of Life Sciences, Kyungnam University, Masan 631-701, Korea
[1]Bioprocess Engineering Division, Korea Research Institute of Bioscience and Biotechnology (KRIBB), KIST, Taejon 305-600, Korea

Received: October 26, 1999

Abstracts *Bacillus cereus* BS229 was identified as a bacteriocin producer with a bactericidal activity against *Bacillus thuringiensis* subsp. *thomsoni* BR40. *Bacillus cereus* BS229 and cerein BS229, named tentatively as the bacteriocin produced by *Bacillus cereus* BS229, showed a narrow spectrum of activity against Gram-positive and Gram-negative bacteria, along with yeast and molds. Production of cerein BS229 in a 5-l fermenter followed typical kinetics of primary metabolite synthesis. The antibacterial activity of cerein BS229 on sensitive indicator cells disappeared completely by α-chymotrypsin or proteinase K, which indicates its proteinaceous nature. Cerein BS229 seemed to be very stable throughout the pH range of 2.0 to 9.0 and it was relatively heat labile, despite the fact that bacteriocin activity was still detected after being boiled for 30 min. Cerein BS229 activity has been changed with some of the organic solvents such as toluene, ethanol, and chloroform. Direct detection of cerein BS229 activity on SDS-PAGE suggested that it had an apparent molecular mass of about 8.2 kDa.

Key words: Bacteriocin, *Bacillus cereus*, cerein BS229

The genus *Bacillus* includes a variety of industrially important species which are commonly used as hosts in the bioindustry. The commercial products today which are produced by *Bacillus* fermentations include enzymes, antibiotics, and insecticides. Other products are nucleotides and nucleosides used mainly for food flavor enhancement and amino acids.

Bacteriocins are proteins or protein complexes with bactericidal activity towards a closely related species to the producer bacteria [23]. The possibility to genetically manipulate an encoding bacteriocin has been considered to be one of the concerns of bacteriocin research. Many bacteriocins in the genus *Bacillus* have been reported [23], and the best characterized ones are subtilisin of *B. subtilis* [2, 6, 9, 11, 12, 16], megacin of *B. megaterium* [25, 26], and some bacteriocins of *B. thuringiensis* [5, 13, 21]. However, an importance of industrial value for *Bacillus* bacteriocins have been strongly underestimated. The possibility of screening for a new *Bacillus* bacteriocin producer is considered to be one of the major interests in the bacteriocin research.

B. cereus is the Gram-positive spore-former and can be easily isolated from a variety of foods, including dairy products, meats, spices, and cereals. The bacteriocin research associated with *B. cereus* is remarkably scarce in comparison with the industrial importance of the subspecies. As far as we know, the only bacteriocin characterized from *B. cereus* is cerein [15].

This paper describes the isolation of bacteriocin-producing *Bacillus* strains from various sources and the identification and partial characterization of cerein BS229 produced from *B. cereus* BS229. The characteristics of cerein BS229 make this bacteriocin potentially interesting as an antimicrobial agent for the control of *B. cereus* and *E. coli* in industrial applications.

MATERIALS AND METHODS

Bacterial Strains and Media

Producer strain *B. cereus* BS229 and indicator strain *B. thuringiensis* subsp. *thomsoni* BR40 were maintained at -70°C in tryptic soy broth (TSB; Difco, Detroit, U.S.A.) to which 20% (v/v) glycerol was added. Working cultures were propagated in TSB with shaking at 30°C. The indicator organisms were obtained from different culture collections and were grown in appropriate media.

Detection of Antimicrobial Activity

B. cereus BS229 was examined for antimicrobial activity against indicator organisms on tryptic soy agar plate by the

*Corresponding author
Phone: 82-551-249-2689; Fax: 82-551-243-8133;
E-mail: hdpaik@kyungnam.ac.kr

modified deferred method [1]. After inoculating the selected producer with a toothpick, *B. cereus* BS229 was grown on a tryptic soy agar at 30°C for 12 h. Five-ml of soft tryptic soy agar (0.7% agar), containing about 10^7 cells of the indicator strain per overlay, was overlaid on 1.5% tryptic soy agar plates and, after 12 h of incubation at 30°C, an inhibition halo was clearly visible. The strength of the antimicrobial activity was expressed by the diameter (mm) of the halo and its results presented are means of duplicate trials.

Bacteriocin Assay

Cerein BS229 activity was assayed by the spot-on-lawn method as follows: The soft tryptic soy agar (0.7% agar), containing 10^7 cells of the indicator organism per overlay, was overlaid on 1.5% tryptic soy agar plates. When the soft agar was hardened. 5 μl of the bacteriocin preparation were spotted on the plate, and. after about 10 h of incubation at 30°C. an inhibition halo was clearly visible. Bacteriocin preparation was made by spotting serial two-fold dilutions of crude bacteriocin, and the reciprocal of the greatest inhibitory dilution was used to calculate arbitrary activity units (AU) per ml of the original cultures. All conditions were tested in duplicate and its results presented are means of duplicate trials [18].

Identification of Bacteriocin-Producing Isolate

A bacteriocin-producing isolate was identified by criteria based on carbohydrate fermentation patterns using an API 50CHB kit (bioMereux, France) with some biochemical characteristics [24].

Table 1. Antimicrobial spectrum of activity of partially purified cerein BS229.

Indicator strain	Modified deferred method	Spot-on-lawn method	
	Inhibition zone diameter (mm)	Supernatant[a]	Partially purified bacteriocin[a]
Gram-positive bacteria			
Propionibacterium acidipropionici P5	-[b]	-	-
Propionibacterium acidipropionici P9	-	-	-
Propionibacterium thoenii P127	-	-	+
Propionibacterium freudenreichii KCTC 1063	-	-	-
Propionibacterium acidipropionici P200910	-	-	-
Lactobacillus delbrueckii ATCC 4797	-	-	-
Pediococcus acidilactici KCTC 1626	-	-	-
Lactococcus lactis BH5	-	-	-
Lactococcus lactis KCCM 40104	-	-	-
Leuconostoc mesenteroides KCCM 11324	-	-	-
Bacillus cereus	4.0	+	+
Bacillus pumilis HTD-1	2.0	+	+
Bacillus subtilis IFO 12113	3.0	+	+
Bacillus thuringiensis BR40	4.0	+	+
Micrococcus flavus ATCC 10240	6.0	+/-[c]	+
Staphylococcus aureus KCCM 32359	-	-	-
Gram-negative bacteria			
Escherichia coli KCCM 32396	2.0	+	+
Escherichia coli JM109	4.0	+	+
Pseudomonas cepacia SBB 9611	6.0	-	-
Pseudomonas fluorescens SBB 9631	-	-	-
Pseudomonas putida	-	-	-
Chryseomonas luteola SBA 9634	-	-	-
Sphingomonas paucimobilis BNJ 9664	1.5	-	-
Xanthomonas maltophila SBC 9611	-	-	-
Zymomonas mobilis KCTC 1535	-	-	-
Yeast and Molds			
Saccharomyces cerevisiae KCCM 11201	-	-	-
Aspergillus niger KCCM 11239	-	-	-
Aspergillus oryzae KCCM 11371	-	-	-
Penicillium chrysogenum KCTC 6933	-	-	-

[a]These data are the average of duplicate trials.
[b]Not inhibited.
[c]Zone of inhibition was hazy, not clear

Production of Cerein BS229

Cerein BS229 production was performed in a 5-l fermenter (3.0-l working volume; Korea Fermenter Co., Inchon, Korea) and the fermentation medium was TSB. *B. cereus* BS229 that was inoculated into 250 ml of sterile TSB and the seed culture (1%, v/v) was transferred into the jar fermenter. Temperature was controlled at 30°C and pH was maintained at 7.0±0.1 by addition of 3 N HCl or 3 N NaOH. Agitation speed was 500 rpm in a 5-l fermenter and aeration rate was 1 vvm. Antifoam agent (silicone oil) was added automatically whenever it was necessary. For production studies, samples were aseptically removed over a 12 h period to determine the cell growth and bacteriocin activity at different time intervals. Cell growth was monitored spectrophotometrically and the bacteriocin activity of the culture broth was evaluated by the method described previously.

Partial Purification of Cerein BS229

Partially purified cerein BS229 was obtained as described previously [17]. Solid ammonium sulfate was slowly added to the culture supernatant (450 ml) up to 75% of saturation at 4°C, with constant stirring for about 5 h. Slow stirring was continued for an additional 30 min at 4°C. Precipitated proteins were pelleted by centrifugation at 10,800 ×g for 30 min at 4°C, resuspended in a 10 mM phosphate buffer (pH 7.0), and extensively dialyzed against 3 l of 10 mM phosphate buffer (pH 7.0) for 12-18 h in Spectra-Por no. 3 dialysis tubing (molecular weight cutoff, 3,500; Spectrum Medical Industries, Gardena, U.S.A.). The dialyzed samples were stored at -70°C.

Inhibitory Spectrum of Activity

The modified deferred and spot-on-lawn methods were used to assess the antimicrobial activity of *B. cereus* BS229 and partially purified cerein BS229 towards several Gram-positive and Gram-negative bacteria, and a yeast and molds (Table 1). All strains were previously subcultured in an appropriate growth agar medium, propagated in a liquid medium, and then inoculated into a soft agar medium (0.7% agar) made up of the same composition.

Sensitivities to Enzymes, pH, Heat, and Organic Solvents

For enzyme stability, partially purified cerein BS229 was treated for 1 h with various enzymes at a final concentration of 1 mg/ml. All enzymes were dissolved in buffers as recommended by the supplier (Sigma, St. Louis, U.S.A.). Untreated bacteriocin plus buffers, buffers alone, and enzyme solutions served as the control [19]. The pH stability was estimated in the partially purified cerein BS229 after 4 h of storage at 4°C in the following buffers: 50 mM citrate buffer, pH 3-6; 50 mM phosphate buffer, pH 7.0; and 50 mM Tris-HCl buffer, pH 8-9 [21]. To determine the effect of heat on bacteriocin activity, aliquots (500 μl) of partially purified cerein BS229 were incubated at various temperatures (40, 50, 60, 70, 80, 90, and 100°C) for 30 min or 121°C for 15 min [21]. Partially purified bacteriocin was treated with 50% organic solvents such as ethanol, methanol, toluene, chloroform, acetone, and isopropanol. The solvent-treated sample was incubated at 30°C for 1 h and residual solvents were evaporated at 30°C for 2 h. Control included bacteriocin in the buffer without the solvents. The residual bacteriocin activity was determined by the spot-on-lawn method. All data are the average of duplicate trials.

Mode of Inhibition

Cells from the log-phase of *B. thuringiensis* subsp. *thomsoni* BR40 were suspended in a sterile 10 mM phosphate buffer (pH 7.0). The test was carried out at 30°C by adding 21,600 AU/ml of partially purified cerein BS229. At various times, the viable cells (cfu/ml) were determined on tryptic soy agar plates by using the standard plate counting method [21].

Molecular Weight Determination by SDS-PAGE

To estimate the molecular weight of partially purified cerein BS229, SDS-PAGE was performed with a 16% discontinuous gel. Twenty microliters of the sample and molecular weight standards were applied to the gel. Protein standards and their molecular weights were as follows: ovalbumin, 43,000; carbonic anhydrase, 29,000; β-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor, 6,200; and insulin, 3,000. The sample was prepared by mixing a 1:1 ratio of cerein BS229 sample and buffer by boiling the mixture at 100°C for 5 min. Electrophoresis was performed in a vertical slab gel apparatus (Protean Cell II; Bio-Rad, Hercules, U.S.A.), with the buffer system at a constant voltage (100 V) for 2 h. Half of the gel was stained with Coomassie Brilliant Blue R-250 reagent as directed by the manufacturer, while the other half was assayed for bacteriocin activity by following the direct method described by Hechard *et al.* [7]. This part of the gel was fixed for 2 h in 20% 2-propanol and 10% acetic acid and soaked for at least 4 h in sterile deionized water. It was then aseptically placed in a sterile petri dish and covered with 20 ml of soft agar containing 10^7 cells of *B. thuringiensis* subsp. *thomsoni* BR40 as the indicator strain. The plate was incubated at 30°C for 12 h and examined for zones of inhibition.

RESULTS AND DISCUSSION

Screening of Antibacterial Activities of *Bacillus* Strains

In recent years, there have been increasing concerns with regards to the implantation into the indigenous microflora of bacteriocin-producing strains of an apparently low virulence with a potential capability of interfering with colonization and infection by more pathogenic species,

since the antibiotic resistant strains have been increased due to widespread overuse of antibiotics [8]. Basically, an effective detection of inhibitory activity depends on environmental conditions (pH, nutrients, temperature, *etc.*) which may facilitate the effective interaction with a susceptible organism, in addition to the detection method that affects the sensitivity. The 2,000 strains of *Bacillus* isolated from various sources were used to screen antibacterial activities against some of the *Bacillus* strains selected. A few isolates showing antibacterial activity was reconfirmed in a liquid culture and the effect of the enzyme on pH-adjusted culture supernatant tested (data not shown). From these data, *Bacillus* strain BS229 was selected as a good bacteriocin producer. This strain BS229 was isolated from the leaves using sporulation agar after heat treatment and maintained in the laboratory culture collections of KRIBB. Antagonism of *B. cereus* BS229 against Gram-positive, Gram-negative bacteria, yeast, and molds is shown in Table 1. Upon the dilution of cerein BS229, the zones of inhibition on lawns of the indicator strain diminished in size without the appearance of plaques, suggesting that the inhibition was not resulted by replication of bacteriophage.

Identification of Strain BS229

Strain BS229, known as a Gram-positive rod, was tentatively identified as *Bacillus cereus* based on its carbohydrate fermentation patterns using the API 50CHB kit and some biochemical characteristics (data not shown).

Production of Cerein BS229

Incubated in TSB medium at 30°C, *B. cereus* BS229 produced extracellular inhibitory activity against *B. thuringiensis* subsp. *thomsoni* BR40 (Fig. 1). Production of cerein BS229 seems to follow typical kinetics of primary metabolite synthesis. Cerein BS229 reached to its maximum activity (2,880 AU/ml) after being incubated for 6 h, maintained for 1 h, and the activity dropped sharply after the late stationary phase. Possible reasons for this rapid decrease in bacteriocin activity are most likely due to the formation of an inhibitor, its degradation by extracellular proteolytic enzymes, the binding of bacteriocin to cells, and inactivating complex formation with other extracellular products [20]. This decrease in the activity could also be associated with the induction of sporulation, as shown in cerein, a bacteriocin produced by *B. cereus* GN105 [15].

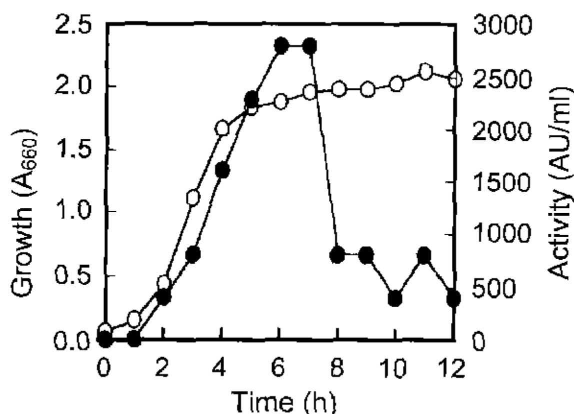

Fig. 1. Growth of *B. cereus* BS229 (●) and production of cerein BS229 (○) in a jar fermenter.

Inhibitory Spectrum of Activity

The supernatants and partially purified cerein BS229 were tested for their antimicrobial activities against various Gram-positive and Gram-negative bacteria, a yeast, and molds by the spot-on-lawn method. Table 1 indicates that cerein BS229 showed a narrow spectrum of activity against all *Bacillus* strains and two *E. coli* strains, *P. thoenii* P127, and *M. flavus* ATCC 10240. No inhibition was observed against lactic acid bacteria, *Staphylococcus aureus* KCCM 32359, Gram-negative bacteria (except for *E. coli*), yeast, and molds used in this study (Table 1). Accordingly, from its inhibitory spectra, cerein BS229 showed similarity with several bacteriocins from *Lactobacillus* sp., whose activity spectra include only strains which belong to the same genus [3, 10, 22], rather than lantibiotic nisin, which inhibits most Gram-positive bacteria [4, 14].

Effects of Various Enzymes, pH, Heat, and Organic Solvents

The effect of various enzymes on the partially purified cerein BS229 was carefully investigated. As shown in Table 2, all the inhibitory substances were completely inactivated by treating with α-chymotrypsin or proteinase K, thus suggesting the proteinaceous nature of cerein BS229. No modification of activity was observed when cerein BS229 was treated with other tested enzymes (trypsin, protease IX, protease XIV, α-amylase, lipase, papain, pepsin, DNase, RNase, lysozyme, and catalase). Buffers and enzyme solutions alone had no effect on the indicator strain. The activity of cerein, other bacteriocin produced by *B. cereus* GN105, was completely lost when

Table 2. Effects of various enzymes on partially purified cerein BS229.

Treatment	Residual activity (AU/ml)	Treatment	Residual activity (AU/ml)
Control	25,600	Lipase	25,600
Trypsin	25,600	Papain	25,600
Protease IX	25,600	Pepsin	25,600
Protease XIV	25,600	DNase	25,600
Proteinase K	0	RNase	25,600
α-Chymotrypsin	0	Lysozyme	25,600
α-Amylase	25,600	Catalase	25,600

Table 3. Effects of pH, heat, and organic solvents on partially purified cerein BS229.

pH	Residual activity (AU/ml)	Heat	Residual activity (AU/ml)	Organic solvents	Residual activity (AU/ml)
Control	25,600	Control	25,600	Control	25,600
2	25,600	40°C[a]	25,600	Ethanol	12,800
3	25,600	50°C[a]	12,800	Methanol	6,400
4	25,600	60°C[a]	6,400	Toluene	25,600
5	25,600	70°C[a]	ND[c]	Chloroform	12,800
6	25,600	80°C[a]	1,130	Acetone	6,400
7	25,600	90°C[a]	400	Isopropanol	6,400
8	25,600	100°C[a]	200		
9	25,600	121°C[b]	0		

[a]Heat treatment for 30 min.
[b]Autoclave for 15 min.
[c]Not determined.

treating with trypsin, chymotrypsin, protease K, but not lost by treating with DNase, ribonuclease A, lysozyme [15]. Partially purified cerein BS229 was pH stable in the range of 2.0 to 9.0 (Table 3). Cerein BS229 was relatively heat labile; the inhibitory activities were detected after treatment at 100°C for 30 min, but the inhibitory activities in the partially purified cerein BS229 were inactivated at 121°C for 15 min (Table 3). This heat stability ruled out the possibility that the inhibitory action is due to bacteriophage. Inhibitory activity of cerein was maintained during heat treatment of up to 75°C and disappeared only after 15 min of incubation at 90°C [15]. Finally, the antimicrobial activity of partially purified bacteriocin was affected by any of the organic solvents shown in Table 3.

Mode of Inhibition

To determine whether cerein BS229 had a bactericidal or a bacteriostatic effect, the partially purified cerein BS229 was added to the indicator cells suspended in 10 mM phosphate buffer (pH 7.0). Cerein BS229 showed a bactericidal mode of action. A decrease in CFU per mililiter was observed after the indicator cells were exposed to the bacteriocin (Fig. 2). However, the intrinsic nature of this inhibition has not been identified and requires further investigation.

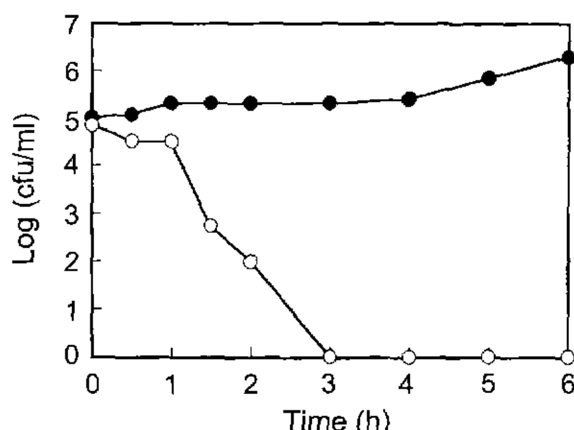

Fig. 2. Mode of inhibition of cerein BS229.
B. thuringiensis BR40 were incubated at 30°C in 10 mM phosphate buffer containing cerein BS229 at 21,600 AU/ml (●). Control (○) had no cerein BS229.

Molecular Weight of Cerein BS229

Several contaminating proteins were detected in the partially purified cerein BS229 (Fig. 3). Partially purified cerein BS229 by 16% polyacrylamide gel was cut into two vertical sections, and the part of the gel containing the sample and of the molecular weight markers was stained, while the remaining part, containing only the sample, was fixed and used for direct detection of antimicrobial activity by the method of Hechard *et al.* [7]. As shown in Fig. 3, the bactericidal activity of cerein BS229 is associated with a band having an apparent molecular mass of about 8.2 kDa.

In conclusion, *B. cereus* strains produce antimicrobials such as bacteriocins, but at present only one bacteriocin (cerein) has been characterized [15]. Cerein was active against other *B. cereus* strains, but, on the other hand, it

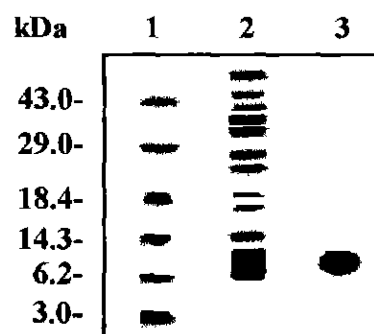

Fig. 3. SDS-PAGE of cerein BS229 and detection of antimicrobial activity.
Lane 1, molecular weight standard; lane 2, partially purified cerein BS229; lane 3, portion of the gel overlaid with the indicator strain as described in Materials and Methods. Inhibition halo was observed overnight after the incubation period at 30°C.

was not active against various Gram-positive and Gram-negative species tested. The activity of partially purified cerein was completely eliminated by trypsin, chymotrypsin, and protease K. Inhibitory activity of cerein disappeared only after 15 min of incubation at 90°C. SDS-PAGE of the partially purified cerein showed that the bactericidal activity of cerein is associated with a band having an apparent molecular mass of about 9 kDa. When compared to these properties of cerein, cerein BS229 appears to differ from cerein, since it is active against all *Bacillus* strains tested, two *E. coli* strains, *P. thoenii* P127, and *M. flavus* ATCC 10240. In fact, the activity was eliminated by α-chymotrypsin or proteinase K; the inhibitory activity was detected during treatments of up to 100°C for 30 min; and it is made up of about 8.2 kDa of the molecular mass.

Acknowledgments

The authors wish to acknowledge the financial support of the Korea Research Foundation for the program year of 1997. N.-K. Lee and K.-H. Lee held a Brain Korea 21 fellowship from the Korean Ministry of Education. We also thank Dr. S.-H. Park with KRIBB for providing *Bacillus* strains in his culture collections.

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