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Identification and partial characterization of tochicin, a bacteriocin produced by *Bacillus thuringiensis* subsp *tochigiensis*

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***Bacillus thuringiensis* subsp *tochigiensis* HD868 was identified as a bacteriocin producer which exhibited a bactericidal effect against closely related species. This bacteriocin designated as tochicin, was partially purified by 75% ammonium sulfate precipitation followed by subsequent dialysis. This partially purified tochicin showed a narrow antibacterial spectrum of activity against most of 20 typical *B. thuringiensis* strains and a strain of *B. cereus*, but not against other bacteria and yeasts tested. The antibacterial activity of tochicin on sensitive indicator cells disappeared completely by proteinase K treatment (1 mg ml^{-1}), which indicates its proteinaceous nature. Tochicin was very stable throughout the range of pH 3.0–9.0 and was relatively heat-stable at 90°C, but bacteriocin activity was not detected after boiling for 30 min. The relationship between cell growth and bacteriocin production was studied in a semi-defined medium. Tochicin activity was detected at the mid-log growth phase, reached the maximum at the early stationary phase, but decreased after the stationary phase. Direct detection of tochicin activity on sodium dodecyl sulfate-polyacrylamide gel suggested it has an apparent molecular mass of about 10.5 kDa. Tochicin exhibited a bactericidal activity against *B. thuringiensis* subsp *thompsoni* HD522 in phosphate buffer (pH 7.0).**

Keywords: bacteriocin; *Bacillus thuringiensis*; tochicin

Introduction

Bacteriocins are proteins produced by a heterogeneous group of bacteria that have a bactericidal effect on closely related organisms [14]. Because bacteriocins are natural products of many microorganisms associated with foods, there is currently an enhanced interest in their use as natural food preservatives.

The genus *Bacillus* includes a variety of industrially important species and has a history of safe use in both food and industry. The products in commerce today that are produced by *Bacillus* fermentations include enzymes, antibiotics, amino acids, and insecticides. Many bacteriocins in the genus *Bacillus* have been reported; the best characterized are subtilin [2,5,7–9,12] of *B. subtilis* and megacin [15,16] of *B. megaterium*.

Bacillus thuringiensis is an insect pathogen producing a proteinaceous crystalline toxin (δ-endotoxin) which causes paralysis of the larval gut and a thermostable β-exotoxin capable of killing flies and other insects [1]. Although certain species of *B. thuringiensis* have very high DNA homology and taxonomic identity with strains of *B. cereus*, which can produce toxins causing food poisoning and human and animal infections, *B. thuringiensis* has been used as a safe bioinsecticide for more than 50 years. However, the bacteriocin research associated with *B. thuringiensis* is remarkably scarce in comparison with the industrial importance of this species. The only bacteriocin characterized from *B. thuringiensis* is thuricin [4]. Krieg [10] described the antagonistic factor produced from *B. thuringiensis* strains as bacteriocin based on the reduction of activity by trypsin treatment. The characterization of thuricin, a bacteriocin produced by *B. thuringiensis* HD2 was performed by Favret and Yousten [4].

The exploitation of bacteriocin in agriculture will be focused in the near future on the retardation of spoilage by plant pathogens and for grain preservation [6]. As *B. thuringiensis*-based bioinsecticides are sprayed with its spores, any additional activities such as bacteriocin would make this bacterium more attractive in agriculture. In this study, we report the identification and partial characterization of tochicin produced from *B. thuringiensis* subsp *tochigiensis* HD868.

Materials and methods

Bacterial strains and media

All *Bacillus thuringiensis* strains were obtained from USDA (Brownsville, TX, USA) and BGSC (Bacillus Genetic Stock Center, Columbia, OH, USA). Producer strain *B. thuringiensis* subsp *tochigiensis* HD868 and indicator strain *B. thuringiensis* subsp *thompsoni* HD522 were maintained at –65°C in nutrient broth to which 20% (v/v) glycerol was added. Working cultures were propagated in tryptic soy broth (TSB) with shaking at 30°C. The strains used as indicator organisms were obtained from different culture collections and indicator strains were grown in appropriate medium as indicated in Tables 1 and 2.

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Table 1 Antagonism of *B. thuringiensis* subsp *tochigiensis* HD868 against various *B. thuringiensis* strains by agar spot assay

Indicator strain[a]	Inhibition zone diameter (mm)[b]
B. thuringiensis subsp *kurstaki* HD1	15
B. thuringiensis subsp *kurstaki* (Thuricide)	16
B. thuringiensis subsp *kurstaki* (Biobit)	15
B. thuringiensis subsp *kurstaki* HD73	15
B. thuringiensis subsp *thuringiensis* HD2	13
B. thuringiensis subsp *kenyae* HDB23	17
B. thuringiensis subsp *galleriae* HD29	9
B. thuringiensis subsp *entomocidus* HD9	14
B. thuringiensis subsp *aizawai* HD11	13
B. thuringiensis subsp *aizawai/pacificus* HD137	15
B. thuringiensis subsp *morrisoni* HD12	17
B. thuringiensis subsp *sandiego*	17
B. thuringiensis subsp *tolworthi* HD537	16
B. thuringiensis subsp *darmstadiensis* HD146	19
B. thuringiensis subsp *thomsoni* HD522	19
B. thuringiensis subsp *israelensis* HD567	17
B. thuringiensis subsp *tochigiensis* HD868	–[c]
B. thuringiensis subsp *colmeri* IS 720	17
B. thuringiensis subsp *moritai*	13
B. thuringiensis subsp *canadensis* HD30	12

[a]All strains were grown in tryptic soy broth at 30°C.
[b]These data are the average of triplicate trials.
[c]Not inhibited.

Detection of antimicrobial activity

Twenty typical strains of *B. thuringiensis* were examined for bacteriocin activity on tryptic soy agar plate by agar spot assay. After inoculating the selected producer with a toothpick, *B. thuringiensis* strains were grown on tryptic soy agar at 30°C for 12 h. Five milliliters of soft tryptic soy agar (0.7% agar), containing about 10^7 cells of the indicator strain per overlay, were overlaid on 1.5% tryptic soy agar plates and after 12 h of incubation at 30°C, an inhibition halo was clearly visible.

Bacteriocin assay

Tochicin activity was assayed by the modified well diffusion method as described by Paik and Glatz [13]. The basal layer of TSA contained 2.5% agar and was 5 mm deep. After pouring the agar layer, plates were incubated for 24 h at room temperature before wells were cut. After 7-mm diameter wells were cut, plates were incubated at 37°C for 2 h or at room temperature for 2 days to dry the plates and to facilitate sample diffusion into the agar. The indicator strain was *B. thuringiensis* HD522, which was added to 5-ml soft agar (0.7% agar) overlays of TSB medium at about 10^7 cells per overlay. Serially diluted samples (200 μl) were added to 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 12 h at 30°C before diameters of zones of inhibition were measured. The minimum detectable zone diameter was 9 mm (1 mm beyond well diameter). Activity units (AU) per ml of culture were calculated from the reciprocal of the highest dilution that produced a detectable zone of inhibition. If the inhibition zone at this dilution was large ($>$ 11 mm diameter), additional incremental dilutions were assayed to define the titer more precisely. All assays were performed in duplicate, and results presented are the means of duplicate trials.

Table 2 Inhibitory spectrum of activity of partially purified tochicin

Indicator	Growth medium[a]	Temp. (°C)	Inhibition zone diameter (mm)[b]
Gram-positive bacteria			
B. thuringiensis subsp *kurstaki* HD1	TSB	30	15
B. thuringiensis subsp *thuringiensis* HD2	TSB	30	13
B. thuringiensis subsp *aizawai* HD11	TSB	30	13
B. thuringiensis subsp *thompsoni* HD522	TSB	30	19
B. thuringiensis subsp *israelensis* HD567	TSB	30	17
B. subtilis ATCC 9372	NB	30	–[c]
B. subtilis ATCC 14593	NB	30	–
B. subtilis ATCC 6051a	NB	30	–
B. megaterium IAM 1245	NB	30	–
B. sphaericus KCTC 1184	NB	30	–
B. licheniformis ATCC 14580	NB	37	–
B. cereus KCTC 1013	NB	30	–
B. cereus KCTC 1014	NB	30	18
B. coagulans KCTC 1823	NB	45	–
B. circulans KCTC 1662	NB	30	–
B. macerans KCTC 1822	NB	30	–
B. polymyxa KCTC 1099	NB	30	–
Lactococcus brevis subsp *brevis* KCTC 3498	MRS	30	–
Lac. confusus KCTC 3499	MRS	30	–
Leuconostoc mesenteroides subsp *mesenteroides* KCTC 3505	MRS	26	+/–[d]
Staphylococcus aureus KCTC 1928	NB	37	–
Micrococcus luteus ATCC 4698	NB	30	–
Gram-negative bacteria			
Escherichia coli ATCC 9492	NB	37	–
Escherichia coli KCTC 2191	NB	37	–
Agrobacterium tumefaciens KCTC 2429	NB	30	–
Erwinia herbicola (an isolate)	NB	30	–
Pseudomonas syringae (an isolate)	NB	30	–
Pseudomonas syringae KCTC 2440	NB	30	–
Xanthomonas campestris (an isolate)	NB	26	–
Yeast			
Saccharomyces uvarum IAM 4727	YM	24	–
Saccharomyces cerevisiae IFO 2346	YM	24	–
Candida albicans ATCC 10231	YM	24	–

[a]TSB, tryptic soy broth; NB, nutrient broth; MRS, lactobacilli MRS; YM, yeast malt.
[b]These data are the average of triplicate trials.
[c]Not inhibited.
[d]Zone of inhibition was hazy, not clear.

Production of tochicin

B. thuringiensis HD868 was inoculated (1%, v/v) into 250 ml of sterile TSB and cultured in a shaking incubator at 30°C for 10 h. For production studies, samples were aseptically removed over a 60-h period to determine the absorbance measurement (A_{660}), pH, and bacteriocin activity at different time intervals.

Partial purification of tochicin

Partially purified tochicin was obtained as follows. Solid ammonium sulfate was slowly added to the culture supernatant (450 ml) to 75% saturation at 4°C, with constant stirring, over about 5 h. Slow stirring was continued for an additional 30 min at 4°C. Precipitated proteins were pelleted by centrifugation at $10\,800 \times g$ for 30 min at 4°C, resuspended in 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, 3500; Spectrum Medical Industries, Los Angeles, CA, USA). The dialyzed samples were stored at –65°C.

Inhibition spectrum of activity

The modified well diffusion method was used to assess the antimicrobial activity of partially purified tochicin towards several Gram-positive and Gram-negative bacteria, and yeasts (Table 2). 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.

Sensitivity to enzymes, heat, and pH

For enzyme stability, partially purified tochicin was treated for 1 h with various enzymes at a final concentration of 1 mg ml^{-1}. All enzymes (trypsin, chymotrypsin, proteinase K, α-amylase, lipase II, lipase VII, DNase, RNase, lysozyme, and catalase) were dissolved in buffers as recommended by the supplier (Sigma, St Louis, MO, USA). Untreated bacteriocin plus buffers, buffers alone, and enzyme solutions served as control.

To determine the effect of heat on bacteriocin activity, aliquots (500 μl) of partially purified tochicin were incubated at various temperatures (40, 50, 60, 70, 80, 90, and 100°C) for 30 min or 121°C for 15 min.

The pH stability was estimated in the partially purified tochicin 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; 50 mM Tris-HCl buffer, pH 8–9. The residual bacteriocin activity was determined by the modified well diffusion method. All data are the average of duplicate trials.

Mode of inhibition

Cells from the log-phase of *B. thuringiensis* HD522 were suspended in sterile 10 mM phosphate buffer (pH 7.0). The test was carried out at 30°C by the addition of 200 AU ml^{-1} of partially purified tochicin. At various times, the viable cells (CFU ml^{-1}) were determined on tryptic soy agar plates by the standard plate counting method.

SDS-PAGE

To estimate the molecular weight of partially purified tochicin, sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed on 12% 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, 6200; α and β insulin, 3000 (all from Bethesda Research Laboratories, Gaithersburg, MD, USA). The sample was prepared by mixing a 4:1 ratio of the tochicin sample and buffer and boiling the mixture at 100°C for 3 min. Electrophoresis was performed in a vertical slab gel apparatus (Protean Cell II; Bio-Rad), with the buffer system at constant voltage (100 V) for 2 h. Half of the gel was stained with a silver stain kit (Bio-Rad) as directed by the manufacturer, while the other half was assayed for bacteriocin activity by the direct method described by Daba *et al* [3]. This part of the gel was fixed for 1 h in 20% 2-propanol and 10% acetic acid and soaked for at least 30 min in sterile deionized water. It was then aseptically placed in a sterile petri dish containing 15 ml of TSA and covered with 5 ml of soft agar containing 10^7 cells of *B. thuringiensis* HD522 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 activity of B. thuringiensis strains

The 20 typical *B. thuringiensis* strains were screened for their capacity to produce bacteriocin-like activity against 20 *B. thuringiensis* strains by agar spot assay. *B. thuringiensis* strains tested in this study demonstrated antagonistic activity among nearly all of the strains (data not shown). From these data, *B. thuringiensis* subsp *tochigiensis* HD868 was selected as a good candidate for bacteriocin production. Antagonism of *B. thuringiensis* HD868 against 20 typical *B. thuringiensis* strains is shown in Table 1. All of the *B. thuringiensis* strains used as the indicator strain were sensitive to antimicrobial substances produced by *B. thuringiensis* HD868, except for the producer. Upon the dilution of tochicin, 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.

Production of tochicin

In TSB medium incubated at 30°C, *B. thuringiensis* HD868 produced extracellular inhibitory activity against *B. thuringiensis* HD522 (Figure 1). Tochicin activity was detected

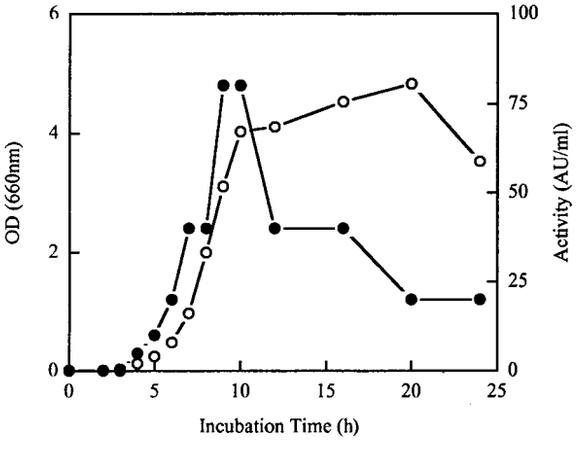

Figure 1 Production of tochicin (●) during the growth (○) of *B. thuringiensis* subsp *tochigiensis* HD868 in TSB medium at 30°C.

at the mid-log growth phase and reached its maximum at the early stationary phase, and decreased after the late stationary phase; this decrease in activity could be associated with the induction of sporulation, as shown in cerein, a bacteriocin produced by *B. cereus* GN105 [11].

Inhibition spectrum of activity

For the antimicrobial spectrum of activity, the partially purified tochicin was tested against various Gram-positive, Gram-negative bacteria and some yeasts by the modified well diffusion method. Tables 1 and 2 indicate that tochicin showed a narrow spectrum of activity against all *B. thuringiensis* strains tested and *B. cereus* KCTC 1014. The other *Bacillus* strains such as *B. subtilis*, *B. megaterium*, and *B. sphaericus* were not inhibited. No inhibition was observed against lactic acid bacteria, *Escherichia coli*, Gram-negative plant pathogens, and yeasts tested in this study (Table 2).

Effects of various enzymes, heat, and pH

The effect of various enzymes, heat, and pH on the partially purified tochicin was investigated (data not shown). All the inhibitory substances were completely inactivated by treatment with proteinase K, thus suggesting the proteinaceous nature of tochicin. No modification of activity was observed when tochicin was treated with other enzymes tested (trypsin, chymotrypsin, α-amylase, lipase II, lipase VII, DNase, RNase, lysozyme, and catalase). Buffers and enzyme solutions alone had no effect on the indicator strain. The activity of thuricin, another bacteriocin produced by *B. thuringiensis* HD2, was drastically reduced by treatment with chymotrypsin, pronase, subtilisin, trypsin, but not by treatment with lysozyme, phospholipase C, papain, and peptidase [4]. Tochicin was relatively stable under heat treatments; the inhibitory activities were maintained during treatment up to 90°C for 30 min, but the inhibitory activities in the partially purified tochicin were inactivated by heat at 100°C for 30 min or at 121°C for 15 min. Inhibitory activity of thuricin was stable at pH 7.0 during heat treatment at 100°C for 30 min [4]. Finally, partially purified bacteriocin was also pH stable in the range of 3.0–9.0.

Mode of inhibition

To determine whether tochicin had a bactericidal or a bacteriostatic effect, two different concentrations of the partially purified tochicin were added to the indicator cells suspended in phosphate buffer (pH 7.0). Tochicin showed a bactericidal mode of action. A decrease in CFU per milliliter was observed after the exposure of the indicator cells to two different concentrations (100 and 200 AU ml^{-1}) of the bacteriocin (Figure 2).

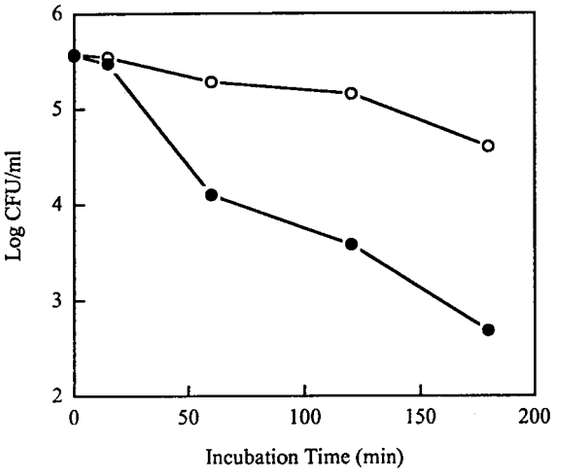

Figure 2 Inhibition of *B. thuringiensis* subsp *thompsoni* HD522 cells with tochicin. ❍, 100 AU ml^{-1}, ●, 200 AU ml^{-1}.

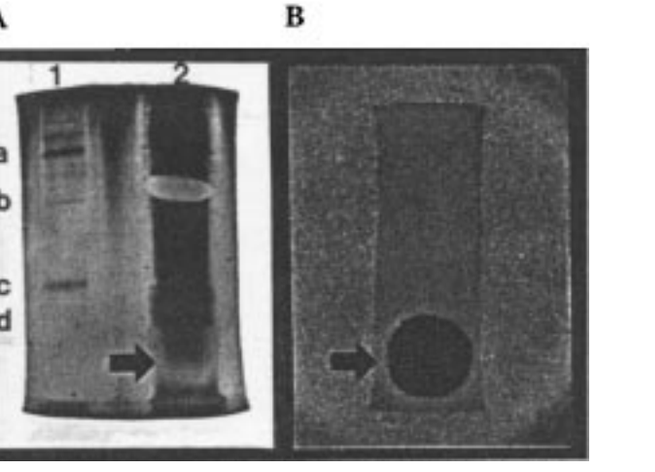

Figure 3 SDS-PAGE of tochicin and detection of antimicrobial activity. (A) Coomassie Blue R-250 stained gel: Lane 1, molecular weight standard (a, ovalbumin, 43000; b, carbonic anhydrase, 29000; c, β-lactoglobulin, 18400; d, lysozyme, 14300); lane 2, partially purified tochicin. (B) Portion of the gel overlaid with the indicator strain as described in Materials and Methods. The arrow indicates the inhibition halo observed after overnight incubation at 30°C.

SDS-PAGE

Several contaminating proteins were detected in the partially purified tochicin (Figure 3). The 12% polyacrylamide gel, containing partially purified tochicin, was cut into two vertical parts. The part of the gel containing the sample and 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 Daba *et al* [3]. As shown in Figure 3, the bactericidal activity of tochicin is associated with a band having an apparent molecular mass of about 10.5 kDa. Thus, the apparent molecular mass of tochicin was estimated to be about 10.5 kDa by direct detection of bactericidal activity after SDS-PAGE.

Conclusion

B. thuringiensis strains produce antimicrobials such as bacteriocins, but to date only one bacteriocin (thuricin) has been characterized [4]. Thuricin was active against several *Bacillus* species (*B. thuringiensis*, *B. megaterium*, *B. polymyxa*, and *B. sphaericus*), but was not active against *B. licheniformis* and *B. macerans* strain. The activity of partially purified thuricin was eliminated or diminished by subtilisin, chymotrypsin, pronase, and trypsin. SDS-PAGE of

the partially purified thuricin showed five major bands ranging from Mw of less than 45 000 to Mw of greater than 200 000. When compared to these properties of thuricin, tochicin seems to differ from thuricin in that it is active exclusively against all *B. thuringiensis* strains tested and a *B. cereus* strain; the activity was eliminated only by proteinase K, and it has about 10.5 kDa of molecular mass.

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