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Weissellicin 110, a Newly Discovered Bacteriocin from *Weissella cibaria* 110, Isolated from Plaa-Som, a Fermented Fish Product from Thailand▽

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***Weissella cibaria* 110, isolated from the Thai fermented fish product plaa-som, was found to produce a bacteriocin active against some gram-positive bacteria. Bacteriocin activity was not eliminated by exposure to high temperatures or catalase but was destroyed by exposure to the proteolytic enzymes proteinase K and trypsin. The bacteriocin from *W. cibaria* 110 was purified, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the purified bacteriocin contained one protein band that was approximately 2.5 kDa in size. Mass spectrometry analysis showed the mass of the peptide to be approximately 3,487.8 Da. N-terminal amino acid sequence analysis was performed, and 27 amino acids were identified. Because it has no similarity to other known bacteriocins, this bacteriocin was defined as a new bacteriocin and termed weissellicin 110.**

Lactic acid bacteria (LAB) have long played important roles in food technology. The LAB include a wide variety of cell types with various physiological and biochemical characteristics. The isolation of LAB from milk products, fermented foods, and plants has frequently been reported. The phylogeny of the bacteria classified currently in the genus *Weissella* was clarified in 1990 (16), and the taxonomy of *Weissella* species was further assessed in 1993 (5). *Weissella* species have been isolated from a variety of sources, and some of them play important roles in fermentation (1). *Weissella cibaria* was first described by Björkroth et al. (1) and later found in various kinds of fermented foods (6, 19).

Bacteriocins are peptides produced by bacteria that kill or inhibit the growth of closely related bacteria. Bacteriocins produced by LAB have attracted special interest as potential safe, alternative food preservatives (4, 8, 15). Many bacteriocins associated with *Lactobacillus*, *Enterococcus*, and *Leuconostoc* species have been described previously (4). However, bacteriocins from *Weissella* species remain rare, and to our knowledge, no bacteriocins from *W. cibaria* (1, 9) have been reported previously.

W. cibaria 110 (AB261010/DDBJ; DNA Data Bank of Japan [http://www.ddbj.nig.ac.jp/]) isolated from plaa-som (17), a fermented fish product from Thailand, was found to produce a bacteriocin active against some gram-positive bacteria. The present paper describes the purification and analysis of this bacteriocin and discusses its similarities to other known peptides. This is the first study to clarify the characteristics of a *W. cibaria* bacteriocin.

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MATERIALS AND METHODS

***W. cibaria* 110.** Ten strains of *W. cibaria* were isolated from plaa-som samples collected from Bangkok, Thailand, and their activities against the indicator strain *Lactobacillus sakei* JCM 1157^T were determined. Only strain 110 showed activity; this strain was identified using the API 50CHL kit, and the identification was confirmed using 16S rRNA sequence analysis. *W. cibaria* 110 was therefore used as the bacteriocin-producing strain in this study. Inhibitory activity was determined using the agar well diffusion assay described by Yanagida et al. (24).

Other bacterial strains and their culture conditions. The culture conditions for strains used for the determination of the antibacterial spectrum are listed in Table 1.

Optimum temperature for growth and bacteriocin production. To study the optimum temperature for growth and bacteriocin production, 100 μl of the overnight bacterial culture was inoculated into 5 ml of lactobacillus Man-Rogosa-Sharpe (MRS) broth (Difco, Sparks, MD) and then incubated at 15, 20, 25, 30, 37, or 45°C for 14 h. After incubation, the optical density at 660 nm was determined and bacteriocin activity was checked by determining the size of the zone of inhibition around the well (8 mm in diameter) in the agar well diffusion assay.

Effects of enzymes and heat on bacteriocin activity. To evaluate heat stability, samples of neutralized supernatant fluid from the *W. cibaria* 110 culture were incubated at 80°C for 30 min, 90°C for 30 min, 100°C for 30 min, 110°C for 15 min, or 121°C for 15 min. To analyze sensitivity to various enzymes, neutralized supernatant fluid was treated with proteinase K (Merck, Darmstadt, Germany), trypsin (Wako, Osaka, Japan), or catalase (Wako, Osaka, Japan) at 30 IU mg^{-1} and 37°C for 5 h. After the treatments described above, bacteriocin activity was determined using the agar well diffusion assay.

Production of the bacteriocin by *W. cibaria* 110. To study bacteriocin production, 5 ml of the overnight bacterial culture was inoculated into 1 liter of MRS medium. At specific time intervals, 1-ml samples were removed and the optical density at 660 nm of the culture and the arbitrary activity units (AU) ml^{-1} (reciprocal of the highest dilution at which activity was still obtained) of the bacteriocin were determined according to the method of Henderson et al. (13). *L. sakei* JCM 1157^T was used as the indicator strain. The incubation temperature was set based on the results obtained from the optimum growth temperature analysis.

Purification of the bacteriocin. Cell-free culture supernatant (2.5 liters) was prepared and then purified by ammonium sulfate precipitation (40%) and column chromatography with a hydrophobic column of phenyl-650M TOYOPEARL (Tosoh, Tokyo, Japan) and Sep-Pak C_{18} cartridges (Waters, Milford, MA) (24). Eluted bacteriocin fractions from Sep-Pak C_{18} cartridges were freeze-dried and then stored at 4°C.

Molecular size approximation. The molecular size of the purified bacteriocin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by following the method described by Yanagida et al. (24). Bac-

TABLE 1. Inhibition spectrum of the bacteriocin produced by *W. cibaria* 110

Indicator strain	Medium	Incubation temp (°C)	Diam (mm) of zone of inhibition[a]
L. sakei JCM 1157^T	MRS	30	13
L. sanfranciscensis JCM 5668^T	MRS	30	10
L. homohiochii JCM 1199^T	MRS	30	10
L. coryniformis subsp. *coryniformis* JCM 1164^T	MRS	30	11
L. brevis JCM 1059^T	MRS	30	–
L. brevis JCM 1170	MRS	30	–
L. acetotolerans JCM 3825^T	MRS	30	12
L. paracasei subsp. *paracasei* JCM 1181	MRS	30	–
L. plantarum subsp. *plantarum* JCM 1551	MRS	30	–
L. jensenii JCM 1146^T	MRS	37	–
L. delbrueckii subsp. *bulgaricus* JCM 1002^T	MRS	37	–
L. gasseri JCM 1131^T	MRS	37	–
L. vitulinus JCM 1143^T	MRS	37	–
L. acidophilus JCM 1132^T	MRS	37	–
Enterococcus faecalis JCM 5803^T	MRS	37	–
Carnobacterium divergens JCM 5816^T	MRS	25	–
Streptococcus salivarius JCM 5707^T	MRS	37	–
W. kandleri JCM 5817^T	MRS	30	10
W. confusa JCM 1093^T	MRS	30	–
W. halotolerans JCM 1114^T	MRS	37	10
W. minor JCM 1168^T	MRS	30	–
W. paramesenteroides JCM 9890^T	MRS	30	13
Leuconostoc mesenteroides subsp. *mesenteroides* JCM 6124^T	MRS	30	–
Leuconostoc lactis JCM 6123^T	MRS	30	11
Listeria monocytogenes NCIMB 13726	PBN[b]	30	–
Escherichia coli JCM 1649^T	PBN	37	–
Bacillus cereus JCM 2152^T	PBN	37	–
Staphylococcus aureus JCM 2151	PBN	37	–

[a] Wells (8 mm in diameter) were filled with 100 μl of supernatant from the *W. cibaria* culture. –, no inhibitory zone observed.
[b] PBN broth (pH 7.3) included the following components: 0.5% peptone, 0.3% beef extract, and 0.8% NaCl.

TABLE 2. Effects of various factors on bacteriocin produced by *W. cibaria* 110

Factor	OD_{660}[a] of *W. cibaria* culture	Diam (mm) of zone of inhibition[b]
Growth temp (°C)		
15	0.125	–
20	1.364	–
25	1.419	12
30	1.502	13
37	1.394	11
45	0.169	–
Treatment with enzyme		
Control		13
Proteinase K		–
Trypsin		–
Catalase		12
Heat		
Control		13
30 min at 80°C		13
30 min at 90°C		13
30 min at 100°C		13
15 min at 110°C		13
15 min at 121°C		13

[a] OD_{660}, optical density at 660 nm.
[b] Wells (8 mm in diameter) were filled with 100 μl of supernatant from the *W. cibaria* 110 culture. *L. sakei* JCM 1157^T was used as the indicator strain. –, no inhibitory zone observed.

teriocin size was estimated using rainbow-colored protein molecular mass markers (Amersham Biosciences, Piscataway, NJ).

Mass spectrometry. The molecular mass of the purified bacteriocin was determined by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) using a mass spectrometer (Microflex; Bruker, Bremen, Germany) (14).

The molecular mass of the purified bacteriocin was also determined by liquid chromatography with an ion trap mass spectrometer (LC/MSD Trap XCT; Agilent, CA).

N-terminal amino acid sequence analyses. The activity of the purified bacteriocin was confirmed on the SDS-PAGE gel, and the gel was then blotted onto polyvinylidene difluoride membranes and stained with CBB R-250 (Wako, Osaka, Japan). The objective bands were cut out and analyzed, and the N-terminal amino acid sequence was determined by Edman degradation on a protein sequencer (model 491; Applied Biosystems, Foster City, CA).

RESULTS

Maximum cell numbers and activity against the indicator strain *L. sakei* JCM 1157^T were observed at 30°C (Table 2). The highest bacteriocin titers (5,120 AU ml^{-1}) were obtained after 10 h of incubation at 30°C, and the highest cell density, based on the optical density at 660 nm, was observed after 10 to12 h of incubation (Fig. 1).

The effects of enzymes and heat on the inhibitory agent from *W. cibaria* 110 are shown in Table 2. The bacteriocin was inactivated by proteinase K or trypsin but not affected by treatment with catalase. The bacteriocin was considered to be heat stable, as activity remained after heating at 121°C for 15 min.

The neutralized supernatant from *W. cibaria* 110 showed activity against some gram-positive bacteria, as listed in Table 1, but had no activity against *Listeria monocytogenes*.

The molecular mass of the purified bacteriocin was approximately 2.5 kDa, according to SDS-PAGE (Fig. 2).

The purified bacteriocin was analyzed by MALDI-TOF MS,

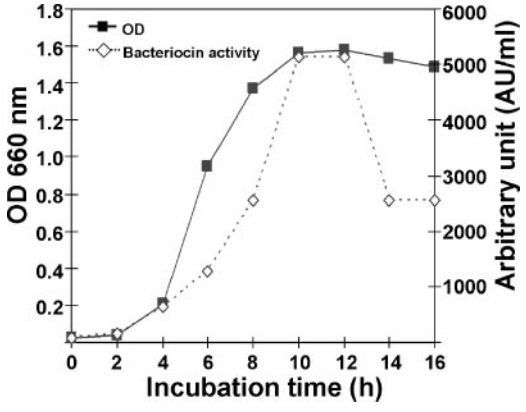

FIG. 1. Production of bacteriocin during the growth of *W. cibaria* 110. OD 660 nm, optical density at 660 nm.

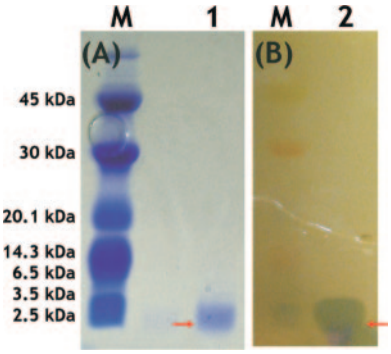

FIG. 2. SDS-PAGE analysis of purified bacteriocin from *W. cibaria* 110. (A) CBB-stained gel. (B) Gel placed onto MRS agar surface overlaid with *L. sakei* JCM 1157^T. Lanes M, low-molecular-mass standards; lanes 1 and 2, purified bacteriocin from *W. cibaria* 110.

and the result revealed a major peak at 3,487.86 Da (Fig. 3). The same sample subjected to liquid chromatography electrospray ionization MS revealed one peak with an identified molecular mass of 3,490.8 Da; this result coincided with the expected molecular mass. N-terminal amino acid analysis of this band revealed the following partial sequence: NH_2-SDKNNV FFQIGKRYVAPVLYXFGKXAE, where X represents unidentified amino acids. MALDI-TOF MS analysis of the protein after tryptic digestion confirmed the identities of amino acids 1 to 12 and 14 to 24 and revealed that the 21st amino acid was tryptophan (W) and the 25th amino acid was glycine (G) (data not shown). No corresponding protein sequence was found in the database (DNA Data Bank of Japan [http://www.ddbj.nig.ac.jp/]) or NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Based on the results described above, we strongly suggest that *W. cibaria* 110 produces a novel, natural bacteriocin and have termed this bacteriocin weissellicin 110.

DISCUSSION

Bacteriocins produced by LAB such as *Lactobacillus* (12, 25), *Enterococcus* (2, 3, 11, 24, 25), *Leuconostoc* (7, 10), *Streptococcus* (22), and *Carnobacterium* (20, 23) species have been frequently reported in previous studies. However, studies of bacteriocins from *Weissella* sp. remain scarce (18). To our knowledge, this is the first study of a bacteriocin from *W. cibaria*.

The optimum growth temperature always has an influence on the production of bacteriocins (7, 21). The results of our investigations of growth temperature and bacteriocin production indicated that *W. cibaria* 110 had higher bacteriocin production when incubated at 30°C for 10 to 12 h than when incubated at other temperatures (Fig. 1). The sensitivity of the substance to proteinase K and trypsin is proof of its proteinaceous nature. In addition, no effect was observed after treatment with catalase; this finding provided evidence that the active agent did not originate from H_2O_2. The same treatments were later performed with the purified bacteriocin, and the results were confirmed (data not shown).

Weissellicin 110 showed a narrow spectrum of inhibition of other LAB. Unlike most class II bacteriocins produced by LAB, weissellicin 110 had no activity against *Listeria monocy-*

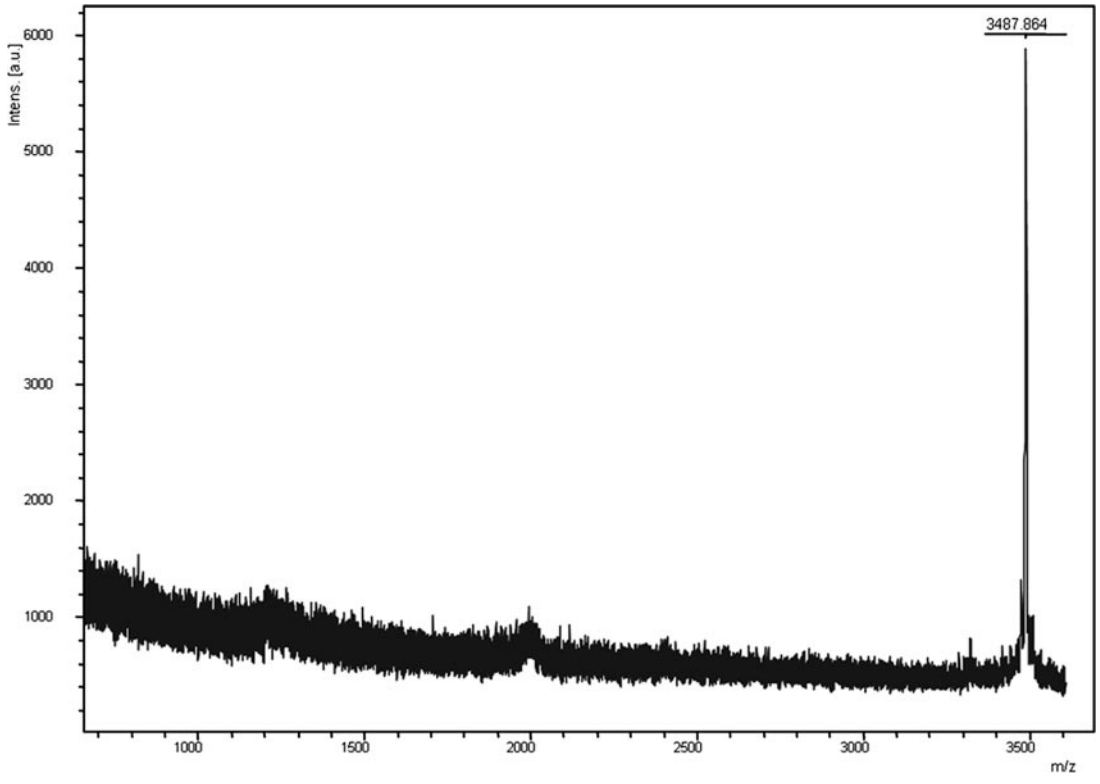

FIG. 3. MALDI MS-determined mass spectrum of weissellicin 110 (mass, 3,487.86 Da).

togenes. This characteristic may limit its potential application in fermented foods.

The molecular mass of the 27-amino-acid peptide was calculated using the tool Compute pI/M_w from the ExPASy proteomics server (http://ca.expasy.org), and a result of 3,205.71 Da was obtained. However, results from MALDI-TOF MS and liquid chromatography electrospray ionization MS indicated that the true molecular mass of the bacteriocin was approximately 3,488 Da (Fig. 3). We therefore believe that two or three of the amino acids remain unknown in this study. To clarify this result, an advanced analysis such as PCR DNA sequencing analysis will be conducted in the future.

In conclusion, the evidence presented in this report indicates that *W. cibaria* 110 isolated from plaa-som produces a novel bacteriocin, which we have named weissellicin 110. Weissellicin 110 is stable after high-temperature treatment but has a narrow spectrum of inhibition of other LAB and does not inhibit *Listeria monocytogenes.* Future work in our laboratory will focus on the clarification of the full amino acid sequence of weissellicin 110 and the possibility of applying weissellicin 110 as a biopreservative.

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