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Purification and Characterization of Plantaricin 163, a Novel Bacteriocin Produced by *Lactobacillus plantarum* 163 Isolated from Traditional Chinese Fermented Vegetables

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ABSTRACT: Presumptive lactic acid bacteria (LAB) strains isolated from traditional Chinese fermented vegetables were screened for bacteriocin production. A novel bacteriocin-producing strain, *Lactobacillus plantarum* 163, was identified on the basis of its physiobiochemical characteristics and characterized by 16S rDNA sequencing. The novel bacteriocin, plantaricin 163, produced by *Lb. plantarum* 163 was purified by salt precipitation, gel filtration, and reverse-phase high-performance liquid chromatography (RP-HPLC). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of plantaricin 163 revealed the molecular weight to be 3553.2 Da. The complete amino acid sequence showed VFHAYSARGNYYGNCPANWPSCRNNYKSAGGK, and no similarity to known bacteriocins was found. Plantaricin 163 was highly thermostable (20 min, 121 °C), active in the presence of acidic pH (3—5), sensitive to protease, and exhibited broad-spectrum antimicrobial activity against LAB and other tested Gram-positive and Gram-negative bacteria. The results suggest that plantaricin 163 may be employed as a biopreservative in the food industry.

KEYWORDS: *Lactobacillus plantarum, bacteriocin, plantaricin, antimicrobial, biopreservative*

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that target other bacteria, either within the same species or across different genera.[1,2] It has been well established that lactic acid bacteria (LAB) have particularly high bacteriocin production.[3] LAB have been defined as "generally recognized as safe" (GRAS) microorganisms[4] and have been used for many centuries in the production of fermented foods and feeds.[5−7] Bacteriocins produced by LAB are capable of inhibiting the growth of specific bacterial species, such as *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus*, and thus represent potential food biopreservatives.[8] For example, nisin, a bacteriocin produced by *Lactococcus lactis*, has been used in several countries to extend the shelf life of food products.[9]

The bacteriocins produced by LAB have been classified into four groups according to the suggested classification scheme for bacteriocins:[10] class I, small, heat-stable lanthionine-containing peptides (<5 kDa); class II, small, heat-stable non-lanthionine-containing peptides (<10 kDa); class III, large, heat-labile proteins (>30 kDa); and class IV, complex proteins composed of one or more chemical moieties, either lipid or carbohydrate. Class I and class II bacteriocins are the most likely candidates for use as biopreservatives in the food industry to at least partially replace chemical preservatives.[11] Potential applications of bacteriocins from LAB in the food industry have attracted tremendous interest, which has led to the search for and identification of novel bacteriocins.

Abundant bacteriocin producers are known to exist in traditional Chinese fermented vegetables, and LAB have been considered to be the dominant microorganisms in fermented food.[12] Many bacteriocin producers have been successfully isolated from fermented vegetables, including *Pediococcus pentosaceus* 05-10,[13] *Lactobacillus sakei* LSJ618,[14] *Lb. sakei* C2,[11] *Pediococcus acidilactici*,[15] *Lactobacillus plantarum* UG1,[5] and *Lactobacillus acidophilus* NX2-6.[16] Although previous studies have identified many effective bacteriocin-producing strains valuable to the food industry, still several bacteriocin producers and a range of bacteriocins that have potential use as biopreservatives remain to be explored.

Therefore, in this study, a novel bacteriocin, plantaricin 163 produced by *Lb. plantarum* 163, which is present in traditional Chinese fermented vegetables, was purified and characterized. Furthermore, the antimicrobial efficacy of plantaricin 163 was evaluated.

MATERIALS AND METHODS

Samples, Media, Strains and Growth Conditions. Samples of traditional Chinese fermented vegetables (fermented cabbage, pickled radish) were collected from Guizhou province, People's Republic of China.

The LAB strains were cultured in MRS medium at 37 °C.[17] Potato dextrose agar was used for the culturing of fungi, whereas nutrient broth was employed for bacteria. The bacterial strains used in this study and the different growth conditions are listed in Table 1.

Screening of Bacteriocin Producers. *Purification of LAB.* Appropriate decimal dilutions of the samples were spread directly on the surface of MRS agar containing 0.3% calcium carbonate[18,19]

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Table 1. Antimicrobial Activity Profile of Plantaricin 163 Produced from the Isolated *Lb. plantarum* 163[a]

strain	source[b]	growth conditions	antimicrobial activity[c]
Staphylococcus ureus	ATCC 25923	nutrient broth/37 °C	+++
Listeria monocytogenes	ATCC 19114	nutrient broth/37 °C	++
Bacillus pumilus	CMCC 63202	nutrient broth/37 °C	++
Bacillus cereus	AS 1.1846	nutrient broth/37 °C	+++
Micrococcus luteus	CMCC 28001	nutrient broth/37 °C	+
Lactobacillus thermophilus	our lab	MRS medium/37 °C	+
Lactobacillus rhamnosus	our lab	MRS medium/37 °C	+
Escherichia coli	ATCC 25922	nutrient broth/37 °C	++
Pseudomonas aeruginosa	AS 1.2620	nutrient broth/37 °C	+
Pseudomonas fluorescens	AS 3.6452	nutrient broth/37 °C	++
Penicillium notatum	AS 3.4356	potato dextrose/30 °C	−
Aspergillus niger	AS 3.6459	potato dextrose/30 °C	−
Rhizopus stolonifer	AS 3.822	potato dextrose/30 °C	−

[a]The partially purified plantaricin 163 preparation from Sephadex LH 20 column chromatography, which was vacuum-evaporated with Christ RVC-2-25 CD plus and pH adjusted to 4.0 with buffer, was used to determine the antimicrobial test. [b]ATCC, American Type Culture Collection; CMCC, China Center of Medicine Culture Collection; AS, China General Microbiological Culture Collection Center. [c]−, no inhibition zone; diameter of inhibition zone +, 5.00—7.00 mm; ++, 7.00—10.00 mm; +++, >10.00 mm; well, 5 mm. Means of three replicate values are shown.

and then incubated at 37 °C for 48 h. Bacterial colonies that exhibited clear zones on MRS agar plates were randomly picked and streaked onto MRS agar plates containing 0.3% calcium carbonate for further purification. The purified colonies were primarily identified by Gram staining and catalase tests. Only Gram-positive, catalase-negative colonies were presumed to be LAB. They were stored in MRS containing 25% (v/v) glycerol at −70 °C.

Preliminary Screening for Bacteriocin Producers. The antimicrobial activity of the selected strains was determined by the agar-spot test with some modifications.[20−23] Briefly, the selected strains were spotted onto the surface of solid MRS agar plates and incubated at 37 °C for 48 h. Then, the spotted plates were overlaid with an appropriate volume of 1.5% nutrient agar seeded with 10^6 cfu/mL pathogenic bacteria (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922). After 20 h, the diameter of the inhibition zone formed by the LAB colonies was measured and scored. Inhibition zones >10 mm were scored positive.[19]

Rescreening of Bacteriocin Producers. Positive stains were subsequently activated in their respective MRS broth. Then, 1% (v/v) of the cultures was inoculated into 100 mL of MRS broth individually and again incubated at 37 °C for 24 h without agitation, after which the bacterial cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C. The pH of the cell-free supernatants and that of the MRS broth were adjusted to 5.5 using 3 N NaOH and lactic acid, respectively.[11] Then, catalase (Sigma, 2.5—5 KU/mL) was added, and the cell-free supernatants were incubated at 37 °C for 12 h to eliminate the effect of hydrogen peroxide, and the same cell-free supernatants without catalase were used as a control. The cell-free culture supernatants were examined for their antimicrobial activity by measuring the diameter of the inhibition zones using an agar well diffusion assay.[24]

Characterization and Identification of *Lb. plantarum* Strain 163. The *Lb. plantarum* strain 163 was first identified on the basis of its morphological, biochemical, and physiological characteristics, in addition to its carbohydrate fermentation profile.[25] Morphological and physiological identification was conducted based on Gram staining, shape, motility, spore formation, and catalase production. Biochemical identification was based on the production of CO_2 and acid from glucose and the ability to grow at different temperatures and under different salt concentrations.

Lb. plantarum strain 163 was finally characterized according to its 16S rDNA sequence analysis. The PCR primers were as follows: 16SF, 5′-AGAGTTTGATCCTGGCTCAG-3′; 16SR, 5′-CTACGGCTACCTTGTTACGA-3′.[26] PCR reactions were conducted using a DNA Engine Peltier Thermal Cycler (Bio-Rad, Mexico). Briefly, genomic DNA was amplified with initial denaturation at 95 °C for 4 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The PCR products were purified using a TakaRa Gel Extraction Kit (Takara, Dalian, China) and sequenced by Genscript Bio (Nanjing, China). Subsequently, these sequences were blasted against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Purification of Plantaricin 163. *Fermentation.* *Lb. plantarum* 163 was activated in 50 mL of MRS broth at 37 °C for 14 h without agitation, and then 1% (v/v) of the above culture was inoculated into 1000 mL of MRS broth and then incubated at 37 °C for 24 h without agitation.

Isolation. Bacterial cells were harvested by centrifugation at 6000*g* for 15 min at 4 °C. Then, the cell-free supernatant was concentrated to 500 mL in a rotary vacuum evaporator. Ammonium sulfate was added very slowly to the supernatant with stirring at 4 °C to 70% saturation. This mixture was then centrifuged at 10000*g* for 20 min at 4 °C to obtain an antimicrobial precipitate (by using *B. pumilus* CMCC 63202 as the indicator). Then, the precipitated fraction was suspended in 20 mL of distilled water.

After a Sephadex G 25 column (1.6 cm × 80 cm) was equilibrated with distilled water, 1 mL of dissolved sample was eluted at a flow rate of 0.3 mL/min with distilled water. The fractions were collected and tested for antimicrobial activity using *B. pumilus* CMCC 63202 as the indicator. Following repeated steps of manual collection with simultaneous monitoring, approximately 10 mL antimicrobial fractions were collected, freeze-dried, and resuspended in 1 mL of distilled water.

Similarly, following the equilibration of a Sephadex LH 20 column (1.6 cm × 120 cm) with 80% methanol, 1 mL of freeze-concentrated fractions was eluted at a flow rate of 0.2 mL/min with 80% methanol. The fractions were collected, vacuum-evaporated in a Christ RVC-2−25 CD plus, and monitored for antimicrobial activity using *B. pumilus* CMCC 63202.

The partially purified fractions containing antimicrobial activity were combined, and their absorption spectra were obtained by using a UV−visible spectrophotometer (UV-2450, Shimadzu Co., Japan). The wavelength displaying the highest absorbance was recorded.

Purification. The antimicrobial fractions from Sephadex LH 20 were further purified by reversed-phase high-performance liquid chromatography (RP-HPLC, UltiMate 3000) by a linear gradient elution using 95% water−acetonitrile (5—95%) containing 0.1% trifluoroacetic acid (TFA) at 277 nm. The eluted fractions purified were screened for antimicrobial activity using *B. pumilus* CMCC 63202. The purified substance with antimicrobial activity was vacuum-evaporated in a Christ RVC-2-25 CD plus for the determination of primary structure.

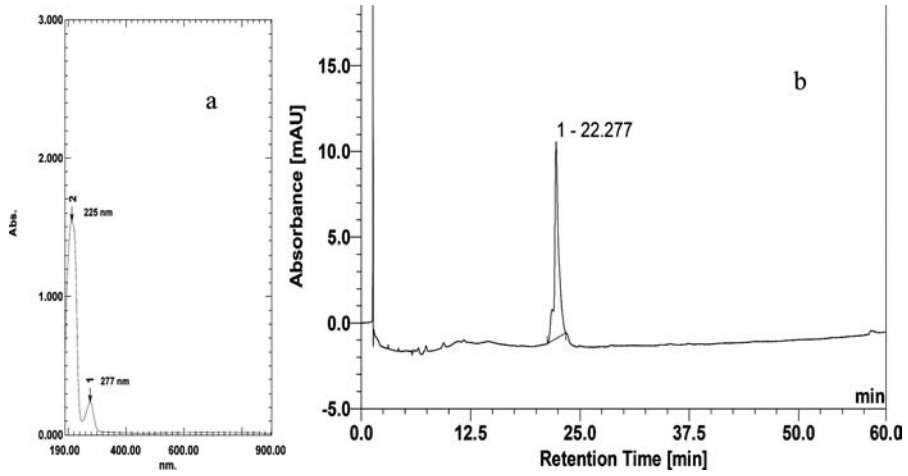

Figure 1. (a) UV−visible absorbance spectrum of the partially purified plantaricin 163 preparations; (b) plantaricin 163 peak with retention time of 22.277 s, as identified by RP-HPLC.

Determination of the Primary Structure of Plantaricin 163. The molecular weight of plantaricin 163 was determined by matrix-assisted laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics, Bremen, Germany) using α-cyano-4-hydroxycinnamic acid as the matrix.[27,28] The N-terminal amino acid sequence of plantaricin 163 was determined by Edman degradation using an ABI Procise 494 Protein Sequencer by GeneCore Bio Technologies (Shanghai, China). The amino acid composition of plantaricin 163 was determined using an amino acid analyzer (L-8900, Hitachi High-Technologies Corp., Tokyo, Japan).

Antimicrobial Spectrum of Plantaricin 163. The partially purified plantaricin 163 preparation from Sephadex LH 20 column chromatography was used to determine the antimicrobial spectrum against indicator organisms (Table 1). The diameter of the inhibition zones was measured by the agar well diffusion assay method.

Effects of Enzyme, Temperature, and pH on the Activity of Plantaricin 163. Plantaricin 163 preparations partially purified by Sephadex LH 20 chromatography were individually treated using the following enzymes (Sigma): trypsin (2.5 KU/mg), protease K (30 U/mg), α-chymotrypsin (1 KU/mg), and pepsin (3 KU/mg), at a final concentration of 5 mg/mL. Moreover, the partially purified plantaricin 163 preparations were adjusted to the optimum pH of the different enzymes and incubated with the enzymes at 37 °C for 3 h. The partially purified preparation, which pH was adjusted to an original pH (pH 4.0) and nonenzymatic treatment, was used as control. To determine the effect of temperature, the partially purified plantaricin 163 preparations from Sephadex LH 20 were individually treated in a thermostatic water bath at 60, 80, and 100 °C for 10, 20, and 30 min, respectively, and in an autoclave at 121 °C for 20 min. Samples without heat treatment (25 °C) were used as control. To determine the effect of pH, the pH values of the partially purified plantaricin 163 preparations were adjusted in a range from 2 to 10, and then the samples were kept at 37 °C for 3 h in a thermostatic water bath. The residual antimicrobial activity was measured after the enzymatic, temperature, and pH treatments by the agar diffusion assay method using *S. aureus* ATCC 25923 as the indicator.

RESULTS AND DISCUSSION

Screening of Bacteriocin Producers. A total of 430 presumptive Gram-positive and catalase-negative LAB strains were isolated from traditional Chinese fermented vegetables, of which 110 presumptive LAB strains exhibited antibacterial activity against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 in agar-spot test (data not shown). However, only the cell-free fermentation broth of five strains exhibited excellent antibacterial activity in the agar well diffusion assay method. Among them, the cell-free fermentation broth of LAB strain 163 (screening from pickled radish) displayed the highest antibacterial activity (data not shown).

Identification of *Lb. plantarum* Strain 163. *Lb. plantarum* strain 163 isolated from traditional Chinese fermented vegetables is characterized as a Gram-positive, nonflagellated, non-spore-forming, rod-shaped bacterium that does not exhibit catalase activity. It grew at 15 °C but not at 45 °C, and it was unable to grow in the presence of 6.5 and 18% NaCl, but grew in the presence of 3% NaCl. In addition, it was capable of producing acids but not CO_2 from glucose. The fermentation profile indicated that it fermented cellulose, esculin, fructose, galactose, glucose, gluconate, lactose, sorbitol, maltose, mannitol, mannose, melibiose, sucrose, raffinose, saligenin, trehalose, xylose, and ribose, but not arabinose and rhamnose.

The isolated strain was next identified and confirmed as *Lb. plantarum* (GenBank accession no. JX524228). We amplified a 1440 bp fragment from the strain's genomic DNA. A neighbor-joining phylogenetic tree was constructed by sequence alignment and comparison using MEGA software ver. 5.0. Analysis of the strain's 16S rRNA nucleotide sequence revealed 100% similarity with those of *Lb. plantarum* WCFS1 (NC_004567.2), *Lb. plantarum* ZJ316 (NC_020229.1), *Lb. plantarum* JDM1 (NC_012984.1), and *Lb. plantarum* JDM1 (NC_012984.1).

Several *Lb. plantarum* strains have been isolated from many fermented foods and vegetables, including *Lb. plantarum* KLDS1.0391,[29] *Lb. plantarum* LB-B1,[30] *Lb. plantarum* LPCO10,[31] *Lb. plantarum* C19,[32] and *Lb. plantarum* KLAB21,[33] which were isolated from traditional fermented cream, koumiss, fermented green olives, fermented cucumbers, and kimchi (a Korean fermented vegetable product), respectively. Similarly to several previous studies, in the present study, strain 163 was identified as *Lb. plantarum*, which has been isolated from fermented vegetables. Therefore, the findings suggest that *Lb. plantarum* isolated from fermented vegetables may have particularly high bacteriocin production.

LAB are GRAS organisms and are widely used as starter cultures for fermentation in food industries. For example, *Lb. plantarum* has been used as a starter culture for a fermented olive preparation.[34] It has been demonstrated that the strains of *Lb. plantarum* are ideal candidates for multifunctional starter culture development because of their functional properties.[35] In

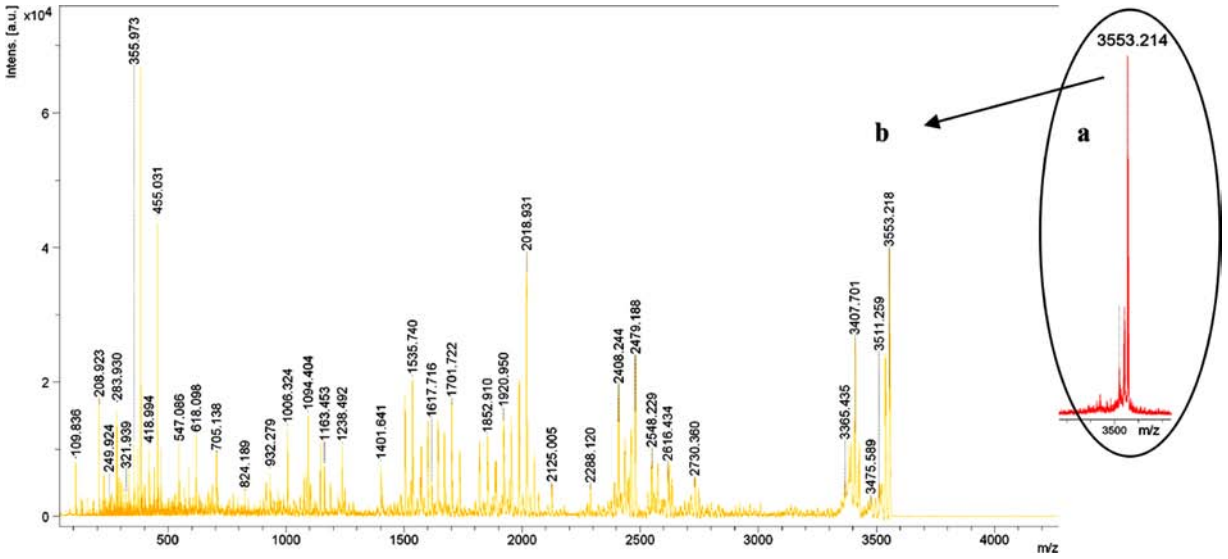

Figure 2. (a) MALDI-TOF-MS of plantaricin 163. The molecular ion peak is represented by a major peak at 3553.2 Da. (b) MALDI-TOF-MS/MS of a fragment of plantaricin 163.

the present study, we demonstrated a high lactic acid yield by *Lb. plantarum* 163 considering that the pH value of its fermented MRS broth was below 3.8 (data not shown). More importantly, we showed that *Lb. plantarum* 163 is capable of secreting bacteriocin, which highlights that *Lb. plantarum* 163 not only is useful as a starter culture in the traditional fermented food industry but also has potential value as a biopreservative in food industries.

Plantaricin 163 Purification. The partially purified plantaricin 163 preparations were obtained by column chromatography using *B. pumilus* CMCC 63202 as the indicator. Notably, the absorption spectra of these partially purified fractions revealed a maximum absorbance at 215 nm and a shoulder at 277 nm (Figure 1). Following the final purification by RP-HPLC, the chromatogram showed only one peak, where the retention time was 22.277 s (Figure 1).

Several classical strategies for the isolation and purification of bacteriocins from cultivation broths to final products were previously described. Biotechnological procedures including salting-out, gel filtration, ion-exchange, and RP-HPLC are among the most usual methods. Many studies have employed these techniques for the purification of piscicocin CS526,[36] enterocin P,[37] lacticin 481,[38] and plantaricin MG.[29] Similarly to previous studies, in the present study, the RP-HPLC technique was used for the purification of plantaricin 163.

Primary Structure of Plantaricin 163. The RP-HPLC chromatogram of the antibacterial fraction revealed a single peak with a retention time of 22.277 s. MALDI-TOF-MS analysis showed that plantaricin 163 has a molecular weight of 3553.2 Da (Figure 2). MALDI-TOF-MS/MS of a fragment of plantaricin 163 is shown in Figure 2. The 10 N-terminal amino acids from purified plantaricin 163 were determined to be VFH-AY-S-A-R-G-N. The amino acid composition was predicted to be V, F, H, A, Y, S, R, G, N, C, P, and K.

On the basis of the peptide fragmentation nomenclature proposed by Roepstorff and Fohlman,[39] the complete amino acid sequence of plantaricin 163 would be determined to be V-F-H-A-Y-S-A-R-G-N-Y-Y-G-N-C-P-A-N-W-P-S-C-R-N-N-Y-K-S-A-G-G-K. The peptide fragmentation of MS used in the deduction of the complete amino acid sequence of plantaricin 163 is shown in Table 2. The calculated MW of this sequence is 3553.9 Da, very close to the experimental value.

A wide range of plantaricins was successfully purified from *Lb. plantarum*, including plantaricin C19 (3.8 kDa) from *Lb. plantarum* C19,[40] plantaricin UG1 (3—10 kDa) produced by *Lb. plantarum* UG1,[5] plantaricin ST8SH (3 kDa) produced by *Lb. plantarum* ST8SH,[41] plantaricin MG (2180 Da) produced by *Lb. plantarum* KLDS1.0391,[29] plantaricin-149 (2.2 kDa) produced by *Lb. plantarum* NRIC 149,[42] and plantaricin ASM1 (5045.7 Da) produced by *Lb. plantarum* A-1.[43] To the best of our knowledge, the present study is the first to report the molecular weight of plantaricin to be 3553.2 Da. Furthermore, the sequence of plantaricin 163 deduced using protein BLAST (BLASTP) against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) showed no remote homology, thus suggesting that plantaricin 163 may be a novel bacteriocin.

Antimicrobial Spectrum of Plantaricin 163. The partially purified plantaricin 163 preparations partially purified by Sephadex LH 20 column chromatography exhibited a good antimicrobial spectrum (Table 1) and significantly inhibited Gram-positive bacteria such as *S. aureus*, *L. monocytogenes*, *B. pumilus*, *B. cereus*, *M. luteus*, *Lb. thermophilus*, and *Lb. rhamnosus* in addition to Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, and *Ps. fluorescens*. However, the partially purified peptide showed no antimicrobial activity against fungi such as *P. notatum*, *A. niger*, and *R. nigricans*.

Bacteriocins have been shown to possess a bactericidal mode of action centered against homologous species.[44] Nonetheless, a few bacteriocins have been found to have similar activity against foodborne pathogens. For example, the bacteriocin produced by *Pediococcus acidilactici* M was shown to inhibit a large number of bacteria, including *S. aureus*, *L. monocytogenes*, *Clostridium perfringens*, *B. coagulans*, *B. cereus*, and *A. hydrophila*.[45] Similarly, sakacin C2, a broad-spectrum bacteriocin produced by *Lb. sakei* C2 isolated from traditional Chinese fermented cabbage, inhibited many Gram-positive and Gram-negative bacteria.[11] Many plantaricins with a broad antimicrobial spectrum that inhibit not only closely related Lactobacilli but also different foodborne pathogens have also also identified. A previous study reported that plantaricin UG1 inhibits various

Table 2. Deduction of the Complete Amino Acid Sequence of Plantaricin 163

no.	m/z[a]	fragment	fracture type[b]	comment
1	208.9	$[His + Ala + H]^+$	b_4-b_2	
2	355.9	$[Phe + His + Ala + H]^+$	b_4-b_2	
3	618	$[Val + Phe + His + Ala + Tyr + H]^+$	b_5	
4	705	$[Val + Phe + His + Ala + Tyr + Ser + H]^+$	b_6	
5	776	$[Val + Phe + His + Ala + Tyr + Ser + Ala + H]^+$	b_7	
6	932	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + H]^+$	b_8	
7	989	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + Gly + H]^+$	b_9	
8	1103	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + Gly + Asn + H]^+$	b_{10}	with N-terminal sequencing results
9	220.9	$[Tyr + Gly + H]^+$	b_{13}-b_{11}	
10	326.9	$[Tyr + Tyr + H]^+$	b_{12}-b_{10}	
11	277.9	$[Asn + Tyr + H]^+$	b_{11}-b_9	
12	383.9	$[Tyr + Tyr + Gly + H]^+$	b_{13}-b_{10}	
13	1486	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + Gly + Asn + Tyr + Tyr + Gly + H]^+$	b_{13}	according to no. 8−12
14	1600.7	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + Gly + Asn + Tyr + Tyr + Gly + Asn + H]^+$	b_{14}	
15	218.9	$[Asn + Cys + 2H]^+$	b_{16}-b_{14}	
16	186	$[Ala + Asn + H]^+$	b_{18}-b_{16}	
17	168.8	$[Pro + Ala + H]^+$	b_{17}-b_{15}	
18	301.9	$[Asn + Trp + 2H]^+$	b_{19}-b_{17}	
19	371.9	$[Ala + Asn + Trp + H]^+$	b_{19}-b_{16}	
20	283.9	$[Trp + Pro + H]^+$	b_{20}-b_{18}	
21	270.9	$[Cys + Pro + Ala]^+$	b_{17}-b_{14}	
22	669	$[Cys + Pro + Ala + Asn + Trp + Pro + H]^+$	b_{20}-b_{14}	
23	2288	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + Gly + Asn + Tyr + Tyr + Gly + Asn + Cys + Pro + Ala + Asn + Trp + Pro + H]^+$	b_{20}	according to no. 13−22
24	260.9	$[Cys + Arg + H]^+$	b_{23}-b_{21}	
25	384	$[Arg + Asn + Asn]^+$	b_{24}-b_{22}	
26	346.9	$[Ser + Cys + Arg + H]^+$	b_{23}-b_{20}	
27	461	$[Ser + Cys + Arg + Asn + H]^+$	b_{24}-b_{20}	
28	2748	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + Gly + Asn + Tyr + Tyr + Gly + Asn + Cys + Pro + Ala + Asn + Trp + Pro + Ser + Cys + Arg + Asn]^+$	b_{24}	According to No.s 23−27
29	228.9	$[Asn + Asn + H]^+$	b_{25}-b_{23}	
30	391.9	$[Asn + Asn + Tyr + H]^+$	b_{26}-b_{23}	
31	291.9	$[Tyr + Lys + H]^+$	b_{27}-b_{25}	
32	277.9	$[Asn + Tyr + H]^+$	b_{26}-b_{24}	
33	519	$[Asn + Asn + Tyr + Lys + H]^+$	b_{27}-b_{23}	
34	213.9	$[Lys + Ser- H]^+$	b_{28}-b_{26}	
35	286.9	$[Lys + Ser + Ala + H]^+$	b_{29}-b_{26}	
36	158.8	$[Ser + Ala + H]^+$	b_{29}-b_{27}	
37	128.8	$[Ala + Gly + H]^+$	b_{30}-b_{28}	
38	185.9	$[Ala + Gly + Gly + H]^+$	b_{31}-b_{28}	
39	312.9	$[Ala + Gly + Gly + Lys + H]^+$	b_{32}-b_{28}	
40	529	$[Lys + Ser + Ala + Gly + Gly + Lys + H]^+$	b_{32}-b_{26}	
41	776	$[Asn + Tyr + Lys + Ser + Ala + Gly + Gly + Lys + H]^+$	b_{32}-b_{25}	
42	3553	$[Val + Phe + His + Ala + Tyr + Ser + Ala + Arg + Gly + Asn + Tyr + Tyr + Gly + Cys + Pro + Ala + Asn + Trp + Val + Ser + Cys + Arg + Asn + Asn + Tyr + Lys + Ser + Ala + Gly + Gly + Lys]^+$		according to no.28−41

[a]All peptide fragments of plantaricin 163 are listed in Figure 2. [b]Fracture type of plantaricin 163: H−(NHCHRCO−)n−1NHCHRnCO+.

other strains of the genera *Lactobacillus* and *Lactococcus*, in addition to foodborne pathogens including *L. monocytogenes*, *B. cereus*, *C. perfringens*, and *C. sporogenes*.[5] Although plantaricin MG showed broad inhibitory activity against Gram-positive and Gram-negative bacteria including *L. monocytogenes*, *S. aureus*, *S. typhimurium*, and *E. coli*,[29] plantaricin LP84 was active against a wide range of bacteria encompassing Gram-positive, Gram-negative, foodborne pathogenic, and spoilage bacteria.[46]

In the work, plantaricin 163 was shown to possess broad-spectrum inhibitory activity against not only LAB but also various other tested Gram-positive and Gram-negative bacteria. The physicochemical properties of plantaricin 163 identified in the present study are in agreement with the characteristic features of antimicrobial peptides, thus indicating the potential value of plantaricin 163 as a biopreservative in the food industry.

Effects of Enzyme, Temperature, and pH on the Activity of Plantaricin 163. The antimicrobial activity of plantaricin 163 was lost after enzymatic treatment with trypsin, protease K, α-chymotrypsin, and pepsin (Table 3). Furthermore, plantaricin 163 was tested at different temperatures (60, 80, 100, and 121 °C) and under pH conditions from pH 2 to 10, at which it displayed remarkable thermal and pH stability. Surprisingly, the peptide recovery was 73% irrespective of

Table 3. Effects of Enzyme, Temperature, and pH on the Antimicrobial Activity of Plantaricin 163

treatment	residual antimicrobial activity[a] (%)
enzymes	
CK	100
trypsin	0
protease K	0
α-chymotrypsin	0
pepsin	0
heat	
CK[b]	100.0
60 °C, 10 min	100.8
60 °C, 30 min	98.4
80 °C, 10 min	97.6
80 °C, 30 min	98.4
100 °C, 10 min	83.6
100 °C, 30 min	74.6
121 °C, 20 min	73.1
pH value	
2	60.9
3	96.9
4 (CK)[c]	100
5	93.8
6	77.8
7	76.1
8	78.5
9	71.8
10	52.5

[a]Means of three replicate values. [b]Temperature: 25 °C. [c]The original pH value of partially purified plantaricin 163 preparations was 4.0.

whether it was treated at temperatures below 80 °C for 10 or 30 min or at 121 °C for 20 min. In contrast, the recovery was 70% at pH 6—9 and increased to 90% in the presence of acidic pH (3—5) for 3 h. Similar effects were previously observed for different plantaricin peptides such as plantaricin MG[29] and plantaricin ASM1.[43] On the basis of the classification scheme for LAB bacteriocins,[47] plantaricin 163 shared similar characteristics with class II bacteriocins and therefore holds great promise for use in food processing and preservation.

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Notes

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