Original paper

Characterization of the Bacteriocinogenic Lactic Acid Bacteria *Lactobacillus curvatus* Strain Y108 Isolated from Nozawana-Zuke Pickles

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Running title

Bacteriocinogenic Strain from *Nozawana-Zuke* Pickles

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Abbreviations: LAB, lactic acid bacteria; MYP, maltose, yeast extract, and peptone; GYP, glucose, yeast extract, and peptone; BLAST, Basic Local Alignment Search Tool; PB, phosphate buffer; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry.

We characterized the antibacterial activity of the *Lactobacillus curvatus* strain Y108 isolated from the traditional Japanese pickle *Nozawana-zuke*, and partially identified the antibacterial agent produced by the strain. The Y108 strain exhibited antibacterial activity against *L. curvatus* JCM1096, *Listeria monocytogenes* JCM7671, *Staphylococcus aureus* subsp. *aureus* JCM20624, and *Serratia marcescens* JCM20012. The antibacterial activity was abolished upon treatment with several proteases and lipase but not catalase, and it was moderately stable against heat treatment for 2 h at 100°C. The Y108 strain showed higher antibacterial activity when grown at 20°C than at 30°C, which is its optimal growth temperature. SDS-PAGE analysis of the purified culture supernatant revealed the presence of two antibacterial peptide agents, F3-I and F3-II, with net molecular weights of 5.5 and 4.5 kDa, respectively. The N-terminal amino acid sequences of F3-I and F3-II were homologous to those of lactocin 705α and 705β, respectively. However, molecular masses and individual antibacterial activities of the two peptides were considerably different from those reported for lactocin 705.

Keywords: *Nozawana-zuke*; bacteriocin; biopreservation; lactocin 705; *Lactobacillus curvatus*

Introduction

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria that are widely used as starter cultures in the manufacture of fermented food products. They impart a characteristic flavor to fermented foods and increase their shelf life (Liu *et al.*, 2008; Ross *et al.*, 2002). In addition to lactic acid production, some LAB strains are known to protect foods from putrefactive bacteria by producing inhibitory agents such as hydrogen peroxide, lactoperoxidase, diacetyl, and bacteriocins (Ross *et al.*, 2002; De Vuyst and Leroy 2007).

Bacteriocins are ribosomally synthesized antibacterial peptides (Riley and Weltz 2002). Although their inhibitory activities are generally limited to the species producing them, some of them are effective against spoilage organisms and food-borne pathogens (Jack *et al.*, 1995). Bacteriocins enhance the organoleptic qualities and nutritional properties of foods, without the need for chemical preservatives and heat treatments (Galvez *et al.*, 2008). Since many LAB-derived bacteriocins meet the generally recognized as safe (GRAS) status, their use as natural food preservatives has been investigated (De Vuyst and Leroy 2007; Gálvez *et al.*, 2007; Settanni and Corsetti 2008). Nisin, a bacteriocin produced by *Lactococcus lactis* strains, has been sanctioned as a food additive worldwide. Other bacteriocins have also received attention as potential preservatives for foods and beverages. In particular, bacteriocins produced by LAB strains used in various pickling processes have been reported (Choi *et al.*, 1999; Jamuna and Jeevaratnam 2004; Huang *et al.*, 2008). However, except for a few reports (Aso *et al.*, 2008), little is known about the characteristics of bacteriocinogenic LAB strains used in locally produced traditional Japanese pickles.

In this study, we report on the bacteriocinogenic *Lactobacillus curvatus* strain Y108 isolated from *Nozawana-zuke*. *Nozawana-zuke* is a low-salt pickle prepared from *Nozawana* (*Brassica campestris* var. *rapa*), a leafy turnip plant primarily grown in *Shinshu*. A bacteriocinogenic LAB strain isolated from *Nozawana-zuke* has thus far not been reported. We characterized the antibacterial activity of the Y108 strain and the antibacterial agents produced by it.

Materials and Methods

Bacterial strains *L. curvatus* strain Y108 was isolated from *Nozawana-zuke* pickles manufactured at Nozawaonsen-mura, Shimotakai-gun, Nagano, Japan using a method modified from that reported by Kawahara and Otani (2006). This strain was preliminarily selected from among 98 strains isolated from *Nozawana-zuke*. Briefly, 0.5 ml of a diluted dipping sauce containing the bacteria was inoculated in 9.5 ml of maltose, yeast extract, and peptone (MYP) medium (pH 6.8) containing 1.2% (w/v) agar and 0.5% (w/v) calcium carbonate ($CaCO_3$) on a culture plate. Sodium azide (0.01 mg/ml) and cycloheximide (0.01 mg/ml) were added as antibiotics. After anaerobic cultivation at 30°C for 48–72 h, colonies forming $CaCO_3$-melting zones around them were transferred to fresh MYP medium. The isolated strain was then cultivated in glucose, yeast extract, and peptone (GYP) medium at 30°C and stored as frozen cultures at –80°C until further use.

The indicator strains used (listed in Table 1) were purchased from the Japan Collection of Microorganisms (JCM; Tokyo, Japan) or the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC; Chiba, Japan) and cultivated under the recommended culture conditions.

Classification The Y108 strain was classified on the basis of 49 sugar fermentation patterns, using the API50CHL identification kit (BioMerieux, Marcy l'Etoile, France). Further, the full-length 16S rDNA sequences of the Y108 strain were compared with the DNA sequences in the National Center for Biotechnology Information (NCBI) database using the standard nucleotide–nucleotide homology search tool BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Preparation of culture supernatants The Y108 strain (1×10^6 CFU/ml) was cultured in GYP medium at 20°C or 37°C for 24 h. Cell-free supernatants were obtained by centrifugation at 8,500 *g* for 30 min, and the pH was adjusted to 6.8 by the addition of 2 M sodium hydroxide; then, the supernatants were sterilized through a 0.2-μm membrane filter (Dismic-13cp; Advantec, Tokyo, Japan). The sterilized supernatants were stored at –20°C until use.

Assay for antibacterial activity Antibacterial activity was qualitatively assessed by the agar-well diffusion method. Wells 5 mm in diameter were made in GYP medium containing 1.2% (w/v) agar and an indicator strain; 50 μl of cell-free supernatant of the Y108 strain was then added to individual wells. After 24–48 h of incubation at the temperature suitable for the growth of the respective indicator strain, the diameters of the inhibition zones of the bacterial lawns were measured.

The antibacterial activity was quantified by the serial dilution method. Two-fold serially diluted cell-free culture supernatant of the Y108 strain was added to the culture of *L. curvatus* JCM1096 strain in 96-well culture plates (Becton Dickinson, Franklin

Lakes, NJ, USA). After 5 h of incubation at 30°C, the reduction in turbidity caused by the inhibition of indicator strain growth was measured at 562 nm using a microtiter plate reader (model 550; Bio-Rad Laboratories, Hercules, CA, USA). The activities were compared by determining the IC_{50}, i.e., the concentration of the Y108 strain supernatant required to inhibit the growth of the JCM1096 strain by 50% compared to the growth under supernatant-free condition.

Preliminary characterization of antibacterial culture supernatant The Y108 strain (1×10^6 CFU/ml) was cultured in GYP medium at 30°C for 24 h, and the culture supernatant was harvested by centrifugation. The centrifuged supernatant was filtered with a Dismic-13cp filter and used as a cell-free crude bacteriocin sample for subsequent characterization. Proteinase K (Merck, Darmstadt, Germany), pronase E (Sigma, St. Louis, MO, USA), trypsin (Sigma), chymotrypsin (Boehringer, Mannheim, Germany), lipase (Elastin Products, Owensville, MO, USA), α-amylase (Elastin Products), and catalase (Sigma) were used for testing the resistance of the supernatant against enzymes. Each enzyme (1 mg/ml in phosphate buffered saline; pH 7.0), was mixed with the culture supernatant at a 1: 1 (v/v) ratio and incubated at 30°C for 2 h. Stability against high temperature was analyzed by incubating the supernatant at 60°C or 100°C for 0, 5, 10, 15, 20, 30, and 60 min in a thermal cycler (PTC-200 DNA Engine; MJ Research, Waltham, MA, USA). Residual activity after the enzymatic or heat treatment was assessed by the serial dilution method and compared with the activity of each untreated control.

Purification of antibacterial agent The antibacterial agent was purified by a

multi-step method modified from the one reported by Jiménez-Díaz *et al.* (1995). The Y108 strain was cultured in 5 L of medium at 20°C for 24 h, and the culture supernatant was obtained by centrifugation at 1,300 *g* for 30 min. The supernatant was sterilized by filtration with a Dismic-13cp filter and its pH was adjusted to 6.8. The supernatant was ultrafiltered through a 30-kDa cut-off membrane (YM30; Millipore, Bedford, MA, USA), using an Amicon-Ultra Ultrafiltration Cell Model 8200 (Millipore).

The fraction positive for antibacterial activity was dissolved in 0.02 M phosphate buffer (pH 6.8) and subjected to hydrophobic-interaction chromatography, using the ATKA Prime system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equipped with a hydrophobic HiPrep Octyl FF column (diameter, 1.6 cm; length, 10 cm; Amersham). The column was equilibrated and washed with 0.02 M phosphate buffer (pH 3.5). Elution was carried out with a reverse linear salt gradient of 20–0% ammonium sulfate at a flow rate of 4 ml/min. The protein concentration of the eluate was determined by measuring absorbance at 214 nm using a spectrophotometer (Ultrospec 3300 pro; Amersham), and antibacterial activity was quantified by the serial dilution method.

The fractions positive for antibacterial activity were pooled and subjected to anion-exchange chromatography on a HiPrep CM FF column (diameter, 1.6 cm; length, 10 cm; Amersham) mounted on the ATKA prime. After equilibration with 0.02 M phosphate buffer (pH 3.5), the active component was eluted stepwise with 1 M NaCl at a flow rate of 4 ml/min.

The active fraction was further purified by reverse-phase high-performance liquid chromatography (RP-HPLC), using a Shimadzu 10A HPLC system (Kyoto, Japan) equipped with a TSK-Gel ODS 80TM column (diameter, 0.6 cm; length, 15 cm;

Tosoh, Tokyo, Japan). After equilibration with 5% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid (TFA), elution was carried out with a linear gradient of 5–90% acetonitrile containing 0.05% TFA for 30 min at a flow rate of 0.8 ml/min. The protein concentration of the eluate was monitored at 280 nm, and 1-ml fractions of eluate were collected.

Assay for antibacterial activity of peptides separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) The RP-HPLC-purified fractions positive for antibacterial activity were loaded onto a tricine-SDS-PAGE slab gel comprising spacer gels of 20% and 10% acrylamide, and a concentrate gel of 4% acrylamide (Schägger and von Jagow 1987). Before loading, the fractions were mixed with an equal volume of a sample buffer containing 30% glycerol and 8% SDS, and heated at 60°C for 15 min. The heated samples were loaded onto the spacer gel and electrophoresed at 5 mA/gel in Tris-Tricine buffer containing 0.1% SDS and 1 mM mercaptoacetic acid, using an electrophoresis cell (AE-6400; Atto, Tokyo, Japan). The Ultra-Low Range Marker (Sigma) was concurrently electrophoresed as a molecular weight marker. After electrophoresis, one half of the gel containing the samples and the molecular weight marker was stained using a silver staining kit (KANTOII; Wako, Osaka, Japan). In order to determine the antibacterial activity of the peptides in the gel, the other half of the gel was immediately fixed with 20% (v/v) 2-propanol and 10% (v/v) acetic acid for 30 min and then washed with distilled water overnight, as described by Bhunia *et al.* (1987). The washed gel was placed in a sterile Petri dish and overlaid with 10 ml GYP medium containing 1.2% agar and the indicator strain JCM1096. The plate was then incubated at 30°C for 24 h, and the inhibition zones against the growth of JCM1096 were examined.

N-terminal amino acid sequence analysis The electrophoresed proteins positive for antibacterial activity were blotted onto a polyvinylidene difluoride membrane (Mini ProBlot; Applied Biosystems, Foster City, CA, USA) at 70 mA for 30 min using a semi-dry type electroblotter system (Horizeblot AE-6678; Atto). The membrane was stained with 0.1 % Coomassie Brilliant Blue R-250 for 5 min and decolorized in a solution of 7% acetic acid and 45% methanol for 15 min. The stained peptides were cut out from the membrane and reduced with 2 μM dithiothreitol at 37°C for 2 h; *S*-carboxyamidomethylation was then carried out with iodoacetamide at room temperature for 30 min. The amino acid sequence of the peptides was determined by direct N-terminal sequencing using automated Edman degradation with a HP G1005A Protein Sequencing System (Hewlett-Packard, Palo Alto, CA, USA).

The compute pI/Mw tool program from the Expert Protein Analysis System website (http://us.expasy.org/tools/pi_tool.html) was used for estimating the molecular masses of the peptides. A homology search was performed using BLAST.

Mass spectrometry For determining the molecular mass of an RP-HPLC-purified active fraction, 1.5-μl aliquots of the fraction were mixed with 0.5 μl of matrix solution consisting of 3,5-dimethoxy-4-hydroxycinnamic acid and spotted on a MALDI plate. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometric (MALDI-TOF MS) analysis in the linear mode using a Voyager-DE STR (Applied Biosystems) in the range of *m/z* 1,000–10,000 was then performed.

Results

Classification of the Y108 strain The Y108 strain was identified as *L. curvatus* by the sugar fermentation pattern in an API50CHL test with 99.9% accuracy. The full-length 16S-rDNA sequence of the Y108 strain showed 100% homology with that of *L. curvatus* (accession no. Tecsrg0011).

Antibacterial spectrum of the Y108 culture supernatant The antibacterial spectrum of the supernatant obtained from the Y108 strain culture is shown in Table 1. The supernatant inhibited the growth of *L. curvatus* strain JCM1096 and *Serratia marcescens* strain JCM20012. *Listeria monocytogenes* strain JCM7671 and *Staphylococcus aureus* subsp. *aureus* strain JCM20624 were also weakly sensitive to the culture supernatant. We used the *L. curvatus* strain JCM1096 as the indicator strain in subsequent studies because this strain was the most sensitive to the culture supernatant.

Preliminary characterization of the antibacterial activity of the culture supernatant The effect of enzymatic or heat treatment on the antibacterial activity of the Y108 culture supernatant is shown in Fig. 1. Incubation of the supernatant with various proteolytic enzymes (proteinase K, pronase E, trypsin, and chymotrypsin) and lipase for 1 h at 37°C completely abolished its antibacterial activity (Fig. 1A). Treatment with α-amylase resulted in partial degradation (approximately 25%) of the antibacterial activity, while catalase had no effect on the antibacterial activity.

Figure 1B shows the changes in the antibacterial activity of the supernatant after heating, over time. The antibacterial activity of the supernatant remained unchanged after incubation at 60°C for 30 min. It decreased by about 13% after incubation at 60°C

for 60 min. At 100°C, the residual antibacterial activity decreased by 34, 39, and 52% after 5, 30, and 60 min of incubation, respectively.

Effect of temperature on growth and antibacterial activity of the Y108 strain

Effect of the cultivation temperature on the growth of the Y108 strain and on the antibacterial activity of the culture supernatant is shown in Fig. 2. When fermentation was carried out at temperatures lower than 10°C, the growth rate of the cultures and antibacterial activities of the supernatants obtained from them were significantly low (Fig. 2A and B). The culture supernatant of the Y108 strain exhibited strong antibacterial activity when the growth temperature was maintained at 20°C (Fig. 2B). Even though the Y108 strain showed 1.7-fold higher growth at 30°C than at 20°C, its antibacterial activity was 1.9-fold higher at 20°C than at 30°C. At 40°C or higher temperatures, the inhibitory activity of the Y108 culture supernatant was almost completely abolished.

Purification of the antibacterial agent The antibacterial agent in the Y108 culture supernatant was purified by a 4-step procedure comprising ultrafiltration, hydrophobic-interaction chromatography, cation-exchange chromatography, and RP-HPLC. After ultrafiltration, the antibacterial activity was observed in only the high molecular-weight fraction; the filtered fraction, i.e., the low molecular-weight fraction did not show any antibacterial activity (data not shown). Hydrophobic-interaction chromatography of the active fraction showed the antibacterial activity-exhibiting major peak (F1) on the chromatogram upon elution with 0–0.25 M ammonium sulfate (Fig. 3A). The cation-exchange chromatogram of the F1 fraction showed that antibacterial

activity was present in a 1-M NaCl-eluted fraction (F2) (Fig. 3B). The RP-HPLC of F2 revealed a single peak positive for antibacterial activity (F3), which was eluted at 52% acetonitrile concentration (Fig. 3C).

Electrophoresis of the antibacterial components To estimate the molecular weight of the antibacterial agent, fraction F3 was subjected to tricine-SDS-PAGE. After electrophoresis, the silver-stained gel showed two distinct bands (Fig. 4A). The molecular masses of the two peptides were estimated to be approximately 5.5 kDa (F3-I) and 4.5 kDa (F3-II). Antibacterial activity was observed in the same molecular-mass range (Fig. 4B). To evaluate the individual antibacterial activity of the two bands, the parts of the gel containing the peptide bands were cut out and separately overlaid with agar inoculated with the JCM1096 strain. As shown in Fig. 4C, both F3-I and F3-II peptides formed inhibition zones independent of each other.

N-terminal amino acid sequences and molecular masses of the antibacterial components N-terminal amino acid analysis identified 30 and 21 amino acids at the N-terminals of F3-I and F3-II, respectively. No amino acid modification was observed in the sequences of F3-I and F3-II. The N-terminal amino acid sequences obtained for each fragment were as follows: F3-I, GMSGYIQGIPDFLKGYLHGISAANKHKKGR; F3-II, GFWGGLGYIAGRVGAAYGHAQ.

MALDI-TOF MS analysis of F3-I and F3-II showed two major peaks at *m/z* 3,585.24 and 3,315.81, respectively (Fig. 5). The molecular masses of F3-I and F3-II were calculated from these major peaks as 3,584.23 Da and 3,314.80 Da, respectively.

Discussion

In this study, we characterized the antibacterial LAB strain *L. curvatus* Y108 isolated from *Nozawana-zuke* pickles. The Y108 strain showed antibacterial activity against some putrefactive bacteria, such as *S. marcescens* JCM20012, *L. monocytogenes* JCM7671, and *S. aureus* subsp. *aureus* JCM20624, without affecting the growth of all the LAB strains tested except for *L. curvatus* strain JCM1096 (Table 1).

The antibacterial activity of the Y108 culture supernatant was highly sensitive to protease and lipase treatment, whereas it was insensitive to catalase (Fig. 1A). In addition, its activity was also relatively heat stable (Fig. 1B). These results indicate that the active agent in the supernatant was not hydrogen peroxide but a heat-stable peptide or triglyceride.

We investigated the effect of cultivation temperature on the antibacterial activity of the culture supernatant of the Y108 strain using JCM1096 as an indicator strain. As shown in Fig. 2, the antibacterial activity of the supernatant of the Y108 strain obtained after cultivation at 20°C was significantly higher than that of supernatants obtained after cultivation at other temperatures. Contrary to our expectation, even though the Y108 strain showed more vigorous growth at 30°C than at 20°C, its antibacterial activity was lower at 30°C than at 20°C. Many studies have indicated that optimal bacteriocin production by LAB strains often depends on the growth temperature (Yang and Ray 1994). Our results suggested that the Y108 strain produces considerably higher amounts of the antibacterial agent at 20°C than at 30°C.

Chromatographic profiles of the Y108 culture supernatant showed that the antibacterial agent had a characteristics net positive charge and was hydrophobic (Fig. 3). SDS-PAGE analysis of the purified active fraction showed two peptide bands (F3-I

and F3-II) with molecular masses of approximately 5.5 kDa and 4.5 kDa, respectively (Fig. 4). According to a currently accepted classification, two-peptide-type bacteriocins are included in class II (Klaenhammer, 1988; Cotter *et al.*, 2005). Thus, we hypothesized that the antibacterial agent produced by Y108 strain was a class II bacteriocin. Most two-peptide-type bacteriocins require the presence of both peptides for optimal antibacterial activity (Oppegård *et al.*, 2007). Therefore, we tested the antibacterial activity of these 2 peptides individually. As shown in Fig. 4C, each peptide independently showed an inhibition zone when incubated with the indicator strain.

Various class II bacteriocins produced by the *L. curvatus* species have been reported (Tichaczek *et al.*, 1992; Sudirman *et al.*, 1993; Garver and Muriana 1994; Bouttefroy *et al.*, 2000; Xiraphi 2006). In order to investigate whether the antibacterial activity exhibited by the Y108 strain was because of the presence of these known bacteriocins, we analyzed the N-terminal amino acid sequences of the F3-I and F3-II peptides. BLAST search of the sequences of F3-I and F3-II revealed that these peptides were partially identical to the mature peptides of the class II bacteriocin lactocin 705α (accession no. Q9RMH7: GMSGYIQGIPDFLKGYLHGISAANKHKKGRLGY) and 705β (accession no. Q9RMH8: GFWGGLGYIAGRVGAAYGHAQASANNHHSPING), respectively. Lactocin 705α and 705β are produced by the *L. casei* strain CRL705, which was subsequently reclassified under the species *L. curvatus* (Palacios *et al.*, 1999; Cuozzo *et al.*, 2000). The MALDI-TOF MS analysis (Fig. 5) showed that the molecular masses of the main peaks of F3-I and F3-II were 3,584.23 and 3,314.80 Da, respectively. Both masses were considerably different from the theoretical masses of mature lactocin 705α (3,578.15 Da) and 705β (3,308.58 Da) considering the generally accepted measurement error in MALDI-TOF MS analysis, i.e., <0.1%. In addition, whereas F3-I

and F3-II exhibit antibacterial activity independent of each other (Fig. 4C), lactocin 705 requires both α and β subunits for exhibiting its antibacterial effect (Cuozzo *et al.*, 2000; 2003). Therefore, F3-I and F3-II may be variants of lactocin 705 that contain distinct amino acid sequences. The molecular masses of F3-I and F3-II, calculated on the basis of N-terminal amino acid sequence analysis, were 339.47 and 1,206.45 Da less than those obtained by MALDI-TOF MS. These results indicate that as yet unidentified amino acid residues may be present in the complete sequence of F3-I and F3-II. Since lactocin 705α and 705β have no modified amino acid (Cuozzo *et al.*, 2000), we think that the difference in masses of F3-I and F3-II from those of lactocin 705α and 705β were because of the presence of distinct amino acid sequences, as seen among other bacteriocin variants (Mulders *et al.*, 1991; Zendo *et al.*, 2003), and therefore, antibacterial spectrum of Y108 culture supernatant shown in Table 1 somewhat differs from that of the CRL705 strain (Vignolo *et al.*, 1993).

The molecular masses of F3-I and F3-II, as indicated by the results of ultrafiltration, SDS-PAGE, and MALDI-TOF MS, were notably different from each other. Whole or partial inactivation of the antibacterial activity of the Y108 culture supernatant by lipase and α-amylase suggests that, F3-I and F3-II associated with certain triglyceride or carbohydrate components, in a manner to class IV bacteriocins. Many researchers have reported that Tween 80, which is used as a surfactant in the culture medium and can act as a substrate for lipase, affected several purification steps of bacteriocins (Carolissen-Mackay *et al.*, 1997). Kawai *et al.* (1994) have reported a strong association between hydrophobic bacteriocin and Tween 80. F3-I and F3-II may have formed a high-molecular-weight complex with such a medium component.

In conclusion, we found that the *Nozawana-zuke*-derived *L. curvatus* strain Y108

showed antibacterial activity against certain Gram-positive and Gram-negative bacteria. The titer of antibacterial activity was highly dependent on the growth temperature of the strain and was higher at 20°C than at 30°C. We found that the antibacterial agent consisted of two component bacteriocins partly homologous with lactocin 705. *Nozawana-zuke* is traditionally known to be a well-preserved food product. The antibacterial property of certain resident bacteriocinogenic LAB strains, such as Y108, may play a partial role in the preservation of *Nozawana-zuke* pickles. Further research is required to understand the role of bacteriocinogenic strains derived from pickles in the preservation of foods and for assessing their broader application in food preservation.

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Table 1. Antibactrial spectrum of culture supernatant of the *L. curvatus* strain Y108

Species	Strain	Activity*
Gram-positive		
Lactobacillus curvatus	JCM1096	++
Lactobacillus acidophilus	JCM1132	-
Lactobacillus acidophilus	JCM1028	-
Lactobacillus johnsonii	JCM2012	-
Lactobacillus johnsonii	JCM2122	-
Lactobacillus delbrueckii subsp. *bulgaricus*	JCM20398	-
Lactobacillus delbrueckii subsp. *lactis*	JCM1248	-
Lactobacillus reuteri	NBRC15892	-
Lactobacillus casei	NRBC15883	-
Lactobacillus rhamnosus	NBRC3425	-
Lactobacillus helveticus	JCM1120	-
Lactobacillus plantarum subsp. *plamtarum*	JCM1149	-
Lactococcus lactis subsp. *lactis*	NBRC100933	-
Lactococcus lactis subsp. *lactis*	JCM7638	-
Lactococcus lactis subsp. *cremoris*	NBRC100676	-
Streptococcus salivarius subsp. *thermophilus*	JCM20026	-
Streptococcus mutans	JCM5705	-
Pediococcus pentosaceus	NBRC3182	-
Leuconostoc mesenteroides subsp. *mesenteroides*	JCM6124	-
Bacillus cereus	JCM2152	-
Listeria monocytogenes	JCM7671	+
Staphylococcus aureus subsp. *aureus*	JCM20624	+
Gram-negative		
Escherichia coli	JCM1649	-
Salmonella enterica subsp. *enterica*	JCM1651	-
Serratia marcescens	JCM20012	++

* Diameter of the inhibition zone: >2 mm, (++); <2 mm, (+); and 0 mm (-).

Figure legends

Fig. 1. Residual antibacterial activity of the crude Y108 culture supernatant after enzyme and heat treatments.

(A) Stability to enzyme treatments: Data are represented as percent residual antibacterial activity against the untreated control. (B) Stability to heat treatment: Data are represented as percent residual antibacterial activity against the untreated control over time. The open and closed circles represent the percent of residual antibacterial activity at 60°C and 100°C, respectively.

Fig. 2. Effect of culture temperature on the antibacterial properties of the Y108 culture supernatant.

(A) Effect of culture temperature on the growth of the Y108 strain. Data are represented as mean percent growth (n = 3) against the growth at 30°C. (B) Effect of culture temperature on the antibacterial activity of the Y108 culture supernatant. Data are represented as percent activity against that at 30°C.

Fig. 3. Chromatographic profiles of the Y108 culture supernatant.

(A) Chromatogram obtained after hydrophobic-interaction chromatography of the ultrafiltered culture supernatant (> 30,000). F1 indicates the fraction positive for antibacterial activity. (B) Chromatogram obtained after cation-exchange

chromatography of F1. F2 indicates the fraction positive for antibacterial activity. (C) Chromatogram obtained after reverse-phase high performance liquid chromatography (RP-HPLC) of F2. F3 indicates the fraction positive for antibacterial activity.

Fig. 4. SDS-PAGE profile and antibacterial activity of the F3 fraction.

(A) Silver-stained PAGE gel, Lane 1, molecular weight marker; Lane 2, RP-HPLC-purified fraction positive for antibacterial activity. (B) Growth-inhibition zone of electrophoresed F3. (C) Growth-inhibition zone of F3-I (a) and F3-II (b).

Fig. 5. MALDI-TOF MS profile of F3.

Data represent the average masses and signal strengths of monovalent ions.

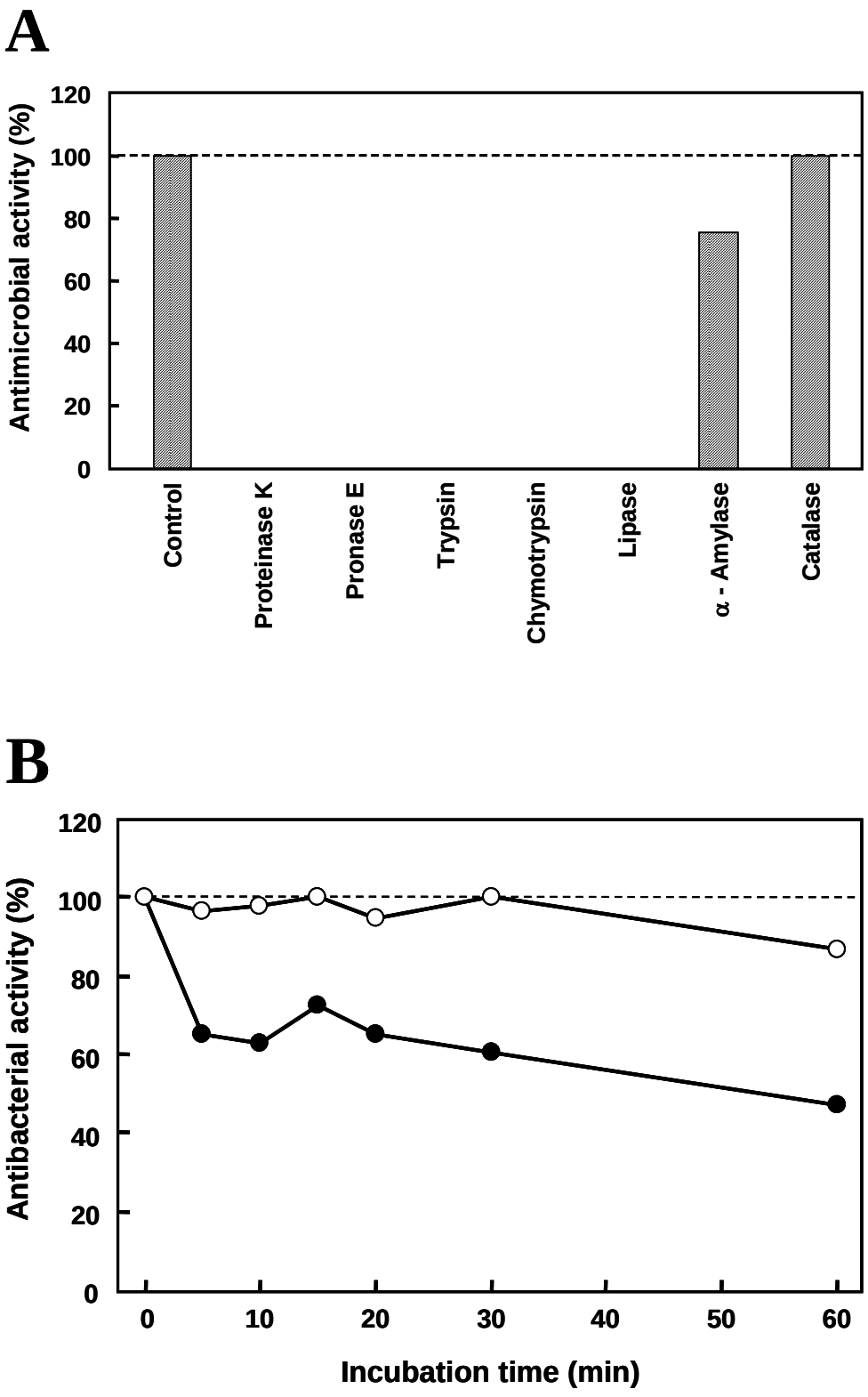
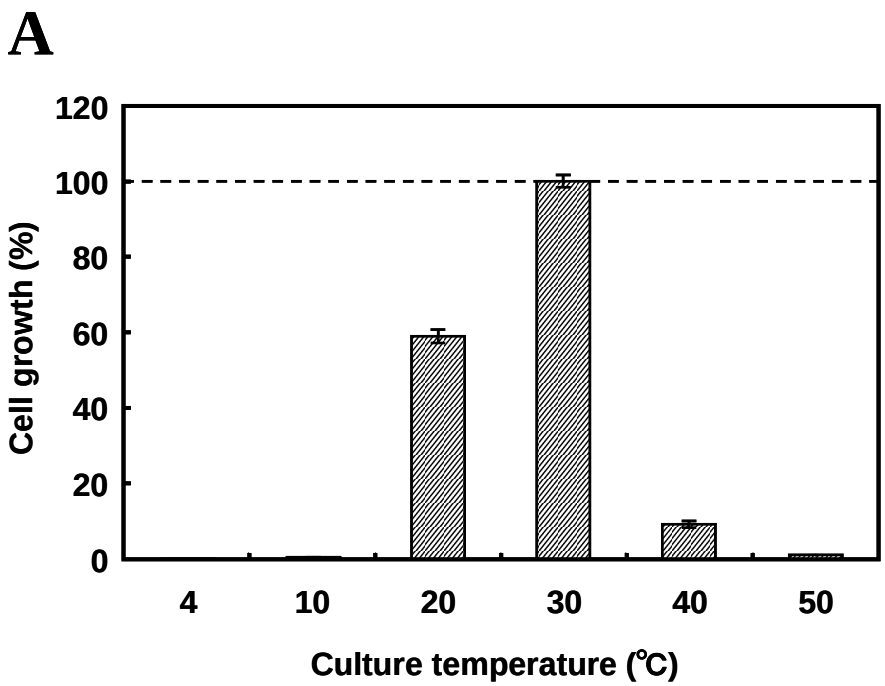

A
Antimicrobial activity (%)
120
100
80
60
40
20
0
Control
Proteinase K
Pronase E
Trypsin
Chymotrypsin
Lipase
α - Amylase
Catalase
B
Antibacterial activity (%)
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Incubation time (min)

Fig. 1


A
120
100
80
60
40
20
0
Cell growth (%)
4
10
20
30
40
50
Culture temperature (℃)

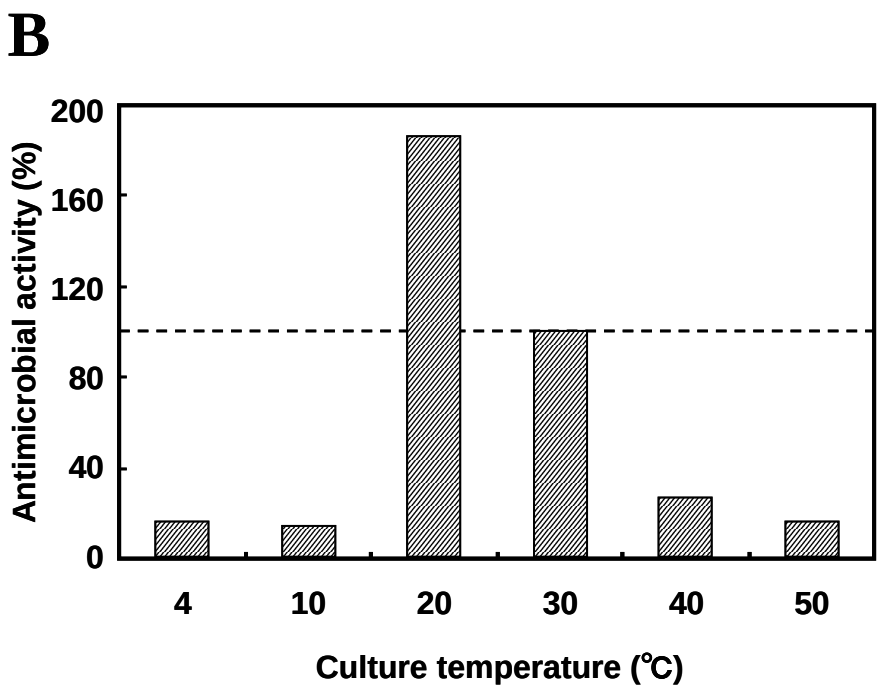

B
200
160
120
80
40
0
Antimicrobial activity (%)
4
10
20
30
40
50
Culture temperature (℃)

Fig. 2

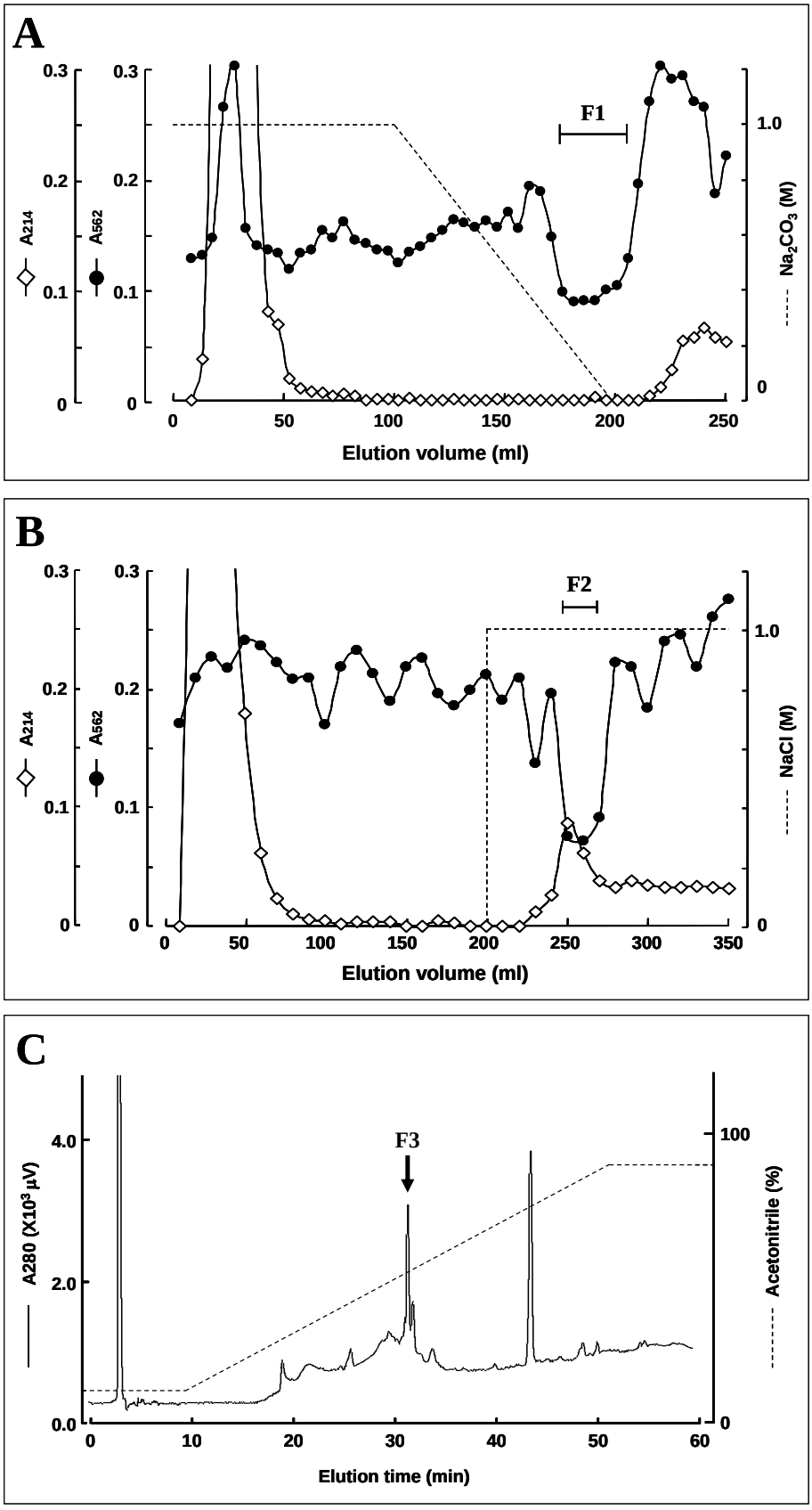

A
0.3
0.2
0.1
0
A214
A562
F1
1.0
Na_2CO_3 (M)
0
50
100
150
200
250
Elution volume (ml)
B
F2
NaCl (M)
300
350
C
4.0
2.0
0.0
A280 (X10^3 µV)
F3
100
Acetonitrile (%)
10
20
30
40
60
Elution time (min)

Fig. 3

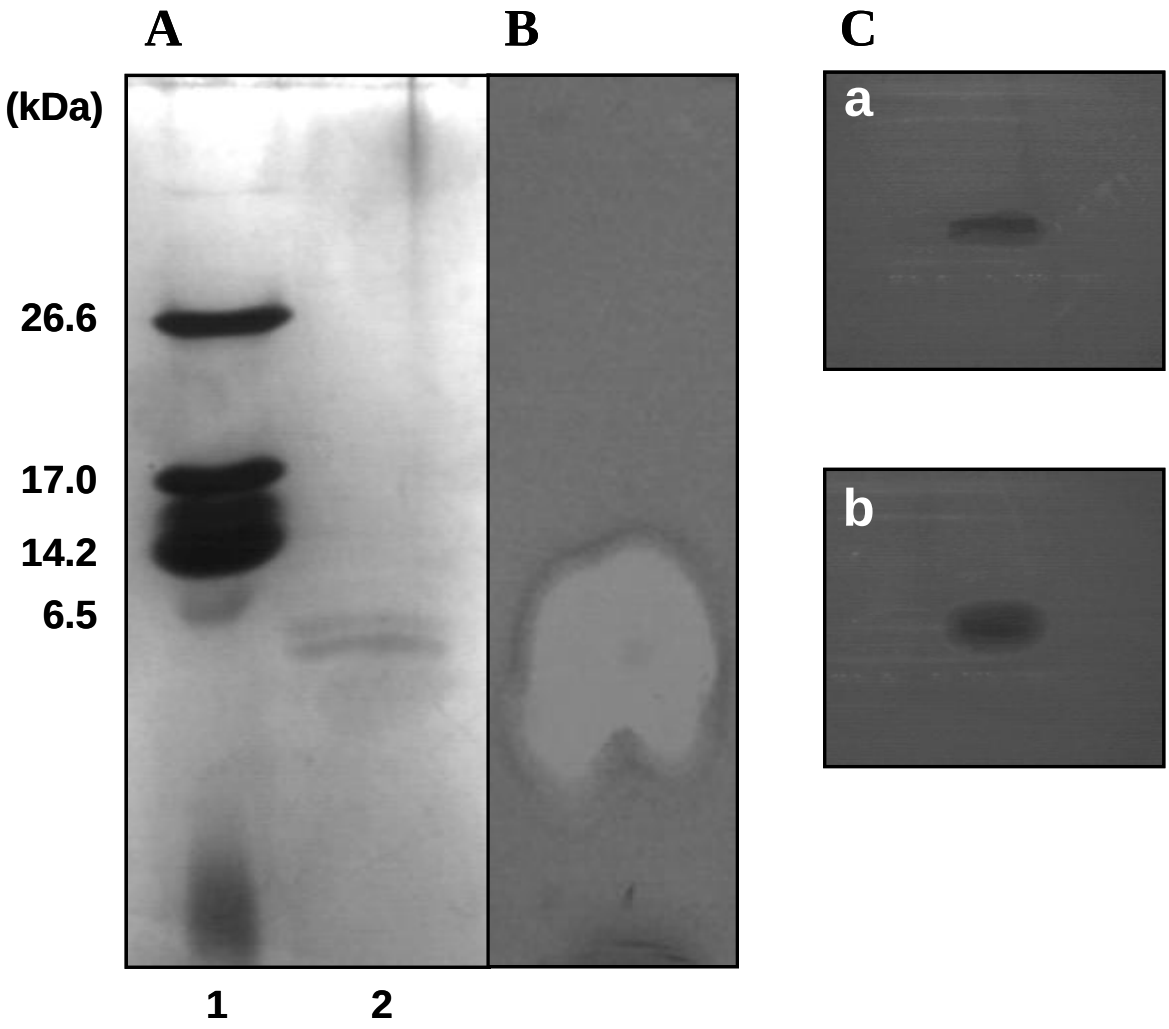


Fig. 4

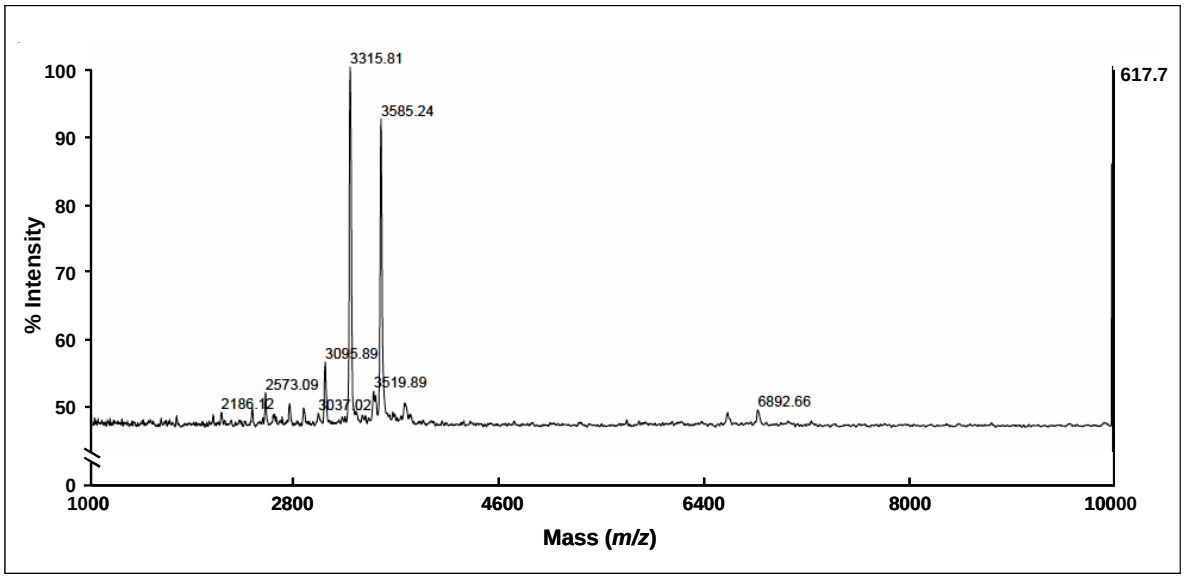

3315.81
617.7
3585.24
100
90
80
70
60
50
0
% Intensity
3095.89
2573.09
3519.89
2186.12
3037.02
6892.66
1000
2800
4600
6400
8000
10000
Mass (m/z)

Fig. 5