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Characterization of Lantibiotic Nisin Z Produced by *Lactococcus lactis* IO–1

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Lactococcus lactis IO–1 isolated in our laboratory produces a peptide antibiotic, a natural nisin variant, nisin Z. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that molecular weight of nisin Z was almost the same as that of nisin A with 3.4 kDa, while reversed–phase high performance chromatography did apparent differences in elution time. Nisin Z was heat stable at acidic pH and showed bactericidal mode of action against an indicator strain, *Bacillus subtilis* C1. The effect of proteolytic enzyme treatment on the activity and the antimicrobial spectrum were tested. These indicated nearly the same characteristics as those of nisin A. In this study, it was found that two nisin variants (nisin Z and nisin A) were practically inactivated by proteinase K and actinase E. Interestingly, nisin–producing strains, *L. lactis* IO–1 and *L. lactis* NCDO 497 (nisin A producer) were sensitive at a high concentration of nisin variants including their own products. The growth of Gram–negative bacterium, *Escherichia coli* was also inhibited by high concentration of nisin, although nisin producers and Gram–negative bacteria are generally resistant to nisin variants.

INTRODUCTION

Lactic acid bacteria are used in the production of many fermented food products. They are responsible for the specific taste and texture during the preparation by fermentation of various kinds of traditional foods. They produce a variety of antimicrobial compounds such as organic acids, hydrogen peroxide and diacetyl. Bacteriocins, proteinaceous compounds, produced by some lactic acid bacteria generally inhibit the growth of closely related species (Klaenhammer, 1988, 1993; Nettles and Barefoot, 1993; Ralph *et al.*, 1995). Some bacteriocins also inhibit the growth of pathogens and spoilage microorganisms during the processing and fermentation of foods. We have had the fermented foods with safety, in which lactic acid bacteria have contributed, for many years. Therefore, it is anticipated that bacteriocins of lactic acid bacteria will be used as natural food preservatives because of the general tendency to decrease the use of chemical food preservatives such as sorbic acid, benzoic acid, nitrate and nitrite. In these years, microbiological, biochemical and genetic characterization of bacteriocins have been well studied. Similarly, it should be also investigated the possibility whether novel bacteriocins isolated would be able to be used as food preservatives.

Nisin, or more precisely nisin A, the most famous bacteriocin is a lanthionine–containing peptide antibiotic, which is especially called as a lantibiotic. Lantibiotics exhibit the heat stability and the strong antimicrobial activity because they have intramolecular mono–sulfide bridiges such as lanthionine and 3–methyllanthionine. Nisin A is also heat stable at acidic pH and has a broad antimicrobial spectrum against

Gram-positive bacteria (Hurst, 1981) and is accepted to be used as a food preservative in many countries (Delves-Broughton, 1990). Since nisin A is instable and inactivated at neutral pH, however, the use as a preservative is limited at present. Nisin has two natural variants, nisin A (Gross and Morell, 1971) and nisin Z (Mulders *et al.*, 1991). Nisin Z is replaced histidine in nisin A with asparagine at position 27 (Fig. 1).

L. lactis IO-1 isolated in our laboratory produces a peptide antibiotic, which was

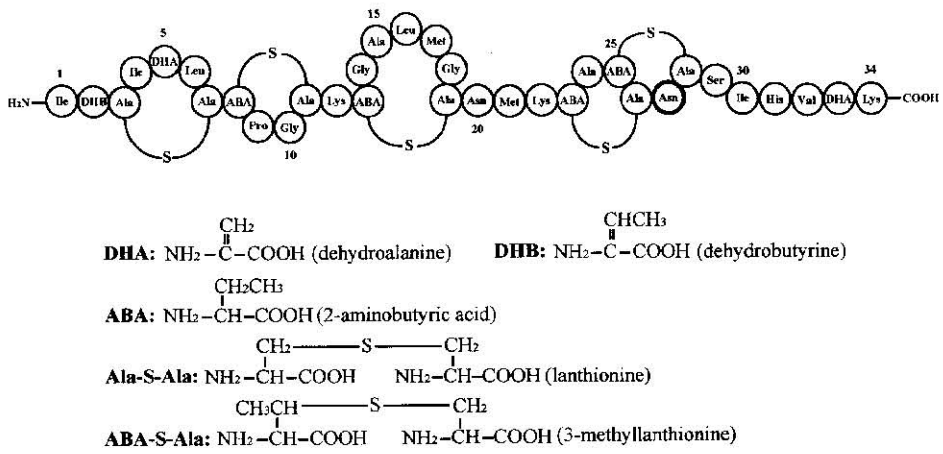

Fig. 1. Structure of nisin Z. The structure of nisin Z was determined by Mulders *et al.* (1991). The amino acid residue at position 27 is histidine in nisin A (Gross and Morell, 1971).

purified and identified to be nisin Z (Matsusaki *et al.*, 1996a). We also investigated the relationship between the primary metabolite production of nisin Z and lactate and cell growth, which had so far not been clarified in detail. This study resulted in 3-fold activity of nisin Z in the culture broth under the optimal conditions (Matsusaki *et al.*, 1996b). Nisin Z has the possibility to be used as food preservative similarly to nisin A. Since there are only a few reports on characterization and properties of nisin Z (De Vuyst and Vandamme, 1994), however, we describe here in detail some characteristics of nisin Z purified from strain IO-1. Protein engineering and molecular design would enable the improvement of stability and antimicrobial activity of nisin Z as a food preservative. Therefore, it is necessary that the characterization of pH and heat stability, sensitivity for proteolytic enzyme and antimicrobial spectrum of wild-type nisin Z is well investigated. The characterization and the property of nisin Z elucidated in this study will also provide many information for the commercial application and the effective production of nisin Z.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Lactococcus lactis IO–1 isolated in our laboratory was used for nisin Z–producing strain (Matsusaki *et al.*, 1996a). To compare nisin Z with nisin A, *L. lactis* NCDO 497, nisin A–producing strain, was also used. The indicator strain used for assay of antimicrobial activity was *Bacillus subtilis* C1 as described previously (Matsusaki *et al.*, 1996a). For testing sensitivity to nisin variants, *L. lactis* JCM 5805^T (nisin non–producer), *L. lactis* subsp. *cremoris* TUA 1344L, *Enterococcus faecalis* JCM 5803^T, *Clostridium acetobutylicum* IFO 13948^T, *B. subtilis* C1, *B. subtilis* JCM 1465^T, *B. coagulans* JCM 2257^T, *B. stearothermophilus* JCM 2501^T, *Micrococcus luteus* IFO12708 and *Escherichia coli* JM109 were used. All lactic acid bacteria and *C. acetobutylicum* were grown on thioglycolate medium without glucose (TGC medium, pH 7.2, Difco Laboratories, Detroit, MI, USA) at 30 °C, but *L. lactis* IO–1, *E. faecalis* and *C. acetobutylicum* at 37 °C and *L. lactis* subsp. *cremoris* at 27 °C. Bacilli were grown on bouillon medium consisting of 0.7% meat extract, 1% polypeptone and 0.5% NaCl (pH 7.0) at 30 °C, but *B. coagulans* at 37 °C and *B. stearothermophilus* at 55 °C. *M. luteus* and *E. coli* were grown on LB medium consisting of 1% bactotryptone, 0.5% yeast extract and 0.5% NaCl (pH 7.0) at 30 °C and 37 °C, respectively.

Assay of antimicrobial activity

For assay of antimicrobial activity, growth inhibition of the indicator strain, *B. subtilis* C1, was determined by turbidimetry as described previously (Matsusaki *et al.*, 1996a). The assay medium consisted of 0.05% yeast extract (Difco), 0.05% polypeptone (Nihon Seiyaku, Co. Ltd., Tokyo, Japan), 0.05% NaCl, 0.1% glucose and 20 mM sodium phosphate buffer (pH 7.0). The activity unit of nisin variants produced by IO–1 and NCDO 497 strains was defined as an arbitrary unit (AU) that is equivalent to the activity of 1 μg of commercial nisin (ICN Biomedicals, Inc., Costa Mesa, CA, USA; activity, 1,000 U/mg–solid; nisin A content, 2.5%). Determination of activity was performed in duplicate or more each time.

Nisin preparation

Nisin Z was recovered from the culture supernatant of *L. lactis* IO–1 and was partially purified by acid treatment, ammonium sulfate precipitation and cation–exchange chromatography as described previously (Matsusaki *et al.*, 1996a). The active fraction obtained from cation–exchange chromatography was used to characterize nisin Z. If necessary, the fraction was further purified through the steps of desalting and concentration with tC_{18} Sep–Pak cartridge (Waters, Milford, MA, USA) and lyophilization (Matsusaki *et al.*, 1996a). Nisin A was also purified from the culture supernatant of strain NCDO 497 in a similar manner as above.

Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Tricine SDS–PAGE) was performed according to the method of Schägger and von Jagow (1987) as described previously (Matsusaki *et al.*, 1996a). Molecular weight markers kit

MW–SDS–17 (Sigma Chemical Company, St. Louis, MO, USA) was used as standard proteins.

High performance liquid chromatography

The active solution collected by cation–exchange chromatography was analyzed by a reversed–phase high performance liquid chromatography (HPLC) using Asahipak ODP–50 column (Asahi Chemical Industry Co., Ltd., Tokyo, Japan). HPLC was performed as described previously (Matsusaki *et al.*, 1996a).

Effect of heat treatment and pH on nisin Z activity

The pH of the active fraction (pH 3.6) obtained from cation–exchange chromatography was adjusted with NaOH or HCl to the following pHs: 3.6, 5.0, 7.0 and 9.0. The samples at each pH were autoclaved at 110 °C for 10 min or stood at 4 °C overnight. Thereafter, the samples were adjusted to pH 3.6 again and then assayed for antimicrobial activity against the indicator strain.

Sensitivity of nisin variants to proteolytic enzymes

A 0.2 mg/ml sample of the purified peptides (nisin Z and nisin A) after lyophilization was treated with various proteolytic enzymes at a final concentration of 1 mg/ml. The following enzymes were dissolved in 50 mM potassium phosphate buffer at the respective pHs: α–chymotrypsin (Sigma), pH 7.5; trypsin (Sigma), pH 7.5; papain (Wako Pure Chemical Industries, Ltd., Osaka, Japan), pH 6.0; proteinase K (Merck, Darmstadt, Germany), pH 7.5; ficin (Sigma), pH 7.0; pancreatin (Nacalai tesque, Inc., Kyoto, Japan), pH 7.5; actinase E (Kakenseiyaku, Tokyo, Japan), pH 7.5. Pepsin (Nacalai tesque) was dissolved in 0.01 N HCl (pH 1.8). The reaction mixtures (total volume of 2 ml) were incubated at the following temperatures: α–chymotrypsin, trypsin and papain at 25 °C; pepsin, proteinase K and ficin at 37 °C; pancreatin and actinase E at 40 °C. After incubation for 24 h, the pHs of the reaction mixtures were adjusted to pH 3 with HCl or NaOH. Thereafter, the reaction mixtures were autoclaved at 110 °C for 10 min to inactivate their enzymes. Subsequently, the antimicrobial activities of the reaction mixtures were determined by bioassay and expressed as relative values. The activities of each nisin variant after 24 h of incubation in each reaction solution without any enzymes were expressed as 100%. The tests of enzyme sensitivity were performed in triplicate.

Antimicrobial action

Exponential growth phase cells of *B. subtilis* C1 (indicator strain) were prepared by the incubation at 30 °C for 3 h aerobically in 500–ml shaking flasks with a working volume of 100 ml of the assay medium. Five milliliters of nisin Z purified from strain IO–1 (3 mg/ml in 0.02 N HCl containing 0.75% NaCl) were aseptically added to the culture, and then the flask was subjected to the subsequent incubation. Hydrochloric acid solution (0.02 N, 0.75% NaCl) was just added to the culture as a control. Cell growths were confirmed by measurement of absorbance at 562 nm.

Antimicrobial spectra of nisin variants

Antimicrobial spectra of nisin Z and nisin A purified from strain IO–1 and strain

NCDO 497, respectively, were obtained according to the method described previously (Matsusaki *et al.*, 1996a). Minimum inhibitory concentrations (MICs) of nisin variants were taken as the minimum effective activity of nisin variants per milliliter of the assay medium.

RESULTS AND DISCUSSION

Comparison of nisin variants purified

The peptide antibiotic (nisin Z) produced by *L. lactis* IO–1 was purified (Matsusaki *et al.*, 1996a) and was compared with nisin A purified from *L. lactis* NCDO 497. The purity of nisin variants was evident from Tricine–SDS–PAGE. Both peptide antibiotics showed single bands with identical molecular weights of 3.4 kDa (Fig. 2). However, nisin Z was eluted approximately 2 min later than nisin A by reversed-phase HPLC (Fig. 3). This showed apparent differences in elution time as described by de Vos *et al.* (1993). Thus,

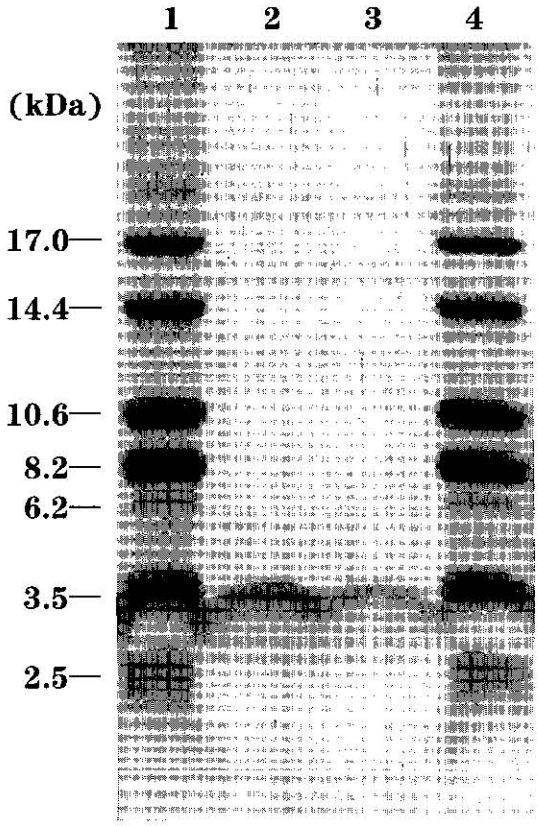

Fig. 2. Tricine–SDS–PAGE of nisin variants. Lanes: 1, 4, low molecular weight standards (kDa); 2, nisin Z purified from *L. lactis* IO–1; 3, nisin A purified from *L. lactis* NCDO 497.

the replacement of only an amino acid residue resulted in different elution times of the peptides. By HPLC, separation of two nisin variants was possible in spite of the same molecular mass by Tricine–SDS–PAGE. A slight difference in molecular mass between two nisin variants was confirmed by fast atom bombardment–mass spectrometry (Matsusaki *et al.*, 1996a).

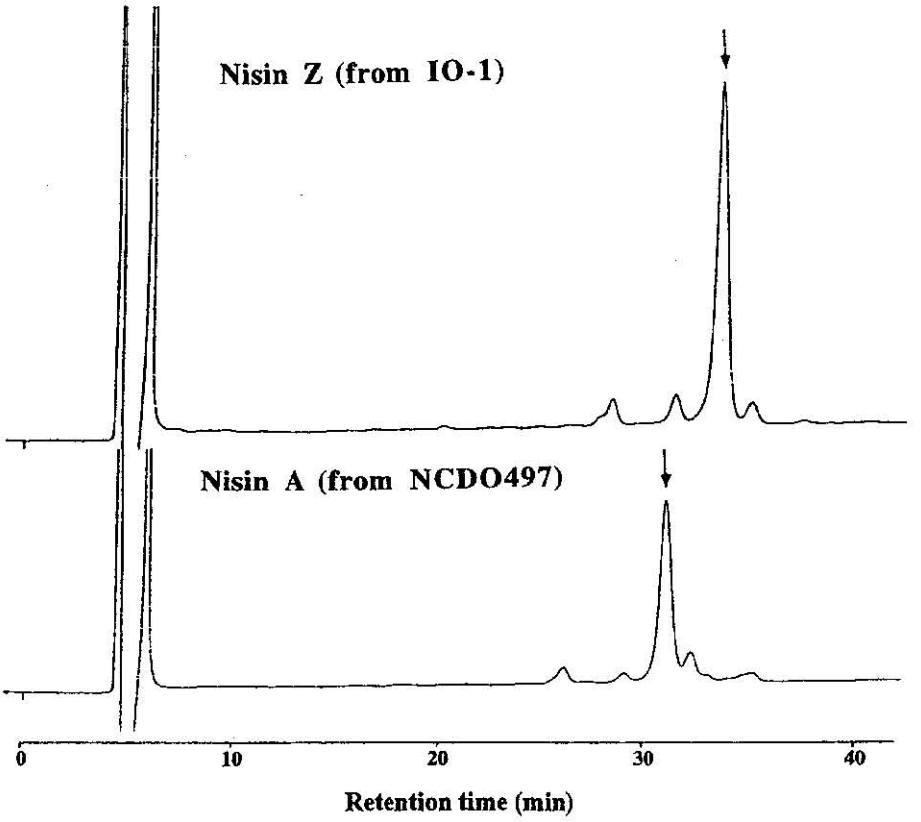

Fig. 3. Reversed-phase HPLC analyses of crude nisin Z and nisin A. The crude antibiotics obtained by cation-exchange chromatography were applied. Arrows indicate the peaks of nisin variants.

pH and heat stability of nisin Z

When autoclaved at 110 °C and pH 3.6 for 10 min, nisin Z was not inactivated as shown in Fig. 4. On the other hand, 60% and more than 85% of the activity were lost by the same treatment at pH 5.0 and above pH 7.0, respectively. The storage at 4 °C overnight resulted in a good stability in the acidic pH range but partially inactivated nisin Z in the neutral and alkaline pH range. These characteristics were similar to those of nisin A (Hurst, 1981).

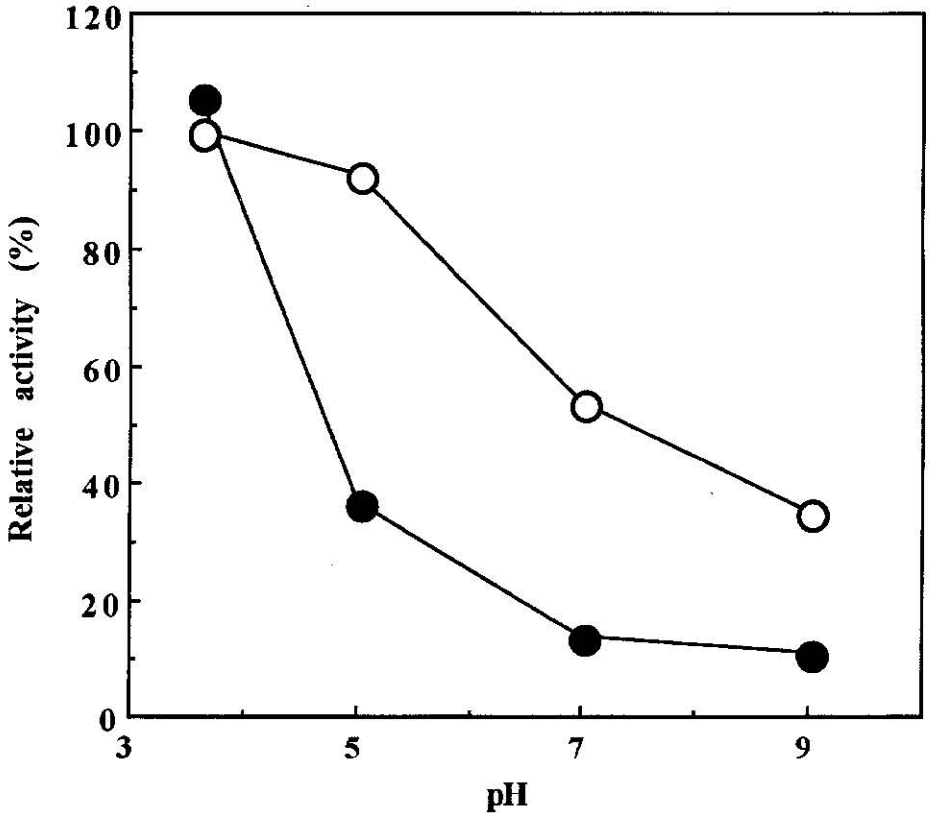

Fig. 4. pH and heat stability of nisin Z. The activities after the treatment were shown as relative values: ●, autoclaved at 110 °C for 10 min; ○, stood at 4 °C overnight.

Effect of proteolytic enzymes on antimicrobial activity

No activity of nisin Z was detected in the range of the concentration examined by treatment with proteinase K and actinase E (Table 1), and the peak of original nisin Z determined by HPLC disappeared after the treatment (data not shown). This means that nisin Z is completely degraded and inactivated by treatment with proteinase K and actinase E. The activity of nisin Z was decreased by treatment with α–chymotrypsin, trypsin, papain, ficin and pancreatin, which still remained. Although the peak of the original nisin Z was not detected by HPLC after treatments (data not shown), the enzymatic digests of nisin Z still had a low antimicrobial activity. Actually, it has been reported that the minimum structure of nisin A for antimicrobial activity is nisin $A^{1\text{-}19}$ (Wakamiya *et al.*, 1991). Therefore, the enzymatic digests seem to retain the structure exhibiting antimicrobial activities. Pepsin was shown to be ineffective on the activity of nisin Z. These results almost corresponded to those of nisin A purified from strain NCDO 497 (Table 1).

Table 1. Effect of proteolytic enzymes on the antimicrobial activity.

Enzyme	Relative activity (%)	
	Nisin Z	Nisin A
Controls[a]	100	100
Pepsin	100	100
α-chymotrypsin	22	23
Trypsin	23	23
Papain	31	57
Proteinase K	<10	<10
Ficin	35	55
Pancreatin	21	34
Actinase E	<5	<5

[a]The activities of nisin variants after 24 h of incubation in each reaction solution without any enzymes were expressed as 100%.

It has been known that nisin A is inactivated by treatment with α-chymotrypsin and pancreatin (Jarvis and Mahoney, 1969; Hurst, 1981). Jarvis and Mahoney (1969) also showed that the nisin-inactivating component of pancreatin was α-chymotrypsin. However, both nisin variants were not inactivated completely by α-chymotrypsin although the activities decreased (Table 1). The enzyme cleaves the peptide bond between residues 31 and 32 (His-Val) of nisin A (Hurst, 1981). Therefore, nisin A^{1-31} formed by the treatment of α-chymotrypsin should have the moderate antimicrobial activity since the enzyme-digested nisin has the minimum structure for antimicrobial activity in a similar manner described above. On the other hand, pepsin and trypsin were reported without effect on nisin A activity (Jarvis and Mahoney, 1969). In this study, trypsin allowed both nisin Z and nisin A to decrease in activity, the trypsin-digests having still less antimicrobial activity as in the case of α-chymotrypsin.

The alteration in activities of two nisin variants after proteolytic enzymes treatment provided different results in this study from those hitherto reported. The differences might be due to the sensitivity of bioassay. The sensitivity of bioassay is the most important for the alteration in antimicrobial activity. Critical dilution assay (Mayr-Harting *et al.*, 1972) is generally used in which the activity is defined as the reciprocal of the highest dilution yielding clear zone of growth inhibition on the indicator lawn (agar plate assay), always under carefully standardized conditions. Since nisin is regarded as difficult to diffuse in agar because of its hydrophobic property, the diffusion would be different among the enzyme-digested nisin and the agar diffusion assay may lead to erroneous results in this investigation. Therefore, turbidimetry is available for the determination of the activity compared with the agar diffusion assay. Additionally, the poor assay medium was used in this study, resulting in high sensitivity in bioassay for detecting subtle activity of the enzyme-digested nisin. Secondly, some papers described the effect of proteolytic enzymes on nisin activity by using crude nisin such as culture supernatant and commercial nisin whose content is only 2.5% (Jarvis 1967; Jarvis and Mahoney, 1969). Impurities in the samples might interfere in the accurate analysis.

Here, pure nisin Z and nisin A were used to investigate the effect of proteolytic enzymes on the activities of two nisin variants. Consequently, we elucidated that both nisin variants were inactivated or affected in activity by the treatment of not only α-chymotrypsin but also other proteolytic enzymes in a manner similar to each other although the structures are slightly different. Thus far it has been reported that the activity of nisin A is affected by α-chymotrypsin only.

Antimicrobial action

The effect of nisin Z on a sensitive indicator was examined to establish whether it demonstrated a bactericidal or a bacteriostatic mode of action. When nisin Z was added to exponential growth culture of the indicator strain, the absorbance value at 562 nm decreased rapidly (Fig. 5). This was due to cell lysis of the indicator strain, which was microscopically confirmed. Thus, nisin Z exhibited a bactericidal mode of action against the indicator, *B. subtilis* C1.

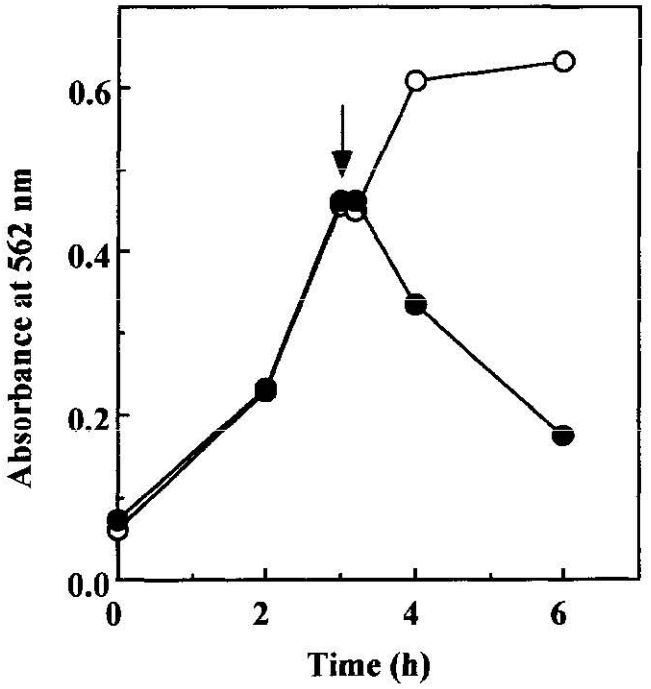

Fig. 5. Bactericidal activity of nisin Z against *B. subtilis* C1. The growth of *B. subtilis* C1 was followed by measuring absorbance at 562 nm. Arrow indicates the time of addition of nisin Z: ●, addition of nisin Z; ○, control (without nisin Z).

Antimicrobial spectrum

Nisin Z as well as nisin A inhibited the cell growths of various Gram-positive bacteria (Table 2). The MICs of nisin Z were almost the same as those of nisin A. The sensitivities of each other test strain toward nisin differed from each other in this study and our previous paper (Matsusaki *et al.*, 1996a). This is probably due to the impurities in the nisin samples as described above.

Bacteriocin-producing strains were generally immune to their own products and also

Table 2. Antimicrobial spectra of the purified nisin variants.

Strains	Minimum inhibitory concentration (MIC)	
	Nisin Z from strain IO-1 (AU/ml)	Nisin A from strain NCDO 497(AU/ml)
B. subtilis C1	10	10
B. subtilis JCM 1465[T]	20	20
B. coagulans JCM 2257[T]	10	5
B. stearothermophilus JCM 2501[T]	2.5	2.5
M. luteus IFO 12708	5	5
E. faecalis JCM 5803[T]	40	50
Cl. acetobutylicum IFO 13948[T]	2	1
L. lactis subsp. *cremoris* TUA 1344L	10	10
L. lactis subsp. *lactis* JCM 5805[T]	20	20
L. lactis NCDO 497	150	150
L. lactis IO-1	200	200
E. coli JM 109	300	300

Nisin Z and A purified from *L. lactis* IO-1 and NCDO 497, respectively, were used in this experiment. All the assay media were 10-fold diluted the usual ones as described previously (Matsusaki *et al.*, 1996a).
JCM, Japan Collection of Microorganisms; IFO, Institute for Fermentation, Osaka, Japan; TUA, Tokyo University of Agriculture, Japan; NCDO, National Collection of Dairy Organisms, Reading, England.

showed similar immunity to the analogous antibiotics, indicating cross-immunity (De Vuyst and Vandamme, 1994). However, the cell growths of strains IO-1 and NCDO 497 were not observed by the addition of nisin variants under the assay condition as described in Materials and Methods section. The MICs of nisin Z against strains IO-1 and NCDO 497 were 200 and 150 AU/ml, respectively as in the case of nisin A (Table 2). Although nisin-producing strains have immunity gene, *nisI* (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Siegers and Entian, 1995), it was found that the growths of these strains were inhibited by high concentration of nisin in this study. It has been reported that the bactericidal action of nisin A occurs in the cytoplasmic membrane of an indicator cells (Henning *et al.*, 1986; Sahl 1991). Cellular damage ranges from the loss of the proton motive force to the disruption of the cellular integrity of the membrane. The growth inhibition of nisin-producing strains by high concentration of nisin may be due to long lag time necessary for the expression of the *nisI* gene, or the repair of the pores of the

cytoplasmic membrane caused by nisin.

Nisin production was shown to be growth-associated for most of the growth of the producing-strains (De Vuyst and Vandamme, 1992; Matsusaki *et al.*, 1996b). The above-mentioned phenomena such as bacteriostatic action probably result from product inhibition. Therefore, it is preferable to remove nisin Z produced from the culture broth when the effective continuous production of nisin Z is carried out. Construction of the integrated bioreactor system with nisin Z separation unit is now in progress.

Furthermore, *E. coli*, Gram-negative bacterium, was also inhibited by nisin variants at the concentration of 300 AU/ml. In Gram-negative bacteria, the outer membrane of the envelope probably acts as a permeability barrier for the cells. Growth inhibition of Gram-negative bacterium, *Salmonella typhimurium* against nisin A using chelating agent EDTA was also reported (Stevens *et al.*, 1991), although Gram-negative bacteria were generally resistant to nisin A. EDTA binds magnesium ions in the lipopolysaccharide layer, resulting in the increase of susceptibility to antibiotics. As described above, Gram-negative bacteria are resistant to nisin, while the cell growth of *E. coli* was slightly affected by nisin variants in this study (Table 2). By the use of high concentration of nisin, Henning *et al.* (1986) noted inhibition of yeasts and only few species of fungi.

After subsequent cultivation for 48 h, the nisin-producing strains and *E. coli* gradually grew and reached to the same level of the controls (without nisin variants). Therefore, this may not be killing effect like bactericidal action observed in Fig. 5 but probably be due to bacteriostatic action. Since nisin Z inactivated by autoclaving at pH 12 did not inhibit the cell growths of nisin-producing strains and *E. coli* (data not shown), it was regarded clearly that the active nisin Z provided the inhibitory function.

In this study, we investigated some characteristics of nisin Z, a natural nisin variant, produced by *L. lactis* IO-1. As a consequence, the characteristics of the peptide was almost the same as those of nisin A. We found the more effective proteolytic enzymes (proteinase K and actinase E) rather than α-chymotrypsin hitherto reported to inactivate nisin variants, and elucidated that α-chymotrypsin could not completely inactivate nisin variants although it affected the decrease in the activity. Furthermore, nisin variants inhibited not only the growths of Gram-positive bacteria but also the growths of *E. coli*, a Gram-negative bacterium and nisin-producing strains. When the fine change in bacteriocin activity is investigated, an assay method for the accurate evaluation should be the most important and be introduced. Here, the assay medium consisting of poor nutrients was used to increase detectable sensitivity, while nutrient-rich medium requires a large amount of the purified antibiotic owing to the higher growth rate of an indicator strain, and the antimicrobial activity of the enzyme-digested antibiotic can not be detected. The poor assay medium contributed to effective bioassay, resulting in apparent findings obtained in this study. The results described here will provide further study on nisin, for example, the development of the bioreactor with a separation system to avoid a product (nisin) inhibition for enhancement of nisin productivity and down-stream processing such as the fractionation and the extraction of nisin. The stability of nisin is related to its solubility (De Vuyst and Vandamme, 1994). If nisin is more soluble and stable at neutral pH, it would be widely applicable as a food preservative. It is expected that nisin Z should be more soluble than nisin A above pH 6 since the asparagine side chain is more polar than the histidine side chain. Therefore, nisin Z should be used rather than nisin A as a

food preservative. However, the property of nisin Z is not necessarily superior to that of nisin A since nisin Z was also instable above pH 7 (Fig. 4). That is, even natural nisin Z, which is substitued with only single amino acid residue in nisin A, is insufficient for the use at neutral pH. The stability of nisin Z should be improved by protein engineering, and the biological and chemical properties of the engineered mutants have to be evaluated in comparison with the wild-type nisin Z. The results of wild-type nisin Z obtained in this study will contribute to make more stable nisin Z mutant with functional properties. Since the minimum structure for antimicrobial activity is nisin$^{1-19}$ (Wakamiya *et al.*, 1991), the C-terminal amino acid residue should be substituted for polar amino acid by the method such as site-directed mutagenesis. If nisin Z mutant with high stability and antimicrobial activity at neutral pH is constructed and the safety is recognized, the use of the mutant would wide-spread as not only a food preservative but also an antibiotic agent.

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