Some Characteristics of Nisin Z, a Peptide Antibiotic 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. Nisin Z was heat stable at acidic pH and showed a 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 results 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 (a nisin A producer) were sensitive to a high concentration of nisin variants including their own products. The growth of a Gram-negative bacterium, *Escherichia coli*, was also inhibited by a high concentration of nisin, although nisin producers and Gram-negative bacteria are generally resistant to nisin variants.

Keywords: nisin, bacteriocin, lantibiotic, lactic acid bacteria, *Lactococcus*

Historically, food fermentations were empirical and fortuitous processes based on the activities of the natural flora contaminating the raw material. Not only lactic acid bacteria are responsible for the specific taste and texture that develop during the preparation by fermentation of various kinds of traditional foods, but also they have prevented the rapid spoilage of dairy, meat and vegetables for a long time. Bacteriocins, proteinaceous compounds, produced by some lactic acid bacteria generally inhibit the growth of closely related species (Klaenhammer, 1988, 1993; Nettles & Barefoot, 1993; Ralph *et al.*, 1995). In these years, microbiological, biochemical and genetic characterization of bacteriocins have been well studied. Similarly, the possibility of whether novel bacteriocins isolated could be used as food preservatives should also be investigated. Nisin, or more precisely nisin A, the most famous bacteriocin, is heat-stable at acidic pH and has a broad antimicrobial spectrum against Gram-positive bacteria (Hurst, 1981); it has been accepted for use as a food preservative in many countries (Delves-Broughton, 1990). Because nisin A is unstable and inactivated at neutral pH, however, its use as a preservative is limited at present. Nisin has two natural variants, nisin A (Gross & 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 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 a 3-fold activity of nisin Z in the culture broth under optimal conditions (Matsusaki *et al.*, 1996b). Nisin Z has the possibility of use as a food preservative similarly to nisin A. Because there are only

a few reports on the characterization and properties of nisin Z (De Vuyst & 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 the stability and antimicrobial activity of nisin Z as a food preservative. Therefore, it is necessary that the characterization of pH and heat stability, sensitivity to proteolytic enzymes and antimicrobial spectrum of wild-type nisin Z is well investigated. The characterization and the properties of nisin Z elucidated in this study will also provide much information for the commercial application and effective production of nisin Z.

Materials and Methods

Bacterial strains and culture conditions *Lactococcus lactis* IO-1 isolated in our laboratory was used as the nisin Z-producing strain (Matsusaki *et al.*, 1996a). To compare nisin Z with nisin A, *L. lactis* NCDO 497, a nisin A-producing strain, was also used. The indicator strain used for the assay of antimicrobial activity was *Bacillus subtilis* C1 as described previously (Matsusaki *et al.*, 1996a). To test the sensitivity to nisin variants, *L. lactis* JCM 5805^T (a 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, bacilli and *C. acetobutylicum* were grown as described previously (Matsusaki *et al.*, 1996a). *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 the

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 & Morell, 1971).

antimicrobial activity, the 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, Detroit, Mich.), 0.05% polypeptone (Nihon Seiyaku, Co., Ltd., Tokyo), 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) equivalent to the activity of 1 μg of commercial nisin (nisin A; ICN Biomedicals, Inc., Costa Mesa, Cal.; activity, 1,000 U/mg-solid; nisin 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 a tC_{18} Sep-Pak cartridge (Waters, Milford, Mass.) and lyophilization (Matsusaki *et al.*, 1996a). Nisin A was also purified from the culture supernatant of strain NCDO 497 in a manner similar to the above.

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 allowed to stand 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 Chemical Company, St. Louis, Mo.), pH 7.5; trypsin (Sigma), pH 7.5; papain (Wako Pure Chemical Industries, Ltd., Osaka), pH 6.0; proteinase K (Merck, Darmstadt, Germany), pH 7.5; ficin (Sigma), pH 7.0; pancreatin (Nacalai Tesque, Inc., Kyoto), pH 7.5; actinase E (Kakenseiyaku, Tokyo), 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 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 the flask was then subjected to the subsequent incubation. Hydrochloric acid solution (0.02 N, 0.75% NaCl) was added to the culture as a control. Cell growths were confirmed by measurement of the absorbance at 562 nm.

Antimicrobial spectra of nisin variants The antimicrobial spectra of nisin Z and nisin A purified from strain IO-1 and strain NCDO 497 were obtained according to the method described previously (Matsusaki *et al.*, 1996a). The minimum inhibitory concentrations (MICs) of nisin variants

were taken as the minimum effective activity of the nisin variants per milliliter of the assay medium.

Results and Discussion

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. 2. 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 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).

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 the 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 A¹⁻¹⁹ (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 nearly corresponded to those of nisin A purified from strain NCDO 497 (Table 1).

It has been known that nisin A is inactivated by treatment with α -chymotrypsin and pancreatin (Jarvis & 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 A¹⁻³¹ formed by the treatment of α -chymotrypsin should have moderate antimicrobial activity because the enzyme-digested nisin has the minimum structure for antimicrobial activity in a manner similar to that described above. On the other hand, pepsin and trypsin were reported to be without effect on nisin A activity (Jarvis & 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 the 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 the bioassay. The sensitivity of the bioassay is 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 a clear zone of growth inhibition on the indicator lawn (agar plate assay), always under carefully standardized conditions. Nisin is regarded as difficult to diffuse in agar because of its hydrophobic property; therefore, the diffusion would be different in 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, a poor assay medium was used in this study, resulting in high sensitivity in the bioassay for detecting subtle activity of the enzyme-digested nisin. Secondly, some papers described the effect of proteolytic enzymes on nisin activity using crude nisin such as the culture supernatant and commercial nisin whose content is only 2.5% (Jarvis 1967; Jarvis & Mahoney, 1969). Impurities in the samples might interfere with the accurate analysis. Here, pure nisin Z and nisin A were used to investigate the effect of proteolytic enzymes on the activities of the two nisin variants. Consequently, we elucidated that both nisin variants were inactivated or affected in activity by the treatment not only with α -chymotrypsin but also with other proteolytic enzymes in a similar manner, although the structures are slightly different. Thus far it has been reported

Fig. 2. pH and heat stability of nisin Z. Nisin Z was autoclaved at 110°C for 10 min (●) or allowed to stand at 4°C overnight (○). The activities after the treatment are shown as relative values. The activity of nisin Z stored at pH 3.6 overnight is expressed as 100%.

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 are expressed as 100%.

that the activity of nisin A is affected only by α -chymotrypsin.

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. 3). 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.

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 test strain toward nisin differed from the others 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 showed similar immunity to the analogous antibiotics, indicating cross-immunity (De Vuyst & 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 conditions as described in the 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 the immunity gene, *nisI* (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Siegers & Entian, 1995), it was found that the growths of these strains were inhibited by the 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 cell (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 a high concentration of nisin may be due to the

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 & 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 the nisin Z produced from the culture broth when the effective continuous production of nisin Z is carried out. Construction of an integrated bioreactor system with a nisin Z separation unit is now in progress.

Furthermore, *E. coli*, a Gram-negative bacterium, was also inhibited by nisin variants at a 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 an increase in susceptibility to antibiotics. As described above, Gram-negative bacteria are resistant to nisin, although the cell growth of *E. coli* was slightly affected by the nisin variants in this study (Table 2). Using a high concentration of nisin, Henning *et al.* (1986) noted the inhibition of yeasts and only a few species of fungi.

After subsequent cultivation for 48 h, the nisin-producing strains and *E. coli* gradually grew and reached the same level as the controls (without the nisin variants). Therefore, this may not be a killing effect like the bactericidal action observed in Fig. 3 but is probably due to bacteriostatic action. Nisin Z inactivated by autoclaving at pH 12 did not inhibit the cell growths of nisin-producing strains and *E. coli* (data not shown); therefore, it was clearly regarded that the active nisin Z provided the inhibitory function.

In this study, we investigated some characteristics of nisin

Fig. 3. Bactericidal activity of nisin Z against *B. subtilis* C1. The growth of *B. subtilis* C1 was followed by measuring the absorbance at 562 nm. Arrow indicates the time of addition of nisin Z: ●, addition of nisin Z; ○, control (without nisin Z).

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)
<i>B. subtilis</i> C1	10	10
<i>B. subtilis</i> JCM 1465 ^T	20	20
<i>B. coagulans</i> JCM 2257 ^T	10	5
<i>B. stearothermophilus</i> JCM 2501 ^T	2.5	2.5
<i>M. luteus</i> IFO 12708	5	5
<i>E. faecalis</i> JCM 5803 ^T	40	50
<i>Cl. acetobutylicum</i> IFO 13948 ^T	2	1
<i>L. lactis</i> subsp. <i>cremoris</i> TUA 1344L	10	10
<i>L. lactis</i> subsp. <i>lactis</i> JCM 5805 ^T	20	20
<i>L. lactis</i> NCDO 497	150	150
<i>L. lactis</i> IO-1	200	200
<i>E. coli</i> 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 compared to 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.

Z, a natural nisin variant, produced by *L. lactis* IO-1. As a consequence, the characteristics of the peptide were almost the same as those of nisin A. We found that the more effective proteolytic enzymes (proteinase K and actinase E) rather than α -chymotrypsin hitherto reported inactivated the nisin variants and elucidated that α -chymotrypsin could not completely inactivate the nisin variants although it caused a 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 a fine change in bacteriocin activity is investigated, an assay method for accurate evaluation should be the most important and must be introduced. Here, an assay medium consisting of poor nutrients was used to increase detectable sensitivity, while a nutrient-rich medium requires a large amount of the purified antibiotic due to the higher growth rate of an indicator strain, and the antimicrobial activity of the enzyme-digested antibiotic cannot be detected. The poor assay medium contributed to the effective bioassay, resulting in the findings obtained in this study. The results described here will lead to further study on nisin, for example, the development of a bioreactor with a separation system to avoid product (nisin) inhibition for enhancement of nisin productivity and downstream processing such as the fractionation and the extraction of nisin. The stability of nisin is related to its solubility (De Vuyst & 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 because 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 because nisin Z is also unstable above pH 7 (Fig. 2). That is, even natural nisin Z, which differs from nisin A by only a single substituted amino acid residue, is insufficient for 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 create a more stable nisin Z mutant with functional properties. Because the minimum structure for antimicrobial activity is nisin¹⁻¹⁹ (Wakamiya *et al.*, 1991), the C-terminal amino acid residue should be substituted by a polar amino acid using a method such as site-directed mutagenesis. If the nisin Z mutant with high stability and antimicrobial activity at neutral pH is constructed and its safety is recognized, the use of the mutant could be widespread not only as a food preservative but also as an antibiotic agent.

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