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Purification and Identification of a Peptide Antibiotic Produced by *Lactococcus lactis* IO-1

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Lactococcus lactis IO-1, which was isolated in our laboratory, produces a new peptide antibiotic that was proposed previously to be a variant of nisin (Ishizaki *et al.*, 1992a *J. Fac. Agr., Kyushu Univ.*, 37: l-11). Further purification to homogeneity of the peptide from the culture supernatant allowed us to identify it as a derivative of natural nisin, designated nisin Z, as follows. The culture supernatant of *L. lactis* IO-1 inhibited the growth of various Gram-positive bacteria but not **that** of a nisin A-producing strain, *L. lactis* NCDO *497, as* shown also in the case of a commercial preparation of nisin (nisin A). A nisin-like peptide antibiotic produced by *L. lactis IO-1* was efficiently purified by a rapid and simple procedure, which included acid treatment, ammonium sulfate precipitation, cation-exchange chromatography and reversed-phase high performance liquid chromatography. The specific activity of the purified antibiotic was 122-fold greater than that of the starting material and the recovery was 24%. The molecular mass of this peptide antibiotic was 3337.9 by fast atom bombardment - mass spectrometry (FAB-MS). Amino acid, FAB-MS and 'H-NMR analyses of the peptide antibiotic purified from strain IO-l, and the deduced amino acid sequence of the precursor peptide (Araya *et al.*, 1992 *J. Gen. Appl. Microbiol.*, 38: 271-278) showed that it differed from nisin A by a single amino acid substitution (His27 → Asn27) and was identical to nisin Z, a natural variant of nisin

INTRODUCTION

Lactic acid bacteria are important in the food industry, in particular for the production of traditional fermented foods. They are responsible for the specific taste and texture that develop during the preparation by fermentation of various kinds of traditional food. They produce a variety of antibacterial compounds, such as organic acids, diacetyl and hydrogen peroxide. Some lactic acid bacteria produce bacteriocins, which are proteinaceous compounds that are generally able to inhibit the growth of closely related species (Klaenhammer, 1988). Some bacteriocins can also inhibit the growth of pathogens and spoilage organisms during the processing and fermentation of foods. It is anticipated that they will be used as natural food preservatives in the near future.

Nisin, or more precisely nisin A is a bacteriocin that is produced by *Lactococcus lactis*. It is known as a peptide antibiotic (Mattick and Hirsch, 1944, 1947; Hurst, 1981) and a lantibiotic such as epidermin (Allgaier et al., 1985) and Pep5 (Kellner et **al.,** 1989). Nisin is heat-stable at low pH and has antibacterial actions against a broad spectrum of Gram-positive bacteria. In view of its strong activity against clostridia and listeria, which are food pathogens, and the fact that it can be degraded in the human alimentary canal, nisin A has been approved by the World Health Organization as a food preservative for use in the food industry. Nisin (nisin A), identified by Gross and Morel1 (1971), has a

molecular mass of 3353.8 and is composed of 34 amino acids, which include the unusual amino acids of dehydroalanine (DHA), dehydrobutyrine (DHB), lanthionine and 3-methyllanthionine. Nisin is synthesized on ribosomes as a precursor peptide of 57 amino acids. It is subjected to various modifications, including cleavage of a leader peptide of 23 amino acids, by post-translational processing (Buchman et al., 1988; Kaletta and Entian, 1989), which yields the final product, namely, active nisin. Genes involved in the biosynthesis of nisin have been cloned and sequenced (Engelke *et al., 1992,* 1994; Kuipers et *al.,* 1993; van der Meer et *al., 1993).* However, the mechanism of biosynthesis of nisin has not been satisfactorily characterized. Further information about nisin and nisin-like peptides is required.

L. lactis IO-l, isolated in our laboratory, produces a nisin-like peptide antibiotic (Ishizaki et al., 1990a, b). Araya et al. (1992) cloned and sequenced a DNA fragment from *L. lactis* 10-l that was highly homologous to the structural gene for nisin. From the deduced amino acid sequence of the precursor peptide, the peptide antibiotic from *L. Zactis* IO-1 was deduced to be nisin Z. However, since formation of the dehydro residues and the thioether cross-linkages in nisin is due to stereospecific reactions by as yet unidentified enzymes, genetic analysis is only one of the approaches to the characterization of these peptides. Therefore, the structure of peptides such as nisin must be elucidated after their complete purification. In a previous report (Ishizaki *et al.,* 1992a), we clarified the presence of dehydro residues in the peptide antibiotic from *L. Zactis IO-1* and suggested that it might be a new derivative of nisin. Despite the results of the analysis of the purified peptide in the previous study, the structure of the peptide is still unclear because degradation and/or aggregation of the peptide might have taken place during the previous purification process. In this report, we describe the efficient purification of the peptide antibiotic from *L. lactis* JO-1 with high recovery and we provide detailed identification, including determination of the exact molecular mass by FAB-MS.

MATERIALS AND METHODS

Bacterial strain and production of the antibiotic

The microorganism used for production of the antibiotic was *L. lactis* IO-1 (JCM7638), which had been isolated in our laboratory (Ishizaki et al., 1990a). The indicator strain for assays of antibacterial activity was Bacillus *subtilis* Cl, which was also isolated in our laboratory (Ishizaki et *al.,* 199213). B. *subtilis* Cl was grown in bouillon medium that consisted of 0.7% meat extract, 1% polypeptone and 0.5% NaCl at 30°C.

Unless otherwise noted, the antibiotic was produced as follows. The stock culture of strain IO-1 was subcultured in 10 ml of thioglycolate medium without glucose (TGC medium; Difco Laboratories, Detroit, MI, USA) at 37°C for 18 h in a static culture, which was then transferred to 100 ml of the following medium, referred to CMG medium. CMG medium contained 0.5% yeast extract, 0.5% polypeptone, 0.5% NaCl and 1% glucose in distilled water at pH 7.0 for preculture. An aliquot of 30 ml of the preculture, after growth at 37°C for 3 h, was inoculated into a 5-liter jar fermenter that contained 3 liters of CMG medium prepared with 3% glucose. The fermentation was carried out at pH 6.0,

maintained by a feeding system that supplied 5 N NaOH, at 37°C with agitation (400 rpm). Cell growth was monitored in terms of absorbance at 562 nm and values were converted to dry cell weight by reference to a standard curve.

Bioassay

For assays of antibacterial activity, inhibition of the growth of the indicator strain *B. subtilis Cl* was determined by turbidimetry as follows. An appropriate volume of the assay sample was added aseptically to 5 ml of the assay medium (IO-fold diluted CMG medium). The assay medium in each tube was then supplemented with an aliquot of a culture of the indicator strain that had been grown for 18 h with shaking (180 strokes/min) to give a cell concentration of approximately 10^6 cells/ml and the assay mixture was incubated at 30°C for 15 h. Growth of the indicator strain was measured quantitatively as the absorbance at 562 nm. One unit of activity of the antibiotic produced by *L. Zactis* IO-1 is defined herein as an arbitrary unit (AU) of activity that is equivalent to the activity of 1 pg of commercial nisin (ICN Biomedicals, Inc., Costa Mesa, CA, USA; activity, 1,000 U/mg solid; nisin content, 2.5%).

The antibacterial spectrum was determined as follows. All anaerobic bacteria tested were grown for 24 h in static culture, and all aerobic bacteria were grown for 18 h with shaking (120 strokes/min). Each culture was inoculated into the assay medium (IO-fold diluted CMG medium) to give a cell concentration of approximately 10^6 cells/ml. A simple exception was the assay medium for *Clostridium acetobutylicum*, which was lo-fold diluted RCM medium. RCM medium contained 0.3% yeast extract, 1.0% meat extract, 1.0% trypticase peptone (Becton Dickinson, Cockeysville, MD, USA), 0.5% glucose, 0.5% NaCl, 0.3% sodium acetate, 0.1% soluble starch and 0.05% L-cysteine hydrochloride (pH 6.8). The culture broth of *L. Zactis* IO-1 was centrifuged at 7,300 X g for 30 min to pellet the cells. The pH of the supernatant was then adjusted to 3.0 with concentrated HCl. After the supernatant had stood overnight at 4°C, it was centrifuged at 7,300 X g for 30 min to remove any precipitate that had formed. The resulting supernatant was aseptically added to the assay medium in each tube. After incubation at each optimal growth temperature for 18 h, the minimum inhibitory concentration (MIC) of the culture supernatant from *L. lactis* IO-1 was taken as the minimum effective volume of the supernatant per milliliter of the assay medium. The antibacterial activity of commercial nisin was taken as the control activity.

Separation of the antibiotic

The active supernatant after acid treatment of the culture broth of *L. lactis* 10-l from a jar fermenter was brought to 70% saturation by the slow addition of solid ammonium sulfate and allowed to stand overnight at 4°C. The precipitate was collected by centrifugation at 7,300 × g for 30 min and then suspended in a small amount of 0.01 N HCl. This suspension was dialyzed against 8 M urea-HCl (pH 3.0) in a Spectra/Por membrane (Spectrum Medical Industries, Inc., Houston, TX, USA; molecular weight cut-off, 1,000) to desalt the protein and disrupt any aggregates.

Cation-exchange chromatography

Further purification of the antibiotic was carried out by cation-exchange chromatog-

raphy on a column of CM-Sephadex C-25 (Pharmacia, Uppsala, Sweden; 5 cm i.d. X 20 cm). The dialysate was loaded on the column and washed with 20 mM acetate buffer (pH 3.6) at a flow rate of 1 ml/min for 100 min. The column was subsequently eluted with a 0-1.2 M NaCl-20 mM acetate buffer (pH 3.6), as a linear gradient, at a flow rate of 1 ml/min. Absorbance was monitored at 210 nm.

Reversed-phase high performance liquid chromatography

The pooled active fractions obtained from the cation-exchange column were loaded on a tC_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) and the column was washed with 0.05% trifluoroacetic acid (TFA). The fraction with antibacterial activity was eluted with 50% acetonitrile in 0.05% TFA, desalted and concentrated. The eluate was then lyophilized. The active material was then dissolved in a small quantity of 0.01 N HCl. Further purification was performed by a reversed-phase high performance liquid chromatography (HPLC) on an Asahipak ODP-50 column (Asahi Chemical Industry Co., Ltd., Tokyo, Japan). For the mobile phase, solvent A was 0.05% TFA in distilled water and solvent B was 0.05% TFA in 100% acetonitrile. For preparative purposes, a large column (21.5 mm i.d. × 250 mm) was used with a linear gradient of 30-35% solvent B in solvent A over the course of 50 min at a flow rate of 4 ml/min. Analytical chromatography was performed on a smaller column (6.0 mm i.d. X 250 mm) with a linear gradient of 2633% solvent B in solvent A over the course of 40 min at a flow rate of 1 ml/min. Absorbance was monitored at 210 nm. Each fraction with antibacterial activity was lyophilized.

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) was performed by the method of Schagger and von Jagow (1987) in 16.5% T- 6% C gels. T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide). C denotes the percentage concentration of the crosslinker relative to the total concentration T. A molecular mass marker kit (MW-SDS-17; Sigma Chemical Company, St. Louis, MO, USA) was used as a source of standard proteins. After electrophoresis, the gel was stained with 0.025% SERVA blue G (Serva, Heidelberg, Germany) in 10% acetic acid. The molecular mass of the antibiotic was estimated after calculation of relative mobilities and regression analysis.

Amino acid composition and sequence analysis

The peptide antibiotic (0.3 mg) purified from strain IO-1 was hydrolyzed by incubation with 6 N HCl at 110°C for 24 h in a sealed vial. Amino acid analysis of the hydrolyzate was performed with an amino acid analyzer (model 655A; Hitachi, Tokyo, Japan). The lanthionine and 3-methyllanthionine content was estimated with DL-lanthionine as the standard.

The peptide antibiotic was sequenced by Edman degradation with an automated gas-phase protein sequencer (PSQ-1; Shimadzu, Kyoto, Japan) equipped with an on-line LC-6A HPLC system (Shimadzu).

Determination of molecular mass

The peptide antibiotic purified from strain IO-1 was dissolved in 0.01 N HCl. The

molecular mass of the peptide was determined by fast atom bombardment - mass spectrometry (FAB-MS) with a mass spectrometer (JMS-SX102; JEOL, Tokyo, Japan) equipped with a JMA-DA 6000 data system (JEOL). Glycerol was used as the matrix.

NMR analysis

The 'H-NMR spectrum was recorded at 25°C with a JNM GSX 400 spectrometer (JEOL) operated at 400 MHz. The sample contained approximately 2 mg of peptide in a mixture of H_2O and ^2H_2O (9:1, v/v) and the pH was adjusted to 3.3 with HCl. The solvent resonance was suppressed by presaturation. The chemical shifts given are relative to 3-trimethylsilyl(2,2,3,3-$^2H_4$) propionic acid.

RESULTS AND DISCUSSION

Production of the peptide antibiotic from *L. lactis* 10-l

Figure 1 shows the time course of the production of antibiotic during the growth of *L. lactis* IO-l. The culture supernatant without concentration (pH 6.0) had little antibacterial activity but considerable activity became apparent when the pH was adjusted to 3.0 with concentrated HCl. Antibacterial activity increased in almost direct proportion to the extent of cell growth. Antibacterial activity increased to a maximum at the early stationary phase of growth, after which there was a sharp decrease in activity. Such changes are well known characteristics of the synthesis of bacteriocins including nisin (De Vuyst and Vandamme, 1992) by lactic acid bacteria, and they are due to hydrolytic enzymes that are secreted. Consequently, for the purification of the antibiotic, the culture supernatant was collected at the early stationary phase of growth.

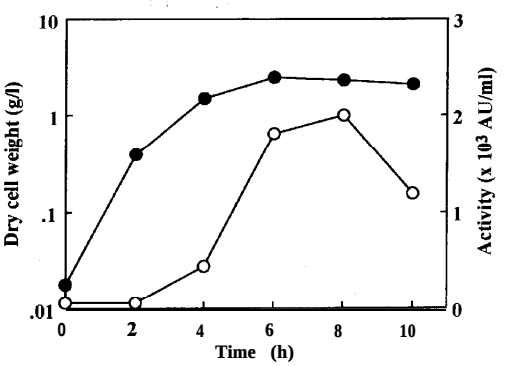


Fig. 1 Batch fermentation profile of the microbial growth of *L. lactis* 10-l (●) and production of the antibiotic (○). Analysis was performed with a 1 -liter jar fermenter with a working volume of 406 ml.

Antibacterial spectrum

The antibacterial spectrum of the antibiotic secreted in the culture broth of *L. Zactis* 10-l was determined and compared with that of commercial nisin. As described in our previous report (Ishizaki et ***al.,*** 1992a), the culture supernatant from strain IO-1 inhibited not only the growth of closely related species, a general characteristic of bacteriocins, but it also inhibited the growth of the various Gram-positive bacteria tested, as did commercial nisin. However, *L. lactis* NCDO 497, a nisin A-producing strain and *L. lactis* IO-l were insensitive both to commercial nisin and to the culture supernatant from strain IO-1. This result indicates that the antibiotic-producing strains were immune to their own products. In addition, these producer strains showed similar immunity to each of the antibiotics, namely, cross-immunity. On the basis of these results, the antibiotic produced by strain IO-1 was expected to be a derivative of nisin.

Purification of the peptide antibiotic produced by *L. lactis* IO-1

The peptide antibiotic produced by *L. Zactis* IO-l, was partially purified by acid treatment, ammonium sulfate precipitation and cation-exchange chromatography. The activity of the supernatant after acid treatment was approximately 2,000 AU/ml (Table 1). Ammonium sulfate precipitation resulted in a 13-fold increase in the specific activity with recovery of approximately 80% of the activity. After dialysis, the active material was fractionated by cation-exchange chromatography on a column of CM-Sephadex C-25, as described in Materials and Methods (Fig. 2). Fractions 84-96 with clear antibacterial activity were collected after the bioassay. Cation-exchange chromatography resulted in a 68-fold increase in the specific activity and recovery of the activity was approximately 70% (Table 1).

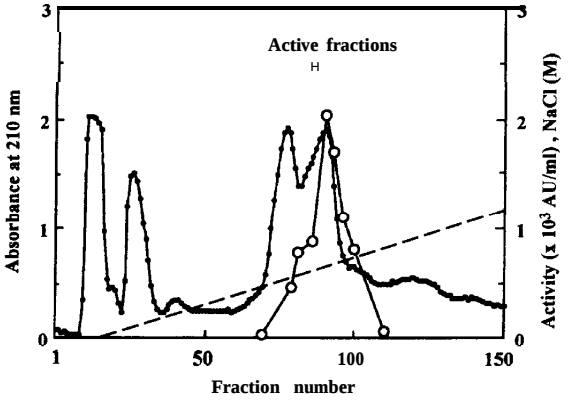


Fig. 2. Purification of the antibiotic by cation-exchange chromatography. Symbols: ●, absorbance at 210 nm; ○, antibacterial activity (AU/ml); -------, NaCl gradient. Fractions 84-96, indicated by the bar, were collected for further purification.

When the active fractions from cation-exchange chromatography were analyzed by reversed-phase HPLC, four peaks were obtained, as shown in Fig. 3. The four peak fractions, nos. 1, 2, 3 and 4, had different antibacterial activities. The major peak fraction, no. 3, was regarded as that of the desired antibiotic produced by *L. lactis* IO-l. It included approximately 70 % of the activity in the total eluate. Since the other peak fractions (nos. 1, 2 and 4) had low activity and were also found in the analysis of the original culture supernatant, they were probably not generated by degradation of the mature peptide during our purification process.

The purity of the antibiotic was assessed by Tricine-SDS-PAGE. Peak fraction no. 3,

Table 1. Purification of the peptide antibiotic produced by *L. lactis* IO-l.

Sample	Volume (ml)	Activity (AU/ml)	Total protein[b] (mg)	Total activity ($\times 10^3$ AU)	Specific activity (AU/mg)	Recovery (%)	Purification (-fold)
Culture supernatant[a] (pH 6)	600		1,470				
Culture supernatant (pH 3) after acid treatment	600	1,960	1,330	1,180	886	100	1
Ammonium sulfate precipitate (70% saturation)	8.00	122,000	82.4	978	11,900	83	13
Cation-exchange eluate	130	6,650	14.3	864	60,400	73	68
C_{18}-HPLC extract eluate	130	2,140	2.58	278	108,000	24	122

[a] The culture supernatant was obtained from the culture broth of *L. lactis* IO-1 after 13 h cultivation, as described in Materials and Methods.

[b] Protein was estimated by Lowry's modified method (1951).

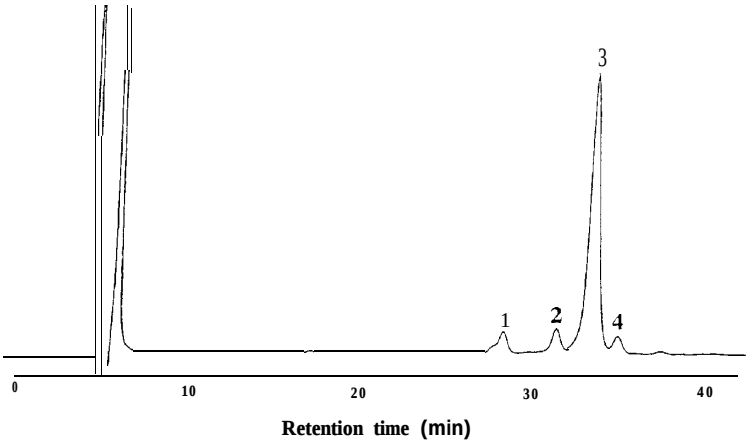


Fig. 3. Analysis by reversed-phase HPLC of the crude antibiotic. The crude antibiotic obtained by cation-exchange chromatography was subjected to HPLC without concentration.

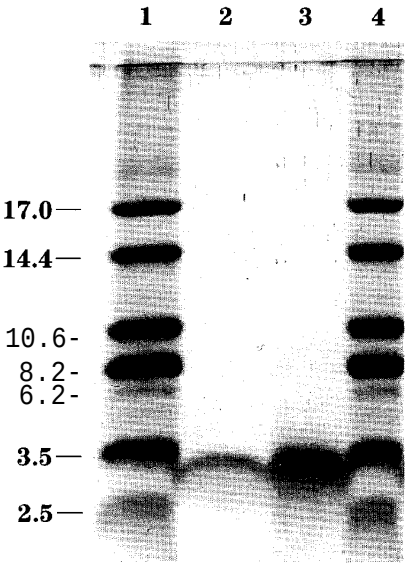


Fig. 4. Tricine-SDS-PAGE of the purified antibiotic. Lanes: 1, 4, low-molecular-mass standards (kDa); 2, peak fraction no. 3 obtained by HPLC; 3, commercial nisin.

obtained by HPLC, was concentrated with a tC_{18} Sep-Pak cartridge. After lyophilization and dissolution in a small quantity of 0.01 N HCl, the active material was subjected to Tricine-SDS-PAGE. A single band was detected on the gel, and the molecular mass of the peptide was estimated to be approximately 3.4 kDa (Fig. 4). Analysis by Tricine-SDS-PAGE indicated that the molecular mass of the peptide antibiotic from strain IO-1 was almost the same as that of commercial nisin, namely, nisin A.

Finally, the peptide antibiotic was highly purified by HPLC. The product had a specific activity that was 122-fold greater than that of the culture supernatant and the recovery was 24% (Table 1).

Bacteriocins and nisin easily form aggregates (Mørtvedt et al., 1991; Muriana and Klaenhammer, 1991; Jarvis *et al.,* 1968; Piard *et al., 1992).* After ammonium sulfate precipitation of the culture supernatant from strain IO-l, the antibacterial activity was eluted in the void fractions during gel filtration chromatography on a column of Sephadex G-25 (l,OOO-5,000 Da; Pharmacia) or G-50 (1,500-30,000 Da; Pharmacia), as a result of the aggregation of the peptide antibiotic with proteins. This phenomenon is probably the cause of the confusing analytical data that was used for the putative identification of the peptide in our previous report (Ishizaki et *al.,* 1992a). Eight molar urea was used to solve this problem. No inactivation of the peptide antibiotic was observed when the ammonium sulfate precipitate was dialyzed against 8 M urea-HCl (pH 3.0). Fractionation of the active material by cation-exchange chromatography without urea was unsuccessful and the recovery was very low (data not shown).

Amino acid composition and sequence

Analysis of the amino acid composition of the peptide antibiotic purified from strain IO-1 indicated that it could have an excess of Asx and a deficiency of His, as compared with nisin A (Table 2), being similar to nisin Z. It contained lanthionine and/or 3-methyllanthionine, which are characteristic of lantibiotics. However, unsaturated amino acids such as DHA and DHB could not be detected because of acid hydrolysis of the purified peptide, as reported by Gross and Morell (1976).

The N-terminal amino acid of the peptide antibiotic purified from strain IO-1 was identified as Ile by automated Edman degradation, and it was identical to that of nisin A. Further degradation after Ile was not successful. This result suggested that an unusual amino acid could be adjacent to Ile.

Table 2. Amino acid composition of the purified peptide antibiotic from *L. lactis* IO-l.

Residue	Nisin A[a]	Peptide from strain IO-1
	mol/mol	mol/mol
Asx		1.9
His	2	0.6
Gly	3	2.7
Ala	2	1.8
Val	1	1.3
Leu	2	2.0
Ile	3	2.3
Ser	1	1.3
Met	2	1.5
Lys	3	2.4
Pro	1	1.2
DHA	2	N.D.
DHB	1	N.D.
Lan	5	4.8

DHA, Dehydroalanine; DHB, dehydrobutyrine; Lan, denotes DL-lanthionine and represents an estimate of the amount of lanthionine plus 3-methyl-lanthionine content; N. D., not detected.
[a] Data from Gross and Morel1 (1971).

Molecular mass of the peptide antibiotic

After HPLC, four peaks were detected and peak fraction no. 3 was regarded as the peptide of interest (Fig. 3). The molecular mass of the peptide antibiotic (peak fraction no. 3) purified from strain IO-1 was 3337.9 by FAB-MS, as shown in Fig. 5, being very similar to the theoretical molecular mass (3330.7) of nisin Z and corresponding to the replacement of histidine in nisin A (molecular mass, 3353.8) with asparagine in the peptide from strain IO-l. The other peak fractions, nos. 1, 2 and 4 (Fig. 3) could be oxidized forms or degradation product of the original peptide (peak fraction no. 3)

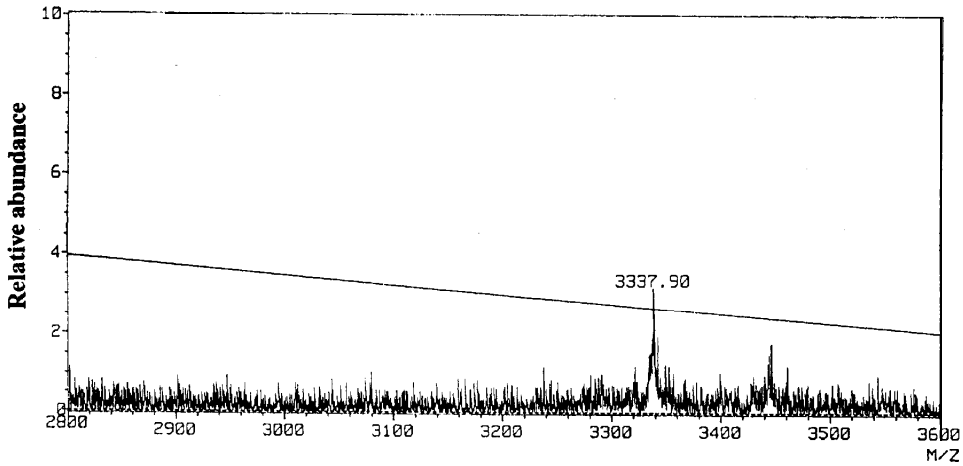


Fig. 5. FAB-MS spectrum of the peptide antibiotic purified from *L. lactis* 10-1

generated during the course of the fermentative process, as deduced from the molecular mass determined by FAB-MS.

NMR analysis

The chemical structure of the peptide antibiotic purified from strain IO-1 was investigated by ^{1}H-NMR spectroscopy. As shown in Fig. 6, the low-field part of the spectrum of the peptide antibiotic showed that it contained three unsaturated amino acids, namely, DHA at positions 5 and 33 and DHB at position 2. Furthermore, the spectrum revealed the presence of additional Asn27 resonances and the lack of His27 resonances, as compared with that reported for nisin A (Mulders et *al.*, 1991).

Consequently, the results in this study and the deduced amino acid sequence of the precursor peptide (Araya et *al.*, 1992) suggest that the structure of the peptide antibiotic produced by *L. Zactis* 10-1 is that proposed in Fig. 7. The structure is the same as that of nisin Z, a natural variant of nisin isolated from the culture broth of *L. lactis* NIZO 22186 (Mulders et al., 1991).

Since nisin and other lantibiotics contain several unusual amino acids, such as DHA and DHB, amino acid sequencing by Edman degradation is not very successful. It is best to determine the chemical structure of purified lantibiotics by NMR analysis. Furthermore, we determined directly the molecular mass of the peptide antibiotic purified from strain IO-1 by FAB-MS. Although the molecular mass of, this peptide antibiotic was determined to be almost the same as that of commercial nisin (nisin A) by SDS-PAGE (Fig. 4), we were able to find a difference between these peptides by FAB-MS.'

In this study, the efficient purification and the identification of the peptide antibiotic produced by *L. Zactis* IO-1 were accomplished. Although many studies have dealt with nisin A, there is little in the literature about natural variants of nisin such as the present peptide. It is expected that this peptide should prove to be more soluble than nisin A above pH 6 since the asparagine side chain is more polar than the histidine side chain that

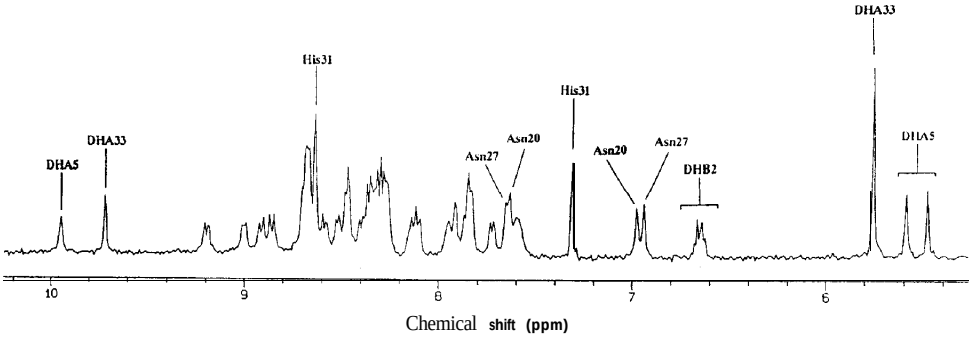
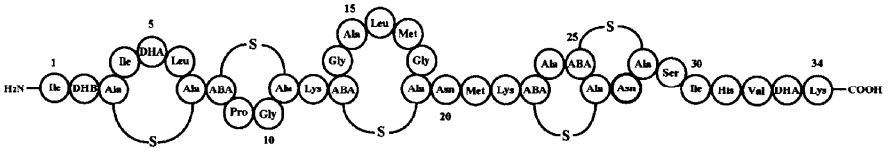


Fig. 6. NMR analysis of the peptide antibiotic purified from *L. lactis 10-l.* The low-field part of the ^{1}H-NMR spectrum of the peptide antibiotic at pH 3.3 is shown. The assignments of some relevant resonances are indicated.



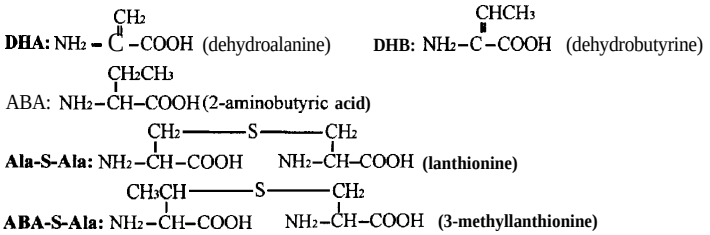


Fig. 7. Proposed structure of the peptide antibiotic purified from *L. lactis* IO-1. The amino acid residue at position 27 is a His residue in nisin A. The structure of nisin A was determined by Gross and Morel1 (1971).

it replaces. Furthermore, this peptide also has a broad antibacterial spectrum, as shown in this study. These facts suggest that nisin Z could be used as a food preservative instead of nisin A.

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