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Ishizaki, Ayaaki
Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Matsusaki, Hiromi
Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Uryu, Kazuko
Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

Inui, Takako
Department of Food Science and Technology, Faculty of Agriculture, Kyushu University

他

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Biochemical Properties of a New Lantibiotic Produced by *Lactococcus lactis* IO-1

Ayaaki Ishizaki, Hiromi Matsusaki, Kazuko Uryu, Takako Inui, Kayo Kurematsu, Satoe Ichii, Tomoyuki Hayami, Michio Tonokawa and Yojiro Koba

Department of Food Science and Technology, Faculty of Agriculture, Kyushu University 46-09, Fukuoka 812, Japan

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A new peptide antibiotic was isolated from the culture broth of ***Lactococcus*** *lactis* IO-1 and biochemical properties of this compound were investigated. The peptide had a strong inhibitory effect on the growth of ***Bacillus subtilis Cl*** and the antibacterial spectrum and minimum inhibitory concentration (MIC) for various type strains showed that this antibiotic resembled nisin. Automated Edman degradation indicated the N-terminal amino acid of this peptide was isoleucine, the N-terminal amino acid for nisin. ^{1}H NMR spectra showed the presence of DHB,. However, hydrolysis using carboxypeptidase Y released isoleucine as the C-terminal amino acid which is not the C-terminal amino acid for nisin. The molecular weight of the IO-1 peptide is smaller than nisin and the amino acid composition was slightly different. The IO-1 peptide did not contain His and Ser and the Ile, Lys and Met content was less than that in nisin; these amino acids are the members of the C-terminal amino acid sequence of the nisin peptide. Thus, the peptide produced by strain IO-1 was a new lantibiotic with a different amino acid sequence from that of nisin.

INTRODUCTION

It is generally known that lactic acid bacteria produce a variety of antibiotics and bacteriocins (Klaenhammer, 1988). However, few studies have been carried out on these interesting antagonistic materials until recent years. Lactic acid bacteria develop during the fermentation processing of various kinds of traditional foods and these bacteria are common inhabitants of the mammalian gut. These antibiotics produced by lactic acid bacteria may influence man's life and health. Recently much attention has been focused on this field of research with investigations being concentrated in two main areas: one is the investigation of new antibiotics including that structure differentiation, and taxonomic positions of the producer organism; the other is the genetics of antibiotic synthesis and related subjects including plasmid biology and genetic manipulations (Dodd et al., 1990, Donkersloot and Thompson 1990 and Gonzalez and Kunka 1987). Since the *nisA* gene was isolated as the gene coding pro-nisin peptide (Kaletta and Entian 1989), genetic studies on nisin should provide further information on the matured peptide, the biosynthesis of lantibiotic, the secretion of active antibiotic, and the immunity of the producer cell to the antibiotic.

Lactococcus lactis IO-1 was isolated in our laboratory (Ishizaki *et al.*, 1990 and Ishizaki *et al.*, 1990) and was shown to produce the antibiotics which were strongly antagonistic to the growth of *Bacillus subtilis* Cl also isolated in our laboratory

(Tamura et al., 1991). The antibacterial spectrum and other properties of the antibiotic indicated that it may be a variation of nisin (Ishizaki et al., 1990). Although there are a few nisin producers as variant strains of *Lactococcus* ***Zactis,*** no nisin producer has been found in Japan. We isolated a peptide from the culture broth of this microorganism. The peptide contained dehydrobutyrine (DHB) so that the peptide is lantibiotic but differed from nisin in its molecular weight, amino acid composition and C-terminal amino acid. Therefore, further investigation of our peptide should provide valuable information on the genetic expression and biochemistry of nisin related lantibiotics produced by lactic acid bacteria (Kaletta et ***al.,*** 1989).

MATERIALS AND METHODS

Microorganism

The microorganism used for antibiotic production was *Lactococcus lactis* IO-1 isolated in our laboratory (Ishizaki et ***al.,*** 1990). The microorganism used for bioassay was ***Bacillus*** *subtilis* Cl which was also isolated in our laboratory (Tamura et ***al.,*** 1991). Microorganisms used for the antibacterial spectrum determination were ***Lactococcus Zactis*** JCM 5805^T, ***Lactococcus Zactis*** subspecies *cremoris* TUA 1344L, ***Enterococcus*** *faecalis* JCM 5803^T, ***Bacillus*** *coagulans* JCM 2257^T, ***Bacillus*** *stearothermophilus* JCM 2501^T, *Clostridium acetobutylicum* IFO 13948^T and ***Pseudomonas*** *aeruginosa* KYU-1. The media and culture conditions for the individual microorganisms are listed in Table 1.

Antibiotic production

Culture of strain 10-l for antibiotic production was carried out by using a medium consisting of yeast extract 5 g, polypeptone 5 g, NaCl 5g and glucose 50 g in 1 l of distilled water which was sterilized by autoclaving at 110°C for 10 min. The stock strain of IO-1 was subcultured in 10 ml of thioglycolate-without-dextrose liquid medium (containing 0.75 g/l of agar agar) (TGC, Difco Laboratories, USA) for an overnight static culture which was then transferred to 100 ml of YMG broth (yeast extract 1%, polypeptone 1%, NaCl 0.5% and glucose 1%, pH 7) for inoculum prepara-

Table 1. Media and culture conditions employed for the various bacterial strains.

Strains	Media and culture conditions	
Lactococcus *lactis* JCM 5805^T	TGC Difco, static	at 30°C
Lc. Zactis sb. *cremoris* TUA* 1344L	〃 〃	27°C
Enterococcus ***faecalis*** JCM 2501^T	〃 〃	37°C
Bacillus *subtilis* ***Cl***	Bouillon, shaken	at 37°C
B. coagulans JCM 2257^T	〃 〃	***37°C***
B. *stearothermophilus* JCM 2501^T	〃 〃	***55°C***
Clostridium ***acetobutylicum*** IFO 13948^T	RCM bouillon, gas pak	at 37°C
Pseudomonas *aeruginosa* KYU-1	Bouillon, shaken	at 37°C

* Abbreviation TUA means type culture collection of lactic acid bacteria in Tokyo University of Agriculture.

tion (18 h, 37°C). The fermentation was carried out in a 2 l jar with a working volume of 1.5 1 at pH 6.0 controlled by NaOH feeding and 37°C.

Separation of the antibiotics

Culture broth was centrifuged at 12,000 g for 20 min (0°C) to separate the cells and the pH of the supernatant was adjusted to 3.0 with HCl. The supernatant was stood overnight at 0°C and centrifuged to remove any precipitate formed. Then the solution was salted out with 70% $(NH_4)_2SO_4$ and stood overnight at 0°C. The precipitate formed was collected by centrifugation at 12,000 g for 20 min. (0°C) and then dissolved in 0. 001N HCl.

Column chromatography

Further purification of the separated antibiotics was carried out by CM Sephadex C-25 column chromatography (column size 15 mm I.D. *X* 600 mm L, equilibrated by 0. 05M acetate buffer, pH 3.6). Elution was carried out using a concentration gradient of O-O.25 M KCl. The active fraction was detected by UV absorbance at 210 and 280 nm and confirmed by bioassay using *Bacillus subtilis* **Cl.** The active fraction was then filtrated through a YM-2 ultra filtration membrane under nitrogen pressure (2 kgf/ cm*). The filtrate was then subjected to Bio-Gel P-10 column chromatography and eluted with 0.001N HCl without a gradient. The active fraction was subjected to NMR analysis and C-terminal amino acid determination. However, active fraction was purified further by HPLC before amino acid sequence analysis.

Bioassay

Growth inhibition of ***B. subtilis*** Cl was observed by strain IO-1 on plates divided into quadrants. Strain IO-1 was inoculated on two quadrants facing each other and incubated at 37°C for two days. The remaining two quadrants were then inoculated with strain Cl and incubated overnight. Growth of strain Cl was observed whether the quadrants with strain IO-1 hinder the growth of strain Cl from his territory to the territory for strain IO-l.

The antibacterial spectrum of IO-1 was determined by the following procedure. The cells of strain 10-l were cultured in YMG medium (glucose 2.5%) for 18 h and harvested by centrifugation of 12,000 g for 20 min. The supernatant was acidified with HCl to pH 3. and centrifuged to remove any precipitate formed. The supernatant was kept at 4°C and filtered through a DISMIC25cs membrane filter (Toyo Roshi Kaisha Ltd., Japan). The filtrate was added to the bioassay media (10% YMG) aseptically with appropriate dilution. The assay medium was then inoculated with the test strain to give a cell concentration of 10^6/ml and incubated for 18 h at 37°C. Growth was measured quantitatively by the optical absorbance at 562 nm. The minimum volume of supernatant added to the medium at which no growth was observed was regarded as the minimum inhibitory concentration (MIC) expressed as 1 supernatant/ml.

HPLC

High purity of the peptide was confirmed by obtaining a single peak after HPLC analysis, The antibacterial active fraction from the Bio-Gel P-10 column chromatography was injected into an Asahipak GS-320 column (7.6 mm I.D. *X* **500** mm L)

installed in the HPLC system consisting of Hitachi 638-30 and Shimadzu SPD-GA. The mobile phase was 50 mM phosphate buffer (NaH_2PO_4 + Na_2HPO_4, pH 7.0)/ acetonitrile = 80/20. Optical absorbance at UV 210 nm was determined.

Purification of the peptide for amino acid sequence analysis was carried out using a HPLC Shimadzu LC-GA system employing a column, COSMOSIL $10C_{18}$ (4.6 mm I. D. *x* 250 mm L). Elution was done using a mixture of 0.1% TFA in water and 0.05% TFA in acetonitrile which varied from 25 to 55% acetonitrile against water for the gradient.

Amino acid analysis

The isolated active fraction was hydrolyzed by autoclaving at 120°C for 2h in 6N -HCl in a sealed ampoule. The quantitative determination of the amino acid composition of one mole of the peptide was completed using an amino acid analyser (Hitachi model 655A).

FAB-MS

Fast atom bombardment (FAB) mass spectra were recorded on a JMS-SX102 mass spectrometer equipped with a JMA-DA 6000 data system (JEOL, Tokyo Japan) using glycerol as the matrix.

Amino acid sequence analysis

Amino acid sequences were determined on a PSQ-1 automated gas-phase protein sequencer equipped with an on-line LC-GA HPLC system (Shimadzu, Kyoto, Japan) for identification of PTH amino acid. Reagents were purchased from Wako Chemicals (Osaka, Japan).

NMR

^1H nuclear magnetic resonance (NMR) analysis using a JEOL GSX 400 spectrometer was carried out to determine the presence of dehydroalanine (DHA) and dehydrobutyrine (DHB) in the peptide. Purified nisin peptide was used for obtaining a reference spectrum. The ^1H NMR spectra of the peptides were recorded in D_2O.

C-terminal amino acid determination

C-terminal amino acid was determined by paper chromatography of the peptide digested by carboxypeptidase Y purchased from Oriental Yeast Co. Ltd., Tokyo Japan. The IO-1 peptide for digestion was purified according to the procedure already stated and the nisin peptide was refined by the method stated below. Digestion was carried out in 0.2 M citrate buffer (pH 5.0), 37°C for 30 min. Amino acids in the digested samples were identified by ordinary paper chromatography with the solvent consisting of butanol, acetic acid and water 4:2:1.

Standard nisin

Standard nisin, 1,000,000 Reading units/g (Hurst 1981), was purchased from ICN Biomedicals Inc., N.Y., USA. Purification was carried out by CM cellulose column chromatography with a pH gradient elution in the range from pH 3 to 1. The active fraction was monitored by UV absorbance at 210 and 280 nm and confirmed by

bioassay using ***Bacillus*** *subtilis* Cl. Very high purity of the peptide in the active fraction was confirmed by obtaining a single peak after HPLC analysis as stated previously.

RESULTS

Antibacterial activity

The antibacterial activity of strain IO-1 was confirmed by the plate assay (Fig. 1).

Antibacterial spectrum

The antibacterial spectrum of the antibiotics excreted in the culture broth of strain IO-1 grown on YMG medium was determined in comparison with nisin as a standard. Fig. 2 indicates the MICs for the various strains tested and shows that the antibacterial spectrum for strain IO-1 was almost the same as the spectrum for nisin.

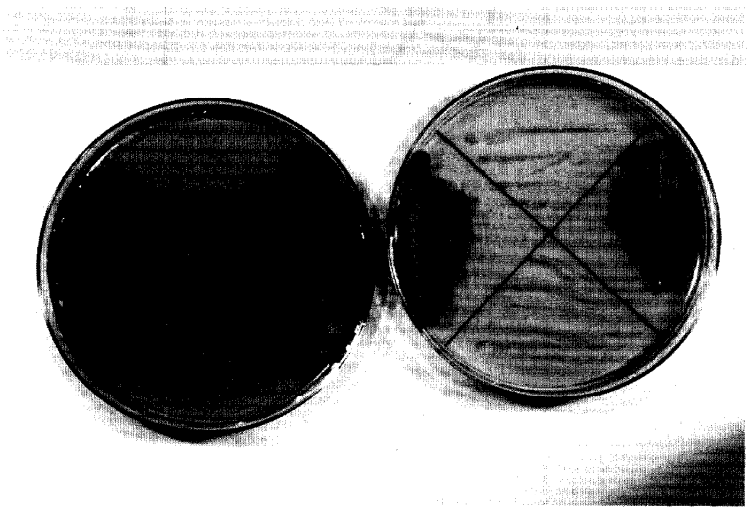

Fig. 1. Inhibition for microbial growth of Bacillus *subtilis* Cl by *Lactococcus lactis* IO -1. Left; *Bacillus subtilis* Cl without strain IO-l, right; strain IO-1 was cultured prior to the inoculation of *Bacillus subtilis Cl.*

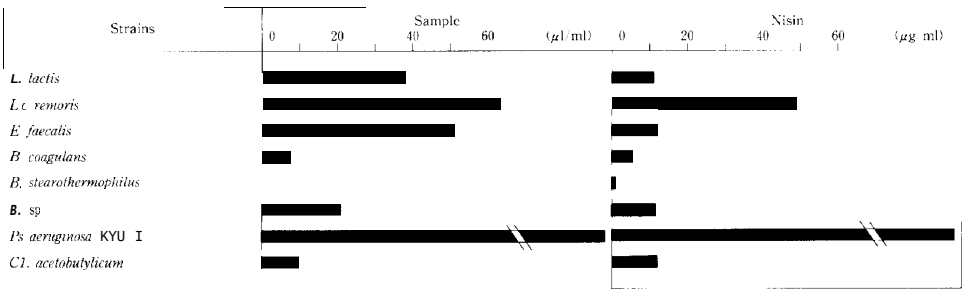

Fig. 2. Antibacterial spectrum and MIC for various strains. Activity of nisin used was 1,000,000 Reading units/g.

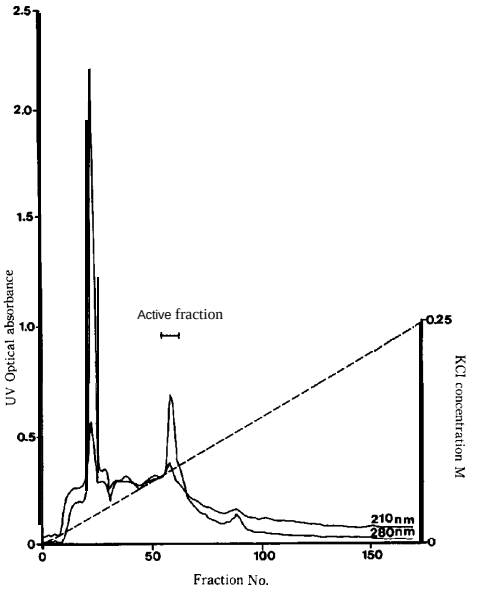

Fig. 3. Elution profile of the IO-1 peptide from CM Sephadex C-25 column chromatography with KC1 gradient O-O.25 mol/l. Antibacterial activity was observed in fractions no. 51-60.

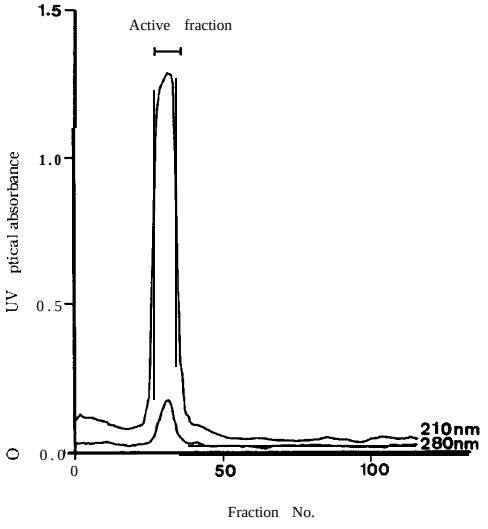

Fig. 4. Elution profile of the IO-1 peptide from Bio-Gel P-10 column chromatography. Antibacterial activity was observed in fractions. no. 32-35.

According to the results, the activity of the supernatant of the broth of strain IO-1 was equivalent to 1,000 Reading units/ml of nisin.

Separation and purification of antibiotics

Cell free broth (adjusted to pH 3 with HCl) was fractionated according to the procedure described in the methods section. Fig.3 shows the UV absorbance of the fractions collected by the CM Sephadex C-25 column. Antibiotic activity was detected in fractions No. 51-60. These fractions were collected and filtered by ultra filtration and then applied to a Bio-Gel P-10 column. Fig. 4 shows the results of the UV absorbance of the fractions. Antibiotic activity was observed in the fractions No 32-35. These fractions gave a single peak on HPLC analysis described in the method section.

Amino acid composition

The fractions in which antibacterial activity was found were hydrolyzed with HCl and the amino acid composition was determined using an amino acid analyser. The data are presented in Table 2. The system could not detect unsaturated amino acids such as DHA and DHB and sulfur bonding amino acids such as lanthionine and β-methyllanthionine.

Molecular weight

Molecular weights of the 10-l peptide and nisin were determined by FAB-MS and the spectra are shown in Fig. 5. As shown in these spectra, protonated molecular ion (MH^+) at m/z 3354.9 agreed perfectly well with the theoretical molecular weight of nisin ($C_{143}H_{230}O_{37}N_{42}S_7 = 3354.2$) while, in the FAB mass spectrum of the IO-1 peptide, MH+ was observed at m/z 2697.98.

Edman degradation and NMR analysis

The N-terminal amino acid of the IO-1 peptide was identified by automated Edman degradation to be isoleucine, the same N-terminal amino acid for the nisin peptide. Since DHA/DHB stops Edman degradation, the fact that further degradation of the IO-1 peptide after isoleucine did not occur indicates DHA/DHB is adjacent to isoleucine, the N-terminal amino acid in the IO-1 peptide. To confirm this, ^1H NMR analysis was carried out. Since Liu and Hansen reported the chemical shift region of the vinyl proton for DHB_2, DHA_5 and DHA_{33} in nisin peptide (Liu and Hansen 1990),

Table 2. Amino acid composition of the IO-1 peptide

Amino acid	Mol%	Relative abundance	Nearest integer
Gly	18.9	3.18	3
Ala	12.7	2.14	2
Val	6.3	1.07	1
Leu	12.5	2.10	2
Ile	11.8	1.99	2
Asx	12.3	2.09	2
Met	5.9	1.00	1
Lys	11.5	1.94	2
Pro	7.4	1.25	1

it is possible to confirm the presence of DHB/DHA in the IO-1 peptide by observing the NMR spectra of the IO-1 peptide and nisin peptide. As shown in Fig. 6, the spectrum of the 10-l peptide mirrored the spectrum for the chemical shift of DHB_2 of nisin. However, the spectrum for DHA,, of the nisin peptide was not observed in the IO-1 peptide and it was not confirmed that DHA, was present in the 10-l peptide.

C-terminal amino acid

C-terminal amino acid was determined by paper chromatography of the digested product of the IO-1 peptide using carboxypeptidase Y (Fig. 7). Isoleucine and valine were isolated as digested products. However, isoleucine was determined as the C-terminal amino acid by quantitative amino acid analyser.

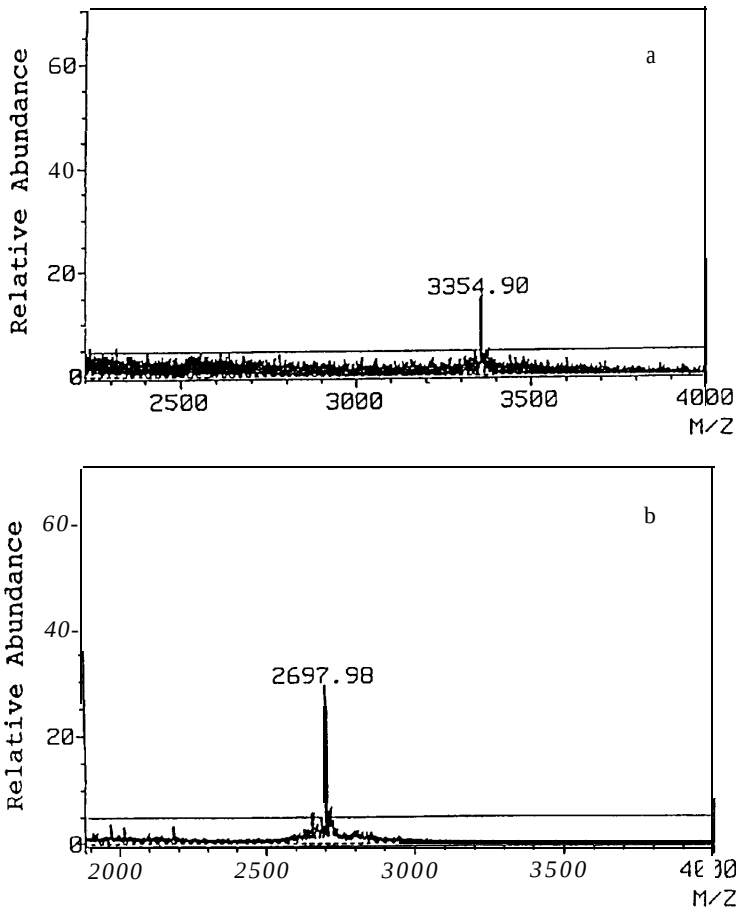

Fig. 5. Determination of molecular mass by FAB-MS spectra. Above (a); nisin, a standard, below (b); the IO-1 peptide.

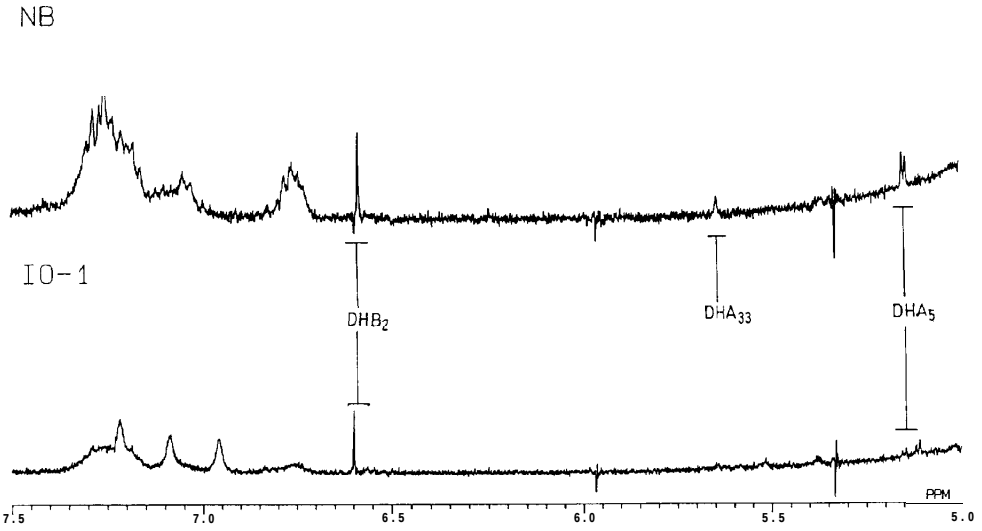

Fig. 6. 400 MHz ^1H NMR spectra for nisin, above (NB) and the IO-1 peptide, below (10-l) in D_2O.

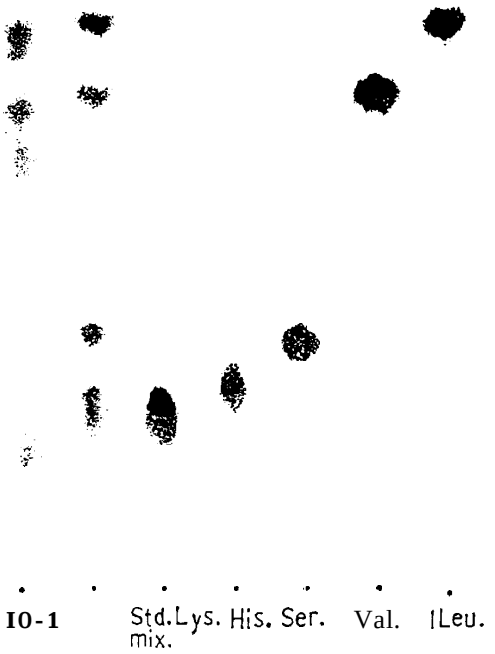

Fig. 7. Results of C-terminal amino acid determination by paper chromatography for the IO-1 peptide which hydrolyzed by carboxypeptidase Y.

DISCUSSION

Further study with respect to the amino acid sequence of the IO-1 peptide was carried out by analyzing the information and data obtained from the experiments. Since the amino acid sequence of nisin has been determined by Gross (Gross and Morel1 1971), we can estimate the amino acid sequence of the IO-1 peptide by comparing the molecular weight and amino acid composition of the IO-1 peptide with those of nisin. Since it was confirmed that DHB was present in the IO-1 peptide, this peptide must be a lantibiotic containing unsaturated amino acids and sulfur bonding amino acids. The N-terminal amino acid in this peptide was shown to be isoleucine adjacent to which was DHB. This sequence is exactly the same as that found in nisin. The antibacterial spectra of the IO-1 peptide resembled nisin, however C-terminal amino acid was isoleucine but not lysine which is the C-terminal amino acid for nisin. FAB-MS determination revealed that the molecular weight of the IO-1 peptide was smaller than that of nisin. From these facts, it is claimed that the IO-1 peptide must be a lantibiotic which is similar to nisin. Assuming that the 10-l peptide is consists of the amino acids listed in Table 2 plus the same mole ratio of DHA, DHB, lanthionine (Ala-S-Ala) and β-methyllanthionine (Ala-S-ABA) as those in nisin, the molecular weight of the IO-1 peptide was estimated as 2734. However it does not agree with the observed molecular weight for the 10-l peptide of 2698.

The amino acid composition of the IO-1 peptide was compared with that of nisin. It was found that histidine and serine were absent from the IO-1 peptide. Isoleucine, lysine and methionine were less frequent in the IO-1 peptide than in nisin while aspartic acid/asparagine were more frequent than in nisin. It is interesting that His, Ser, Ile and Lys are composing the C-terminal sequence of the nisin peptide. From these facts and observations, the IO-1 peptide must be a new lantibiotic which is closely resemble nisin but has a different amino acid sequence.

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