[Chem. Pharm. Bull. 20(7)1551—1557(1972)] UDC 615.322.011.5 : 615.277.3.011.5.076.9

Antitumor Activity of *Bacillus natto*. III.[1] Isolation and Characterization of a Cytolytic Substance on Ehrlich Ascites Carcinoma Cells in the Culture Medium of *Bacillus natto* KMD 1126[2]

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(Received February 9, 1972)

There were at least two kind of cytolytic substances on Ehrlich ascites carcinoma cells in the culture medium of *Bacillus natto* KMD 1126. One was adsorbed on carbon, eluted with EtOH, and large molecule by Sephadex G 25 gel filtration, but the other was not adsorbed on carbon and small molecule by Sephadex G 25 gel filtration. The former was stable on wide range of pH and temperature. This cytolytic substance was isolated and its chemical structure was examined by elementary analysis, infrared spectrum, nuclear magnetic resonance, and mass spectroscopy and study of decomposed products. As the results, the cytolytic substance was proved to be identical with surfactin which was potent coltting inhibitor in the thrombin fibrinogen system obtained from culture medium of *Bacillus subtilis* by Kakinuma, *et al.*

In a previous paper of this series,[4] the authors reported that a strain of *Bacillus natto* (tentatively called KMD 1126), which was newly isolated from "Natto" (fermented beans), has a contact antitumor effect on solid type Ehrlich carcinoma cells. Then, it was reported[1] that *Bacillus natto* KMD 1126 cell suspension had no remarkable cytolytic effect on Ehrlich ascites carcinoma cells, but cytolytic substances were formed when the suspension was incubated at 37° for 2 hr in the buffer. It was assumed from these results that the cytolytic substances may be also present in the culture medium of *Bacillus natto* KMD 1126. The cytolytic activity in the culture medium was measured according to the method of Shimizu *et al.*[5] and it was shown that there were much amounts of cytolytic substances in the culture medium.

Then, purification of cytolytic substances in the medium was carried out in order to see what compounds have the cytolytic activity. It was observed that there are at least two kinds of cytolytic substances in the culture medium by treatment with carbon and Sephadex G 25 gel filtration. That is, one substance was adsorbed on carbon, eluted with EtOH, and large molecule by Sephadex G 25 gel filtration, which constitute approximately 10% of the cytolytic activity in the culture medium. On the other hand, about 50—60% of cytolytic activity in the culture medium was not adsorbed on carbon and this compound was small molecule by Sephadex G 25 gel filtration. The former substance was also demonstrated to be stable over a wide range of pH and temperature. The purpose of the present paper is to describe the isolation and characterization of this stable cytolytic substance on

1) Part II: Y. Kameda, H. Sagai, T. Yamada, S. Kanatomo, and K. Matsui, *Chem. Pharm. Bull.* (Tokyo), **19**, 2572 (1971).
2) This work was reported at 91st Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, April 1971 and 33rd Meeting of Hokuriku Branch, Pharmaceutical Society of Japan, Toyama, November 1971
3) Location: *13-1, Takaramachi, Kanazawa.*
4) Y. Kameda, S. Kanatomo, Y. Kameda, and Y. Saito, *Chem. Pharm. Bull.* (Tokyo), **16**, 186 (1968).
5) R. Shimizu, N. Nishita, K. Banto, S. Koshimura, A. Hayashi, and T. Kobayashi, *Annual Report of the Research Institute of Tuberculosis Kanazawa University*, **22**, 27 (1964); M. Hatano, R. Shimizu, O. Morita, and T. Yamagishi, *Medicine and Biology*, **74**, 293 (1967).

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Ehrlich ascites carcinoma cells in the culture medium of *Bacillus natto* KMD 1126. As the results, this substance was proved to be identical with surfactin which was potent clotting inhibitor in the thrombin fibrinogen system obtained from culture fluids of *Bacillus subtilis* by Kakinuma *et al.*[6]

Material and Method

Animal——Female ICR-SLC mice, weighing 18—22 g, were used for all experiments. All animals were fed on standard laboratory diet and given water *ad libitum.*

Tumor——Ehrlich ascites carcinoma was maintained by weekly intraperitoneal transplantation in ICR-SLC mice.

Buffer——Buffer, containing 8 g of NaCl, 0.2 g of KCl, 2.9 g of $Na_2HPO_4 \cdot 12\ H_2O$, 0.4 g of KH_2PO_4 in 1000 ml of H_2O and adjusted to pH 7.2, was used for all experiments.

Bacterial Strain and Culture——*Bacillus natto* KMD 1126 is the stock strain kept in this laboratory on nutrient agar slants. Culture was carried out by the following manner. To 3 liters shaking flasks, 800 ml of bouillon (pH 7.2) were added, sterilized in an autoclave under 1.0 kg/cm^2 pressure for 20 min, and inoculated with 50 ml of seed culture of the bacteria, which was prepared by shaking culture of the organisms at 37° for 15 hr in the same medium. The culture was carried out at 37° with reciprocal shaking (130 c/min, stroke 7 cm) for 24 hr.

Preparation of Tumor Cell Suspension——The ascites fluid, aspirated from mice bearing 6—9 day old Ehrlich ascites carcinoma by sterile syringe, was suspended in chilled buffer and centrifuged at 700 rpm for 3 min. The sedimented tumor cells were washed twice with chilled buffer and resuspended in appropriate volume of the buffer and then the tumor cell count was made in a hemocytometer by the standard method for blood leucocytes. The tumor cell suspension was further diluted, if necessary, to give a desired cell concentration (usually 2.5—3.5×10^7 cells/ml).

Cytolytic Activity——The reaction mixtures, containing 0.2 ml of tumor cell suspension and 0.2 ml of appropriately diluted samples, were incubated in water bath at 37° for 2 hr. After incubation, they were diluted with 3.6 ml of the buffer, and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 260 nm after 10 fold dilution (HITACHI Model 101 Spectrophotometer). At the same time, 2 control experiments were run using following 2 mixtures and absorbance of the controls were deducted from that of sample. 1) A mixture of 0.2 ml of tumor cell suspension and 0.2 ml of the buffer. 2) A mixture of 0.2 ml of the sample and 0.2 ml of the buffer. One unit of the cytolytic activity was defined as the amount of cytolytic substance which gave an E_{260} reading of 1 under above conditions. Therefore, total activity was expressed as follows.

Total Activity (unit) $= E_{260} \times \text{Dilution} \times 5 \times \text{Volume}$

Sephadex G 25 Gel Filtration——Sample was introduced into a column of Sephadex G 25 (2×40 cm) equilibrated with the buffer and filtration through the gel bed was carried out with the same buffer. The effluents were fractionated in 10 ml portion at a rate of approximately 120 ml per hr. Absorbances of the fractions were determined spectrophotometrically at 280 nm and cytolytic activity were also measured.

Isolation of Cytolytic Substances——*Bacillus natto* KMD 1126 was cultured at 37° for 24 hr in 2 flasks (dispense volume in a flask: 800 ml) by a reciprocal shaker. The culture broth was collected and centrifuged at 10000 rpm for 20 min. To the supernatant (1.6 liters), 24 g of carbon (Wako Pure Chem.) was added. After stirring for 1 hr, the carbon was separated by filtration and eluted 3 times with 150 ml of EtOH under reflux. The active eluate was concentrated to dryness under reduced pressure and the residue was dissolved in 10 ml of the buffer (pH 7.2). The cytolytic activities of this solution and carbon filtrate were measured. Then, these two solution were concentrated by lyophilization and carried out Sephadex G 25 gel filtration respectively.

Purification of the Cytolytic Substance——*Bacillus natto* KMD 1126 was cultured at 37° for 24 hr in 12 flasks (dispense volume in a flask: 800 ml) by a reciprocal shaker. The culture broth were collected and contineously centrifuged at 15000 rpm for 200 ml/min. The supernatant was acidified with HCl and the resulting precipitate was separated by centrifugation at 10000 rpm for 20 min. The precipitate was extracted with AcOEt and the AcOEt extract was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was washed with petr. ether and the residue was dissolved in 20 ml of the buffer. Twenty ml of the solution was poured over a column of Sephadex G 25 (3×50 cm) equilibrated with the buffer. The effluents were fractionated in 20 ml portion at a rate of approximately 150 ml per hr. Absorbance at 280 nm and cytolytic activity of the fractions were measured. The active fractions were collected, acidified with HCl, and extracted with AcOEt. The AcOEt extract was evaporated under reduced pressure and the residue was dissolved in small amount of acetone. This solution was poured over a

6) K. Arima, A. Kakinuma, and G. Tamura, *Biochem. Biophys. Res. Commun.*, **31**, 488 (1968); A. Kakinuma, M. Hori, M. Isono, G. Tamura, and K. Arima, *Agr. Biol. Chem.*, **33**, 971 973, 1523, 1669 (1969).

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column of Sephadex LH 20 (2×30 cm) equilibrated with acetone, and filtration through the gel was carried out with the same solvent. The effluents were fractionated in 10 ml portion at a rate of approximately 100 ml per hr. The solvent was evaporated under reduced pressure and the weight of residual material and cytolytic activities in each fraction were measured. The active fraction were collected, concentrated to dryness, and washed with petr. ether. The residue was recrystallized from acetone–H_2O. 0.16 g of the cytolytic substance was obtained from 10 liters of culture medium as a white powder, mp 136—138°, $[\alpha]_D$: +39° (c=1, chloroform), $[\alpha]_D$: −39° (c=1, MeOH). *Anal.* Calcd. for $C_{53}H_{93}O_{13}N_7$: C, 61.42; H, 9.05; N, 9.46. Found: C, 61.02; H, 9.10; N, 9.11.

Determination of Amino Acid Composition of the Cytolytic Substance——Ten mg of sample and 1 ml of 6N HCl were added to a sealed tube and heated at 110° for 20 hr. After cooling, a content of the tube was taken out and extracted with ether. The aqueous layer was evaporated under reduced pressure to dryness. The residue was dissolved in a small amount of H_2O and applied to amino acid analyzer (Hitachi Amino Acid Analyzer Type KLA-2).

Methanolysis of the Cytolytic Substance——Sample (0.5 g) was dissolved in 50 ml of 30% HCl–MeOH and heated at 90° in a sealed tube for 14 hr. After methanolysis, the HCl-MeOH was removed by evaporation and the residue was extracted with petr. ether. The extract was washed with H_2O, dried over Na_2SO_4 and evaporated under reduced pressure. The oily material thus obtained, was dissolved in chloroform and poured over a column of silicic acid (2×30 cm) and the column was developed with chloroform. The chloroform eluate was concentrated by evaporation and obtained about 93 mg of fatty acid methylester. After that, the column was eluted with chloroform–MeOH (4:1). The chloroform–MeOH eluate was concentrated to dryness and obtained 10 mg of oily material. The structure of chloroform eluate was examined by gas chromatography, nuclear magnetic resonance (NMR) and mass spectroscopy. The chloroform–MeOH eluate was examined amino acid composition after acid hydrolysis by amino acid analyzer.

Determination of Amino Acid Sequence of the Cytolytic Substance——The cytolytic substance (0.44 g) was dissolved in 5 ml of acetone, added 0.6 ml of 10% NaOH, and allowed to stand overnight at room temperature. The solution was evaporated to dryness under reduced pressure. The residue was dissolved in H_2O, acidified with HCl, and extracted with AcOEt. The AcOEt extract was evaporated to dryness and the residue was recrystallized from acetone–H_2O. An acid (0.28 g) which has opened a lactone ring present in the cytolytic substance, was obtained as white powder, mp 174—175°, $[\alpha]_D$: +14° (c=1, MeOH). *Anal.* Calcd. for $C_{53}H_{95}O_{14}N_7$: C, 60.37; H, 9.08; N, 9.29. Found: C, 59.98; H, 8.97; N, 8.99. Permethylation of this acid was carried out by the procedure of Hakomori.[7] Sodium hydride oil suspension (30 mg) was rinsed 3 times with dry ether. Dimethyl sulfoxide (0.3 ml) was added and heated at 100° under a nitrogen stream, until H_2 evolution ceased, the cooled to room temperature. To this solution, 3 mg of the acid was added and allowed to stand under a nitrogen stream for 10 min at room temperature. Then, 0.3 ml of methyl iodide was added and further allowed to stand for 1.5 hr at room temperature. The reaction mixture was diluted with H_2O and was followed by extraction of the methylated products with chloroform. The chloroform extract was washed with H_2O, dried with anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was subjected to measurement of mass spectrum without further purification, (Nihondenshi Mass Spectrometer Type JMS-01SG).

Gas Chromatography——Gas chromatography of fatty acid methyl ester was carried out using a 2 m column of 1.5% SE-30 chromosorb w at 180°. The flow rate of the He carrier gas was 60 ml/min. (Shimadzu Gas Chromatograph GC-4B).

Result and Discussion

Cytolytic Substances in Culture Broth

Bacillus natto KMD 1126 was cultured in bouillon at 37° for 24 hr and the supernatant (S) of the culture broth was obtained by centrifugation. Carbon was added to a portion of the supernatant. After stirring for 1 hr, the mixture was filtered to obtain adsorbed carbon and filtrate (F). The adsorbed carbon was eluted with EtOH under reflux and the EtOH extract was concentrated to dryness. The residue was dissolved in the buffer and this solution was called EtOH extract (E). Cytolytic activities of each fractions (S, F, and E) were measured and the results were shown in Table I. As shown in Table I, about 50—60% of cytolytic activity in the culture medium was not adsorbed on carbon, but the remaining activity was adsorbed on carbon. About 10% of cytolytic activity in the culture medium was contained in EtOH extract. These two substances were concentrated by lyophilization and applied to Sephadex G 25 gel filtration. As shown in Fig. 1, EtOH extract was eluted

7) S. Hakomori, *J. Biochem.* (Tokyo), 55, 205 (1964).

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Table I. Assay of Cytolytic Activity of Culture Medium, EtOH Extract, and Carbon Filtrate

	Cytolytic activity[a)]	Relative activity
Culture medium (S)	1050—2000	100%
EtOH extract (E)	120— 220	10—15%
Carbon filtrate (F)	650—1000	50—60%

a) Cytolytic activity was indicated by total activity (unit) obtained from 1 liter medium.

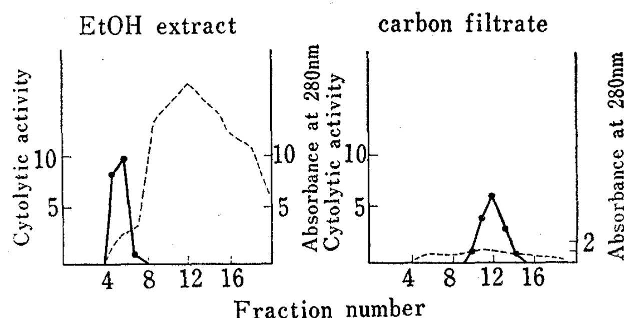

Fig. 1. Sephadex G 25 Gel Filtration of the EtOH Extract and Carbon Filtrate

EtOH extract or concentrated carbon filtrate by lyophilization were applied to a column of Sephadex G 25 (2×40cm) equilibrated with the buffer. The effluents were fractionated in 10 ml portion and measured absorbance at 280 nm (---------) and cytolytic activity (—●—).

Table II. Effect of Cultural Time on the Production of Cytolytic Substances

Cultural time (hr)	Cytolytic Activity[a)]		
	Culture medium (S)	EtOH extract (E)	Carbon filtrate (F)
12	1070	140	600
18	1210	140	720
24	1280	150	850
36	1720	160	1050
48	1850	180	1220
72	1600	160	1090

a) Cytolytic activity was indicated by total activity (unit) obtained from 1 liter medium.

Table III. Stability of EtOH Extract on Temperature

	Cytolytic activity u/ml
Control	19.4
100° 10 min	19.0
100° 20 min	18.8
100° 40 min	18.8
100° 60 min	19.0

Table IV. Stability of EtOH Extract on pH

		Cytolytic activity (u)
Control		20.5
pH 1	10 min—pH 7.2	20.1
pH 13	10 min—pH 7.2	20.0
pH 1	AcOEt extract H_2O—pH 7.2	0.0
	AcOEt extract	17.5

after void volume, but the active substance in carbon filtrate (F) was eluted slower than EtOH extract. It was suggested from these results that there were at least two kind of cytolytic substances in the culture medium.

Then, the cytolytic activity in culture medium (S), carbon filtrate (F), and EtOH extract (E) were measured at several culture time. As shown in Table II, the cytolytic activity in EtOH extract was almost same value in 12—48 hr culture. Then we used 24 hr culture broth in the following experiments.

Stability of the EtOH Extract

EtOH extract was heated in boiling water bath for several min. As shown in Table III, cytolytic activity in EtOH extract was stable on heating at 100° for 60 min. EtOH extract was acidified with HCl to pH 1 or alkalized with NaOH to pH 13, after 10 min, they were adjusted to pH 7.2 with NaOH or HCl, and measured cytolytic activities. As shown

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in Table IV, the EtOH extract was also demonstrated to be stable over a wide range of pH value (1—13). On the other hand, when EtOH extract was acidified with HCl, precipitate occurred and this precipitate could be extracted with AcOEt. The AcOEt extract was concentrated to dryness, the residue was dissolved in the buffer and measured cytolytic activity. As shown in Table IV, the cytolytic substance in EtOH extract could be extracted with AcOEt at pH 1. As the AcOEt extract process were found to be more efficient for extraction of the cytolytic substance than the carbon adsorption process, it was used in the following experiment for isolation of the cytolytic substance.

Purification of the Cytolytic Substance in the Culture Medium

Bacillus natto KMD 1126 was grown in bouillon at 37° for 24 hr on a reciprocal shaker. After the elimination of cells by centrifugation, the culture medium was acidified with HCl, the resulting precipitate was collected by centrifugation, and the precipitate was extracted with AcOEt. The AcOEt layer was washed with H_2O, dried over Na_2SO_4, concentrated to dryness and the residue was dissolved in the buffer. This solution was poured over a column of Sephadex G 25 and eluted with the buffer. Active fraction was collected, acidified with HCl, and extracted with AcOEt. The AcOEt extract was evaporated to dryness and the residue was treated with petr. ether. The residue was dissolved in acetone, and applied to a Sephadex LH 20 column pretreated with acetone. The eluate containing active fraction was concentrated and added H_2O to make the solution slightly turbid. When this solution was preserved in cold, crystalline precipitate was obtained. Chemical or physical data of this compound were as follows: C, 61.02%; H, 9.10%; N, 9.11%. mp 136—138°, $[\alpha]_D=+39°$ ($c=1$, chloroform), $[\alpha]_D=-39°$ ($c=1$, MeOH). These results were almost identical to surfactin which was potent clotting inhibitor in the thrombin fibrinogen system obtained from culture fluids of *Bacillus subtilis* by Kakinuma, *et al.*[6] The results of purification of the cytolytic substance are summarized in Table V.

Table V. Purification of the Cytolytic Substance

Purification steps	Weight (mg/l)	Total activity (u/l)	Specific activity (u/mg)	Recovery (%)
EtOH extract	106	180.2	1.7	100
Sephadex G 25	37	140.6	3.8	78
Sephadex LH 20	30	135.0	4.5	75
Crystalline Substance	16	76.8	4.8	43

Characterization of the Cytolytic Substance

On thin-layer chromatography using Silica gel GF_{254} and EtOH, MeOH, chloroform, petr. ether, or acetone–AcOH–H_2O (20: 6: 74), the cytolytic substance gave single spot respectively. Ultraviolet (UV) absorption spectrum showed the absence of absorption maximum in the range from 230 nm to 400 nm. On the other hand, presence of peptide bonds were clearly demonstrated from infrared (IR) spectrum (Fig. 2).

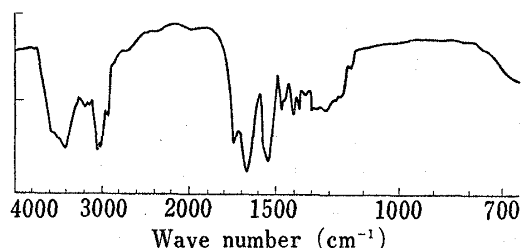

Fig. 2. IR Spectrum of the Cytolytic Substance (KBr)

1. Amino Acid Composition——When this cytolytic substance was subjected to acid hydrolysis in a sealed tube at 110° for 20 hr, aspartic acid, glutamic acid, valine, and leucine were detected by amino acid analyzer in the molecular ratio of 1: 1: 1: 4. Simultaneously, yellowish brown-colored HCl-insoluble oily ma-

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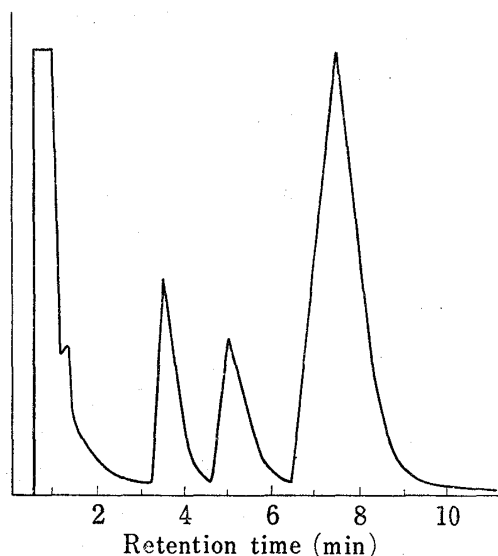

Fig. 3. Gas Chromatogram of Fatty Acid Methyl Ester on SE 30

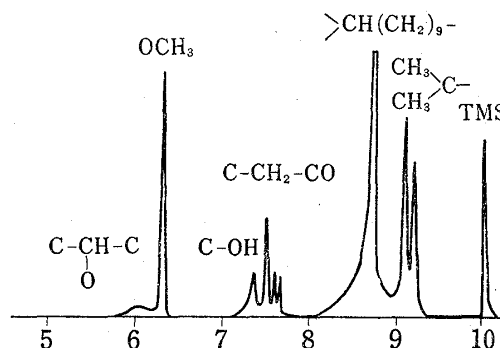

Fig. 4. NMR Spectrum of Fatty Acid Methyl Ester ($CDCl_3$)

terial was liberated with the recovery of about 20% of original substance. This oily material did not contain N and showed in IR spectrum typical features characteristic to fatty acids. From these data, this material is confirmed to be a peptidelipid composed of amino acids and fatty acids.

2. Structure of Fatty Acid——The cytolytic substance was dissolved in 30% HCl–MeOH and heated at 90° for 14 hr in a sealed tube. After methanolysis, HCl–MeOH was removed by evaporation and the residue was extracted with petr. ether. Separation of fatty acid methyl ester and acyl amino acid methyl ester from the petr. ether extract was accomplished by silicic acid column chromatography, using chloroform and chloroform–MeOH (4: 1) as the developing solvent. The fatty acid methyl ester, which was obtained from chloroform eluate, was consisting of 3 component on gas chromatogram (Fig. 3).

That is, main peak which had the longest retention time and two minor peaks were present on gas chromatogram. The main peak was determined to be methyl 3-hydroxy-13-methyltetradecanoate by the following NMR and mass spectrum.

NMR spectrum (Fig. 4). Doublet at 9.2 τ (6H, J=6.0 cps) is regarded to indicate the presence of CH_3-CH-CH_3 in this methyl ester. The presence of OH was further verified from the fact that the signal at 7.35 τ (1H, singlet) corresponding to OH disappeared after treatment of this preparation in D_2O.

Mass spectrum (Fig. 5). From the parent peak (*m/e*, 272) and the dehydration peak thereof (*m/e*, 254), the molecular weight of this methylester was determined to be 272. The base peak at *m/e*: 103 due to the fragment -CH(OH)-CH_2-$COOCH_3$ together with the peak at *m/e*: 199 due to $(CH_3)_2$-CH-$(CH_2)_9$-CH(OH)-, shows clearly the attachment of OH to the C_3 carbon. These data was completly identical to the data reported by Kakinuma, *et al.*[6] Two minor peaks were proved to be C_{13}- and C_{14}-3-hydroxy acids from comparison of retention time with authentic normal 3-hydroxy acid of C_{11}-C_{14}. That is, the cytolytic substance composing fatty acid was determined to be 3-hydroxy-13-methyltetradecanoic acid (C_{15} iso β-OH acid) and C_{13}- and C_{14}-β-OH acids were also detected by gas chromatography as minor components. On the other hand, the acyl amino acid methyl ester, which was isolated by silicic acid chromatography after methanolysis was subjected to acid hydrolysis, and glutamic acid was detected by amino acid analyzer. This results suggested that there was amide linkage between carboxyl group of the fatty acid and amino group of glutamic acid.

3. Amino Acid Sequence——Recently mass spectrometry has found promissing appli-

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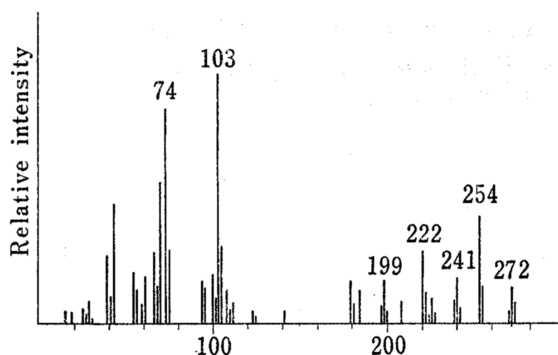

Fig. 5. Mass Spectrum of Fatty Acid Methyl Ester

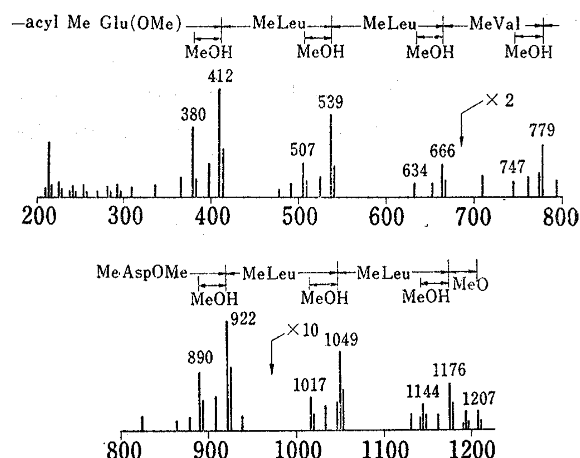

Fig. 6. Mass Spectrum of the Permethylated Derivative of Alkali Treated Cytolytic Substance

cation in the elucidation of the primary structure of oligopeptides.[8] We have applied this method to elucidate the amino acid sequence of the cytolytic substance. Sample was treated with alkali to open the lacton ring, permethylated according to the method of Hakomori,[7] and measured mass spectrum. From the parent peak (*m/e*: 1207) and the demethoxy peak thereof (*m/e*: 1176), the molecular weight of this material was determined to be 1207. The next peak at *m/e* 1144 is due to the further loss of MeOH from the fatty acid moiety of I. The mass difference of 127 between *m/e* 1176 and *m/e* 1049 corresponds to the loss of an N-methylated leucine (MeLeu) indicated that the C-terminal amino acid in the cytolytic substance is leucine. The peaks at 922, 779, 666, 539, and 412 can be regarded to have derived by the further successive elimination of MeLeu, MeAsp-OMe, MeVal, MeLeu, and MeLeu from *m/e* 1049, respectively. The remaining amino acid, Glu, is therefore directly linked to the fatty acid moiety. From Fig. 6, the structure of permethylated derivative is assumed as I. From these results, the cytolytic substance was proved to be identical with surfactin.

Formula 1

Acknowledgement The authors are grateful to Dr. A. Kakinuma of Takeda Chemical Industries, Ltd., for the gift of surfactin and to Prof. Dr. M. Hiramoto of this Faculty, for the gift of authentic 3-hydroxy fatty acids. The authors also wish to thank Mr. Y. Itatani of this Faculty for helpful suggestion on NMR spectrum. Thanks are also due to members of the Central Analysis Room of this Faculty for elementary analysis, NMR and Mass spectral measurements.

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