[Original] Matsumoto Shigaku 12 : 139~144, 1986
key words : B. matruchotii — antimicrobial agents — Matrucin

Isolation and Properties of Low Molecular Weight Antimicrobial Agents (Matrucin) from an Oral Bacterium *Bacterionema matruchotii*

TAKESHI NAKAMURA and SETSUO FUJIMURA
Department of Oral Microbiology, Matsumoto Dental College
(Chief : Prof. T. Nakamura)

Summary

Antimicrobial agents, designated Matrucin-1 and Matrucin-2, were isolated from culture supernatants of *Bacterionema matruchotii* IBN6, obained from dental plaque. They seem to be peptides with molecular weights less than 1,000. Matrucin-1 and Matrucin-2 were hardly soluble in polar solvents. Both inhibited the growth of various oral bacteria.

Introduction

Antimicrobial agents produced by oral bacteria have been investigated by many investigators, since they may function as controling factors of the oral ecosystem[20]. However, the agents from oral aerobic bacteria have not been so extensively studied. In a previous paper[10], we described the production of antimicrobial agent by some strains of *Bacterionema matruchotii,* isolated from dental plaque. After that, we confirmed that two kinds of antimicrobial agents were released into the surrounding medium by these organisms, which were separable from each other by thin-layer chromatography. In this paper, we report on the purification and characterization of these antimicrobial agents.

Materials and Methods

Bacterial strains and cultivation The producer strain of antimicrobial agents, *B. matruchotii* IBN6 was isolated from dental plaque[10]. *B. matruchotii* ATCC 14266 was used as an indicator strain for assaying antimicrobial activity. Strain IBN6 was cultivated on a reciprocating table (120 strokes per min) at 37°C for 2 days in a medium of 3.7 per cent brain-heart infusion and 0.2 per cent yeast extract.

Assay of antimicrobial activity Antimicrobial activity was measured by the diffusion method on agar plates as described earlier[9]. The number of activity units per milliliter was expressed as the reciprocal of the highest dilution which gave clear inhibitory zones of the growth of the indicator strain.

Thin-layer chomatography (TLC) TLC of antimicrobial agents was carried out on Merck F254 silica gel plates (Merck, Darmstadt, W. Germany) using a solvent system of ethyl acetate-methanol (2 : 1 v/v). After chromatography was finished, UV-absorbing areas were decteted by irradiation

(accepted for publication on June 9, 1986)

with a UV-lamp. Then the growth-inhibitory zone on the same plate generated by the antimicrobial agents was detected by bioautography technique[2]; briefly, developed and dried chromatogram plates were overlaid with brain-heart infusion-yeast extract agar containing cells (2×10^5/ml) and kept for 2 days at 37°C for incubation of the indicator. For a better recognition of the inhibitory zones on the chromatogram plates, 2, 3, 5-triphenyltetrazolium chloride was added into the agar plate which is reduced to a formazan of a red coloration, thus inhibitory zones are better visible on a red background[16].

Chemical analyses Quantitative determination of the antimicrobial agents were carried out copper-acetate reagent for lipids[6], anthrone-sulfuric acid reagent for carbohydrates[11], modified Dittmer reagent for phosphate[17], ninhydrin reagent for free amino acisds[14], Dragendroff reagent for choline[3], sulfuric acid reagent for sterols[8], alkaline hydroxylamine reagent for ester bonds[13], 2, 4-dinitrophenylhydrazine for carbonyl compounds[15], and starch-potassium iodine reagent for peptide bonds[12]. For amino acid analysis, 0.1mg of the samples were hydrolyzed in 6 N HCl at 110° C for 16 h in sealed, evacuated tubes. The amino acid composition was determined with a JEOL aminio acid analyzer, model JLC-6AH.

A rough estimation of molecular size was carried out by the method of Hale and Hinsdill[7], using a "specra pore 6" dialysis tubing (Spectrum Medical Industries Inc. Los Angeles, Calif.) for m. w. 1,000. This membrane permits only substance with molecular weight lower than 1,000 to pass through.

Results

Purification The culture supernatant contained two antimicrobial agents, which were separated from each other by TLC in various solvent systers (Table 1 and Fig. 2, lane 1). The antimicrobial agents with high and low Rf values were referred to as Matrucin-1 and Matrucin-2, respectively and we undertook to purify both agents from the culture supernatant. A flow sheet of the purification procedure is shown in Fig. 1.

(i) Fractionation with ammonium sulfate. Solid ammonium sulfate was added slowly to 3 liter of the culture supernatants of *B. matruchotii* IBN6 to give 50 per cent saturation. After stiring for 1 h, the resulting precipitate was collected by centrifugation at 10,000 ×g for 20 min. When we attempted to dissolve the precipitate in 0.05 M phosphate buffer (pH 7.0), a clear solution could not be obtained, even after centrifugation of this sample at 20,000 ×g for 30 min. The sample showing turbidity was dialyzed using a spectra pore 6 dialysis tubing against 0.05 M phosphate buffer (pH 7.0) over night at 4°C.

(ii) Extraction with chloroform-methanol. From the dialyzed sample, antimicrobial agents were extracted by the method of Bligh and Dyer[4]. To 450 ml of the ammonium sulfate fraction, 1,125 ml of methanol and 563 ml of chloroform were added and stirred vigorously for 3 h. Then 563 ml of chloroform and an equal volume of water were added, followed by stiring for 15 min. After the mixture seperated into a chloroform phase and a water phase, the chloroform phase was withdrawn and evaporated to dryness. The dried material was dissolved again in a solution of chroloform-methanol (2 : 1, v/v).

(iii) Silicic acid column chromatography. The sample from the previous step was applied to a colum (1.5 by 30 cm) of silicic acid, equilibrated with chloroform-methanol (100 : 1, v/v). Matrucin-1 was eluted from the column with the eluting solvent chloroform-methanol (100 : 3, v/v), and Matrucin-2 with chloroform-methanol (100 : 15).

(iv) Rechromatography on a silicic acid colum. The fraction containing Matrucin-1 was applied to a silicic acid column (1.0 by 20 cm), equilibrated with chloroform. After the column was washed with chloroform-ethylacetate (1 : 1, v/v), Matrucin-1 was eluted with etylacetate. The fraction containing Matrucin-1 activity was combined and dried with an evaporater in vacuo. The powder was dissolved in a small volume of chloroform and then 10 times the volume of petroleum ether was

Table 1. Thin-layer chromatography of antimicrobial agents, Matrucin-1 and Matrucin-2, in various solvent systems

Solvent system	Rf values	
	Matrucin-1	Matrucin-2
chloroform	0	0
methanol	0.88	0.88
chloroform-methanol (95 : 12, v/v)	0.48	0.06
chloroform-methanol-water (65 : 25 : 3, v/v)	0.66	0.47
chloroform-methanol-acetic acid (65 : 25 : 4, v/v)	0.89	0.80
chloroform-methanol-28% ammonia-water (65 : 25 : 5, v/v)	0.88	0.41
ethyl acetate	0.27	0
ethyl acetate-methanol (100 : 15, v/v)	0.49	0
ethyl acetate-methanol (2 : 1, v/v)	0.68	0.15
n-buthanol-acetic acid-water (3 : 1 : 1, v/v)	0.86	0.78
hexane-diethyl ether-acetic acid (80 : 20 : 1, v/v)	0	0
ethanol-28% ammonia-water (8 : 1 : 1, v/v)	0.96	0.82
propanol-pyridine-acetic acid-water (15 : 10 : 3 : 12, v/v)	0.94	0.84

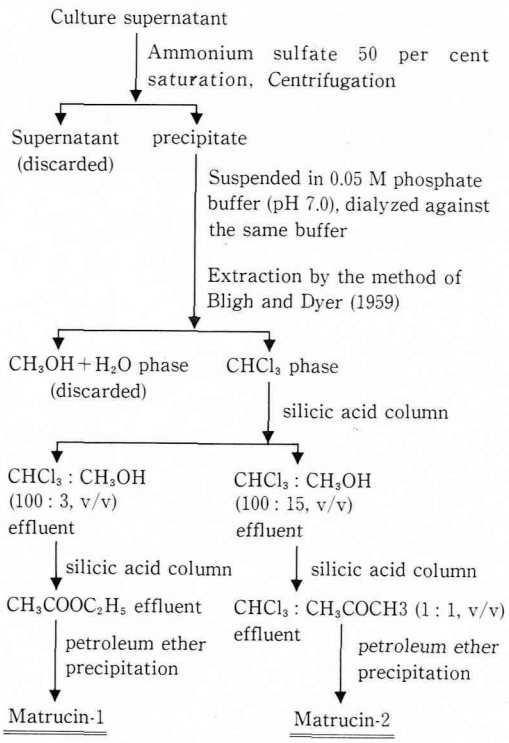

Fig. 1. Flow sheet of purification procedures of Matrucin-1 and Matrucin-2

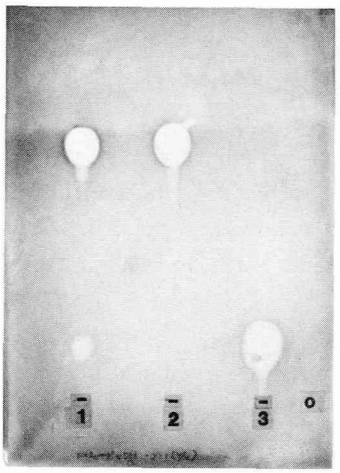

Fig. 2. Growth inhibition of indicator strain on the thin-layer chromatogram of the culture supernatant (ammonium sulfate fraction) (Lane 1), purified Matrucin-1 (Lane 2), and purified Matrucin-2 (Lane 3)

Table 2. Minimum inhibitory concentrations of Matrucin-1 and Matrucin-2 for oral indigenous bacteria

Tested strain	Minimum inhibitory concentration (μg/ml) Matrucin-1	Matrucin-2
Bacterionema matruchotii ATCC 14266	0.1	12.5
Bacterionema matruchotii # 13	0.2	25.0
Actinomyces israelii ATCC 12102	0.05	6.4
Actinomyces naeslundii ATCC 12104	0.05	3.2
Propionibacterium acnes ATCC 6919	0.05	12.5
Propionibacterium acnes ATCC 11827	0.05	6.4
Corynebacterium parvum ATCC 11829	0.05	6.4
Lactobacillus casei ATCC 7469	0.2	12.5
Lactobacillus acidophilus IFO 3205	0.2	12.5
Lactobacillus fermentum IAM 1083	0.2	12.5
Bacterionema matruchotii IBN 6	>100	>100
Actinomyces viscosus ATCC 15987	>100	>100
Streptococcus mutans Ingbritt	>100	>100
Streptococcus sanguis ATCC 10556	>100	>100
Streptococcus sanguis ATCC 10557	>100	>100
Streptococcus mitis ATCC 9896	>100	>100
Streptococcus salivarius ATCC 9759	>100	>100
Fusobacterium necrophorm ATCC 22586	>100	>100
Fusobacterium nucleatum ATCC 25286	>100	>100
Bacteroides melaninogenicus NM2	>100	>100

added to this solution. The resulting precipitate was collected by centrifugation at 2,000 ×g for 10 min and washed twice with diethylether and dried. This was the purified sample of Matrucin-1. Matrucin-2 was applied to a column (1.0 by 10 cm) of silicic acid and eluted with chloroform-acetone (1 : 1, v/v). The active fractions were combined and the Matrucin-2 sample was subsequently prepared similarly.

Purity of both purified Matrucin-1 and Matrucin-2 were examined on TLC. Single UV-absorbing spots were detected from both preparations. Furthermore, the position of these spots coincided with the growth-inhibitory zones of the indicator strain (Fig. 2, lane 2 and 3). Based on these criteria, Matrucin-1 and Matrucin-2 were considered to be pure.

Properties of Matrucin-1 and Matrucin-2. The mobility of Matrucin-1 and Matrucin-2 on TLC with various developing solvents are presented in Table 1.

No difference was found in various quantitative color tests between the preparations; they were positive in starch-potassium iodine reaction and weakly positive in the 2, 4-dinitrophenyl hydrazine reaction. However, they were negative in the following tests: Copper-acetate, anthrone-sulfuric acid, ninhydrin, Dragendroff, sulfuric acid-acetate, and alkaline hydroxylamine.

Solubility of Matrucin-1 and Matrucin-2 in the various organic solvents was also quite similar. Both were soluble in methanol, ethanol, acetone, chloroform, and ethylacetate, but insoluble in petroleum ether, diethylether, and hexane.

In UV-absorbing spectra[18,19] of both preparations in methanol, peaks were found at 205 nm ($E^{1\%}_{1cm}$=549) and 305 nm ($E^{1\%}_{1cm}$=242) with a shoulder at 215.5 nm in Matrucin-1. In Matrucin-2, the peaks were at 205 nm ($E^{1\%}_{1cm}$=864) and 305 nm ($E^{1\%}_{1cm}$=215).

In infrared absorbing test, both preparations had absorption bands at 1,480 cm^{-1}, 1,640cm^{-1}, and 3,280 cm^{-1}. Bands at 2,780 cm^{-1} and 2,830 cm^{-1} in Matrucin-2 were not observed in Matrucin-1.

By amino acid analysis, methionine, tyrosine, phenylalanine and an unidentified amino acid were detected from both preparations.

When Matrucin-1 and Matrucin-2 were heated at 100°C for 1 h at neutral pH, no significant loss of activity occurred. However, incubation at 37°C for 48 h at pH 2 or pH 11 resulted in loss of activity by 60 per cent and 80 per cent, respectively.

Molecular weights of Matrucin-1 and Matrucin-2 appeared to be less than 1,000, since both preparations could pass through the specified dialysis membrane (see Materials and methods).

Minimum inhibitory concentrations of both antimicrobial agents against some species of oral indigenous bacteria were compared and the results are given in Table 2. It is obvious that the inhibitory spectra of Matrucin-1 and Matrucin-2 were quite similar. Matrucin-1 inhibited the susceptible strains at rather lower concentrations than those of Matrucin-2. The producer strain *B. matruchotii* IBN6 was resistant but the negative strains for production, *B. matruchotii* ATCC 14266 and # 13 were readily inhibited.

Matrucin-1 and Matrucin-2 affected susceptible strains bacteriostatically without killing them, as previously reported[10].

Discussion

B. matruchotii IBN6 was found to produce Matrucin-1 and Matrucin-2 in the culture supernatant, which were separable from each other by TLC. We purified them by the sequential procedures of ammonium sulfate precipitation, extraction with chloroform-methanol, and repeated chromatography on silicic acid columns. Although, Matrucin-1 and Matrucin-2 were precipitated from the culture supernatant with ammonium sulfate, this phenomenon may be due to "co-precipitation" with protein fractions in the culture supernatant, since both molecules are too small to be salted out with this reagent at 50 per cent saturation.

The chemical structure of Matrucin-1 and Matrucin-2 seem to be very similar, however, we can not provide reason for their different mobility on TLC at prersent. From the results of amino acid analysis, molecular size, starch-potassium iodine reaction (positive), and ninhydrin reaction (negative), Matrucin-1 and Matrucin-2 are likely to be peptides lacking free 2-amino group, may be cyclic peptide or peptide whose amino group is blocked by, for example, a formyl or acetylgroup. UV-absorbance spectra of antibiotics such as mikamycin B[18,19], estamycin[1], and ostreogrycin[5], show an absorption maximum at 305 nm. This indicates that these antibiotics contain 3-hydroxypicolinic acid[19]. Since Matrucin-1 and Matrucin-2 had an absorbance maximum at this wave length, it is possible that both contain 3-hydroxypicolinic acid as a molecular constituent.

Matrucin-1 and Matrucin-2 seem to inhibit the growth of Gram-positive rods among oral indigenous bacteria. The producer strain of Matrucin-1 and Matrucin-2, viridans streptococci, and Gram-negative species examined were not susceptible to them.

References

1) Arnold, R. B., Johnson, A. W., and Mauger, A. B. (1958) The structure of viridogrisein (estamycin). J. Chem. Soc. 4466—4470.

2) Aszalos, A., and Frost, D. (1975) Thin-layer chromatography of antibiotics. In : Methods in Enzymology, vol 43, J. H. Hash, ed, New York, Academic press, 172—213.

3) Beiss, U. (1964) Zur papierchromatographischen Auftrennung von Pflanzenlipiden. J. Chromatography, **13** : 104—110.
4) Bligh, E. G., and Dyer, W. J. (1959) A rapid methods of total lipid extraction and purification. Can. J. Biochem. Physiol. **37** : 911—917.
5) Eastwood, F. W., Snell, B. K., and Todd, A. (1960) Antobiotics of the E129 (ostreogrycin) complex. Part 1. The structure of E129B. J. Chem. Soc. 2286—2292.
6) Fewster, M. E., Burns, B. J., and Mead, J. F. (1969) Quantitative densitometric thin-layer chromatography of lipids using copper acetate reagent. J. Chromatography **43** : 120—126.
7) Hale, E. M., and Hinsdill, R. D. (1973) Characterization of bacteriocin from *Staphylococcus aureus* strain 462. Antimicrob. Agents Chemother. **20** : 641—648.
8) Jatzkewitz, H., and Mehl, E. (1960) On thin-layer chromatography of cerebral lipid, their conversion and decomposition products. Hoppe-Seyler's Z. physiol. Chem. **320** : 251—257.
9) Nakamura, T., Suginaka, Y., Obata, T., and Yamazaki, N. (1977) Bacteriocin-like activities of human dental plaque flora against oral anaerobic microorganisms. Bull. Tokyo Dent. Coll. **18** : 217—229.
10) Nakamura, T., Kanagawa, N., Taniguchi, H., and Fujimura, S. (1984) Bacteriocin activity of the bacterium *Bacterionema matruchotii* isolated from dental plaque in man. Arch. Oral Biol. **29** : 739—743.
11) Radin, N., Lavin, F. B., and Brown, J. R. (1955) Determination of cerebrosides. J. Biol. Chem.**217** : 789—796.
12) Rydon, H. N. and Smith, P. W. G. (1952) A new method for the detection of peptides and similar compounds on paper chraoatograms. Nature **169** : 922—923.
13) Skidmore, W. D. and Entenman, C. (1962) Two dimensional thin-layer chromatography of rat liver phosphatides. J. Lipid Red. **3** : 471—475.
14) Skipski, V. P., Peterson, R. F. and Barclay, M. (1962) Separation of phosphatidyl serine and other phospholipids by thin-layer chromatography. J. Lipid Res. **3** : 467—470.
15) Skipski, V. P. and Barclay, M. (1969) Thin-layer chromatography of lipids. In : Methods in Enzymology, vol 14, J. M. Lowenstein ed, New York, Academic press, 530—598.
16) Usdin, E., Shockman, G. D. and Toennies, G. (1954) Tetrazolium bioautography. Appl. Microbiol. **2** : 29—33.
17) Vaskovsky, V. E. and Kotetsky, E. W. (1968) Modified spray for the detection of phospholipids on thin-layer chomatograms. J. Lipid Res. **9** : 396.
18) Watanabe, K. (1960) Studies on mikamycin. IV. The isolation and properties of mikamycin. J. Antibiosics (Tokyo) Ser, A, **13** : 57—61.
19) Watanabe, K., Yonehara, H., Umezawa, H. and Sumiki, Y. (1960) Structure of mikamycin B. J. Antibiotics (Tokyo) Ser, **13** : 293—294.
20) Weerkamp, A. L., Bongaerts-Larik L. and Vogels G. D. (1977) Bacteriocin as factors in the in-vitro interactions between oral streptococci in plaque. Infect. Immun. **16** : 773—780.