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Author(s)	Kurihara, Hideyuki; Goto, Yurie; Aida, Masahiko et al.
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Antibacterial Activity against Cariogenic Bacteria and Inhibition of Insoluble Glucan Production by Free Fatty Acids Obtained from Dried *Gloiopeltis furcata*

Hideyuki Kurihara, Yurie Goto, Masahiko Aida, Masashi Hosokawa, and Koretaro Takahashi

Department of Marine Bioresources Chemistry, Faculty of Fisheries, Hokkaido University, Minato, Hakodate, Hokkaido 041-8611 Japan

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Antibacterial substance for mutans streptococci was obtained from dried *Gloiopeltis furcata* by chromatographic separation. The substance was determined to be a mixture of free fatty acids (FFAs), from the results of instrumental analyses. The FFAs obtained showed moderate antibacterial activity against mutans streptococci, with minimal inhibitory concentration values between 25 and 50 $\mu g/ml$. These FFAs also inhibited insoluble glucan production catalyzed by glucosyltransferase of *Streptococcus sobrinus*. Experiments carried out by using commercially available fatty acids indicated that unsaturated fatty acids showed more potent inhibition against insoluble glucan production than saturated fatty acids.

Key words: *Gloiopeltis furcata*, mutans streptococci, antibacterial activity, glucosyltransferase, inhibition, free fatty acid

Dental caries is a major infectious disease.[1] Causative bacteria are mutans streptococci which relate to insoluble glucan production from sucrose as well as induction of caries.[1] Adherent insoluble glucan forms dental plaque, a causative matter of promotion of caries, with incorporating bacteria.[1-3] The plaque gives good conditions for promoting caries such as localization of cariogenic bacteria, anaerobic atmosphere, localization of organic acid produced by bacteria and barrier to saliva working as a buffer. Production of adherent insoluble glucan is mediated with glucosyltransferase (GTase) produced by cariogenic bacteria.[1] Inhibition of insoluble glucan production by GTase could decrease a risk of infecting dental caries.[3]

In attempts to find seafoods containing antibacterial substance against mutans streptococci, we have screened extracts of various seafoods (unpublished results). Among them, the extract of dried *Gloiopeltis furcata* showed potent antibacterial activity. In the present paper, we investigated antibacterial substance against mutans streptococci and inhibitor for insoluble glucan production mediated by extracellular GTase from dried *G. furcata*.

Materials and Methods

General

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL-JMN-FX90Q spectrometer. The substance was dissolved in chloroform-*d* with tetramethylsilane as an internal standard. Gas chromatography (GC) was recorded on a HITACHI 163 Gas Chromatograph equipped with G-300 column (film thickness, 0.5 μm; i.d. 1.2 mm × length 40 m; Chemical Inspection and Testing Institute, Japan). Condition of GC analysis was as follows: column temperature, 200°C; injection temperature, 250°C; carrier gas, He; detection, FID. Turbidity caused by insoluble glucan was measured with a HITACHI UV2000 Spectrophotometer at 550 nm. Fatty acids, 14:0, 16:0, 20:0, 16:1, 18:3n-3, 18:3n-6, and 20:4n-6, were purchased from Doosan Serdary Research Laboratories (Englewood Criffs, USA). Fatty acids 18:0 and (−)-epigallocatechin gallate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), 18:1 from Sigma Chemical Company (St. Louis, USA), and 18:2n-6 from Idemitsu Petrochemical Co., Ltd. (Tokyo, Japan). Fatty acid 20:5n-3 was a generous gift from Nippon Chemical Feed Co., Ltd. (Hakodate, Japan). A GLC-standard mixture of fatty acid methyl esters (GLC-68A) was obtained from Nu-Chek-Prep, Inc. (Elysian, USA).

Microbes

Cariogenic mutans streptococci employed in this study were *Streptococcus mutans* JCM 5175, *S. mutans* JCM 5176, *S. mutans* JCM 5705 obtained from Japan Collection of Microorganisms, the Institute of Physical and Chemical Research (RIKEN). *S. sobrinus* GIFU 8819 and *S. rattus* GIFU 8641 were obtained from Laboratory of Microbiology, School of Medicine, Gifu University. A non-cariogenic bacterium *S. salivarius* subsp. *salivarius* JCM 5707 was obtained from Japan Collection of Microorganisms, the Institute of Physical and Chemical Research (RIKEN). *Streptococcus* spp. were individually inoculated into brain heart infusion (BHI, 3.7 g/l, Difco Laboratories, Detroit, USA) broth and cultured at 37°C for 24 h. The culture suspension was transferred into vials in which beads were placed using sterilized pipettes. When bacteria adhered to beads, remaining suspension was sucked up from the vials. The beads in the vials were frozen in a deep-freezer at −75°C until use. Before assay, a bead was

added into BHI broth (10 m*l*). The bacteria were preincubated at 37°C for 24 h.

Paper Disk Method for Screening of Antibacterial Substance

A paper disk method was used for primary screening and separation of antibiotics. This was done by a modified method of Namba *et al.*[4] A BHI agar (BHI, 0.185 g/20 m*l*; agar, 1.2%) plate was prepared in a Petri dish (i.d. 9 cm). Precultured bacterial suspension (0.25 m*l*) was added onto the plate and then spread. A paper disk (thick, ϕ8 mm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) individually impregnated with a test sample was placed on the agar plate. The Petri dish was kept under anaerobic atmosphere at 37°C for 24 or 48 h. Diameter of the inhibitory zone around the paper disk was measured in duplicate tests.

Extraction and Separation of Antibacterial Substance

Dried *G. furcata* (141 g dry wt) was purchased from a grocery store in Hakodate in May 1996, ground with a mill, and then extracted with acetone at room temperature for a week. Extracted solution was filtered and evaporated under reduced pressure to give the primary extract. The residue was re-extracted with 80% acetone for a week to give the secondary extract. The primary and the secondary extracts were combined to make crude extracts (15.6 g). Then the extracts were partitioned between *n*-hexane and 90% methanol. The *n*-hexane-soluble fraction (1.40 g) thus obtained showed antibacterial activity. Through antibacterial activity-guided fractionation, the fraction was separated into antibacterial substance (0.56 g) by silica gel column chromatography followed by thin-layer chromatography (TLC). A comparison of the antibacterial substance with commercially available 18:1 was carried out by analytical silica gel TLC developed with hexane-diethyl ether-acetic acid (75:25:1).

Agar Dilution Method for Quantifying Inhibitory Activity of the Antibacterial Substance

The agar dilution method was performed by a modified method of Ouattara *et al.*[5] Just in brief, test sample (30 mg) dissolved in dimethyl sulfoxide (DMSO, 6 m*l*) was filtered through a 0.2 μm-membrane filter. The filtrate thus obtained (5000 μg/m*l*) was added into sterilized 5 m*l*-vial, and successively diluted with DMSO at concentrations of 2500, 1250, and 625 μg/m*l* into separate sterilized 5 m*l*-vials. BHI agar media (14.7 m*l*) were pipetted into 20 m*l*-vials, autoclaved, then kept at 60°C. The test sample solution (0.3 m*l*) was added into each vial and mixed thoroughly. The mixtures were applied into sterile 3 cm-Petri dishes and left to solidify. One platinum loop of precultured bacterial suspension was inoculated onto the agar and spread. The bacteria were cultured under anaerobic condition at 37°C for 24 h. Each test was performed in triplicate measurements. Minimal inhibitory concentration (MIC) was determined by judging colonies visually on the agar plate.

Preparation of Crude GTase Solution from S. sobrinus[6]

Precultured *S. sobrinus* suspension (1 m*l*) was transferred into BHI broth (300 m*l* × 4) in culture flasks then incubated at 37°C for 24 h. The suspension was centrifuged at 12000 × *g* (4°C) for 20 min. The supernatant was brought to 60% saturation with ammonium sulfate and then standed overnight. The suspension thus obtained was centrifuged at 30000 × *g* (4°C) for 20 min. The precipitate was dissolved in 50 mM potassium phosphate buffer (pH 6.5). This solution was dialyzed twice against 50 mM potassium phosphate buffer (pH 6.5). Finally, it was centrifuged at 30000 × *g* (4°C) for 20 min to give a crude GTase solution.

Inhibition Assay for insoluble glucan production by Free Fatty Acids (FFAs)

The Assay of GTase activity was done by a modified method of Osawa *et al.*[6] The substrate solution, 2.5% sucrose in 50 mM phosphate buffer (pH 6.5, 0.4 m*l*) and aliquots of FFA (5000, 1670, 556, 185, and 61.7 μM) in DMSO (0.1 m*l*) were mixed in a test tube. Saturated fatty acids (14:0, 16:0, and 20:0) in DMSO were sonicated until homogeneous suspension. Then crude GTase solution (0.5 m*l*) was added into this substrate solution. The mixture was incubated at 37°C for 24 h in the test tube kept at an angle of 30° to the horizontal. Insoluble glucan adhered to the glass surface of the test tube was gently washed and dispersed by sonication in 4.0 m*l* H_2O. The amount of adhered insoluble glucan was measured by turbidimetry at 550 nm using a spectrophotometer. The percentage of insoluble glucan production was calculated in comparison with turbidity in the absence of the test compound as a control. Each test was performed in quadruplicate measurements. IC_{50} values were determined by inhibition-log (FFA concentration) plot analysis.

Results

Identification and Antibacterial Activity of the Antibacterial Substance

The yield of the antibacterial substance was about 0.4% of the dried alga. The substance was determined to bear carboxyl group because of a positive response in a bromocresol green test. The data of ^1H and ^{13}C NMR spectra of the antimicrobial substance showed characteristics of FFAs.[7,8] From these results and also from the R_f value coinciding with that of commercially available FFA standard (18:1) on TLC, the antibacterial substance was determined to be a mixture of FFAs. The GC analysis of the antibacterial substance disclosed that major fatty acid was 20:5n-3, and unsaturated fatty acids comprised high proportion among all fatty acids (Table 1). The MIC values of the FFA mixture obtained were 25 to 50 μg/m*l* against *Streptococcus* spp. (Table 2). The antibacterial activity was almost comparable among the six strains examined of *Streptococcus* spp.

Effect of FFAs on GTase Activity

Inhibitory activity of FFA mixture of *G. furcata* was determined against insoluble glucan production mediated by GTase from *S. sobrinus*. The mixture inhibited insoluble glucan production by 61.6% and 13.4% at concentrations of 100 and 10 μg/m*l*, respectively, while they showed no inhibition at a concentration of 1 μg/m*l*. Inhibitory activity of each standard fatty acid demonstrated that saturated fatty acids, such as 14:0, 16:0, and 20:0, had a weak or no

Table 1. Fatty acid composition of the antibacterial substance

Fatty acid	(%)
16:0	15.9
16:1	3.2
18:1	7.5
18:2	1.6
18:3	0.7
20:0	1.4
20:3	0.9
20:4	2.3
20:5	66.4

Table 2. Minimal inhibitory concentration (MIC) values of the antibacterial substance against *Streptococcus* spp.

Strain	MIC (μg/ml)
S. mutans JCM 5705	50
S. mutans JCM 5175	50
S. mutans JCM 5176	25
S. rattus	50
S. sobrinus	25
S. salivarius subsp. *salivarius*	50

Table 3. IC_{50} values of free fatty acids against insoluble glucan production by *Streptococcus sobrinus* glucosyltranserase

Fatty acid	IC_{50} (μM)
14:0	275
16:0	>500
20:0	320
16:1	75
18:1	45
18:2	50
18:3 n-3	85
18:3 n-6	85
20:4	40
20:5	85
(−)-EGCG*	>500

* (−)-Epigallocatechin gallate was employed as a known glucosyltransferase inhibitor obtained as food components.

inhibitory effect on GTase activity, while that of unsaturated fatty acids were notably high, giving IC_{50} values between 45 and 85 μM (Table 3). A well known GTase inhibitor, (−)-epigallocatechin gallate (EGCG),[9,10] from green tea showed weak inhibition against insoluble glucan synthesis at a concentration of 500 μM under the same condition above.

Discussion

The antibacterial substance in an edible dried *G. furcata* against mutans streptococci was disclosed to be a mixture of FFAs. FFAs are known to have antimicrobial activity against various microorganisms.[5,11-19] In general, the antibacterial activity of FFAs is greater toward Gram-positive bacteria than Gram-negative bacteria.[12] Mutans streptococci could have high susceptibility to antibacterial properties of FFAs since they are Gram-positive bacteria. Most unsaturated fatty acids showed potent antibacterial activity while most of saturated fatty acids showed no or weak antibacterial activity against *S. mutans*, as demonstrated by Hattori *et al.*[11] The antibacterial activity of the FFA mixture in this study would be dependent on high proportion of unsaturated fatty acids. The possible mechanism of antibacterial action by unsaturated fatty acids might be attributed either to an alteration of cell membrane properties or to generation of free radicals.[11,16,18,19] At any rate, the true mechanism is not fully understood.

FFAs also showed inhibitory activity for insoluble glucan production. To the best of our knowledge, inhibition of GTase by FFAs has not been reported before. They show considerable multifunctional properties for dental caries prevention like the methanolic extracts of traditional medicines used in Sri Lanka[20] and macrocarpals from the leaves of *Eucalyptus globulus*.[6] Inhibitory potencies on insoluble glucan production by GTase were examined for the individual FFAs. Unsaturated fatty acids showed more potent inhibition against GTase reaction than saturated fatty acids. Although it is not clear, the nature of the fatty acids, especially the dispersibility against buffer solution might be one reason for this. Insoluble glucan catalyzed by GTase of *Streptococcus* spp. contains predominantly $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow6)$ linkages,[1] while glucan catalyzed by (1,3)-β-glucan synthase of yeast *Saccharomyces cerevisiae* contains a $\beta(1\rightarrow3)$ linkage.[21] Ko *et al.*[21] revealed that FFA inhibited yeast (1,3)-β-glucan synthase most effectively among the lipid class examined. IC_{50} values against yeast (1,3)-β-glucan synthase were almost comparable among the FFAs.[21] From their report[21] and our results, we bear out that fatty acid has an inhibitory potency against various glucan production. But the inhibitory mechanisms are not well understood.

A mixture of FFAs, showing both antibacterial activity against mutans streptococci and inhibition of insoluble glucan production by GTase, was obtained from an edible dried alga in this study. Chlorhexidine is well known as an antibacterial agent against mutans streptococci.[22] Tsuchiya *et al.*[23] reported that MIC values of chlorhexidine for *S. mutans*, *S. sobrinus*, and *S. rattus* varied in the range from 3.13 to 6.25 μg/ml. Although antibacterial potency of the FFA mixture is lower than that of chlorhexidine, the moderate antibacterial potency of FFAs would be sufficient to prevent dental caries without undesirable side effects, because of large intake of FFAs as food components. Inhibitory potency of FFAs for insoluble glucan production was similar to that of glycyrrhizin[24] as a known inhibitor and higher than that of other food components such as tea polyphenols.[9,10] It is conceivable that FFAs, food components, have an effect on prevention of dental caries. It appears to be great to explore the potentials of these kinds of foods for the antibacterial activity against mutans streptococci and also the inhibition of insoluble glucan production by GTase.

References

1) S. Hamada and H. D. Slade: Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol. Rev.*, **44**, 331-384 (1980).
2) A. A. Scheie: Mechanisms of dental plaque formation. *Adv. Dent. Res.*, **8**, 246-253 (1994).
3) R. R. B. Russell: Control of specific plaque bacteria. *Adv. Dent.*

Res., **8**, 285–290 (1994).

4) T. Namba, M. Tsunezuka, K. H. Bae, and M. Hattori: Studies on dental caries prevention by traditional Chinese medicines (part I). Screening of crude drugs for antibacterial action against *Streptococcus mutans*. *Shoyakugaku Zasshi*, **35**, 295–302 (1981).
5) B. Ouattara, R. E. Simard, R. A. Holley, G. J.-P. Piette, and A. Begin: Antibacterial activity of selected fatty acid and essential oils against six meat spoilage organisms. *Int. J. Food Microbiol.*, **37**, 155–162 (1997).
6) K. Osawa, H. Yasuda, H. Morita, K. Takeya, and H. Itokawa: Macrocarpals H, I, and J from the leaves of *Eucalyptus globulus*. *J. Nat. Prod.*, **59**, 823–827, (1996).
7) D. Chapman, F. M. Goni, and F. D. Gunstone: Nuclear magnetic resonance spectra, in "The Lipid Handbook" (ed. by F. D. Gunstone, J. L. Harwood, and F. B. Padley), Chapman & Hall, London, 1994, pp. 511-533.
8) F. D. Gunstone, M. R. Pollard, C. M. Scrimgeour, and H. S. Vedanayagam: Fatty acids. Part 50. ^{13}C Nuclear magnetic resonance studies of olefinic fatty acids and esters. *Chem. Phys. Lipids*, **18**, 115–129 (1977).
9) M. Hattori, I. T. Kusumoto, T. Namba, T. Ishigami, and Y. Hara: Effect of tea polyphenols on glucan synthesis by GTase from *Streptococcus mutans*. *Chem. Pharm. Bull.*, **38**, 717-720 (1990).
10) S. Otake, M. Makimura, T. Kuroki, Y. Nishihara, and M. Hirasawa: Anticaries effects of polyphenolic compounds from Japanese green tea. *Caries Res.*, **25**, 438–443 (1991).
11) M. Hattori, K. Miyachi, S. Hada, N. Kakiuchi, F. Kiuchi, Y. Tsuda, and T. Namba: Effects of long-chain fatty acids and Fatty alcohols on the growth of *Streptococcus mutans*. *Chem. Pharm. Bull.*, **35**, 3507–3510 (1987).
12) C. E. Isaacs, R. E. Litov, P. Marie, and H. Thormar: Addition of lipases to infant formulas produced antiviral and antimicrobial activity. *J. Nutr. Biochem.*, **3**, 304–308 (1992).
13) K. M. Minokova. D. I. Georgiev, T. G. Toncheva-Panova, M. I. Sholeva, N. B. Houbavenska: Fatty acid composition of *Porphyridium croentum* and its antibacterial activity. *Comt. Rend. Acad. Bulg. Sci.*, **46**, 79–82 (1993).
14) M. F. Feldlaufer, D. A. Knox, W. R. Lusby, and H. Shimanuki: Antimicrobial activity of fatty acids against *Bacillus larvae*, the causative agent of American foulbrood disease. *Apidologie*, **24**, 95–99 (1993).
15) I. Babic, C. Nguyen-the, M. J. Amiot, and S. Aubert: Antimicrobial activity of shredded carrot extracts on food-borne bacteria and yeast. *J. Appl. Bacteriol.*, **76**, 135–141 (1994).
16) E. J. Giamarellos-Bourboulis, P. Grecka, A. Dionyssiou-Asteriou, and H. Giamarellou: *In-vitro* influence of polyunsaturated fatty acids on nosocomial *Pseudomonas aeruginosa*: a preliminary report. *Int. J. Antimicrob. Agents*, **6**, 47–50 (1995).
17) S. Ohta, Y. Shiomi, A. Kawashima, O. Aozasa, T. Nakao, T. Nagate, K. Kitamura, and H. Miyata: Antibiotic effect of linolenic acid from *Chlorococcum* strain HS-101 and *Dunaliella primolecta* on methicillin-resistant *Staphylococcus aureus*. *J. Appl. Phycol.*, **7**, 121–127 (1995).
18) E. J. Giamarellos-Bourboulis, P. Grecka, A. Dionyssiou-Asteriou, and H. Giamarellou: *In-vitro* inhibitory activity of gamma-linolenic acid on *Escherichia coli* strains and its influence on their susceptibilities to various antimicrobial agents. *J. Antimicrob. Chemother.*, **36**, 327–334 (1995).
19) B. W. Petschow, R. P. Batema, and L. L. Ford: Susceptibility of *Helicobacter pylori* to bacterial properties of medium-chain monoglycerides and free fatty acids. *Antimicrob. Agents Chemother.*, **40**, 302–306 (1996).
20) T. Namba, M. Tsunezuka, D. M. R. B. Dissanayake, U. Pilapitiya, K. Saito, N. Kakiuchi, and M. Hattori: Studies on dental caries prevention by traditional medicines (Part VII) Screening of Ayurvedic medicines for anti-plaque action. *Shoyakugaku Zasshi*, **39**, 146–153 (1985).
21) Y.-T. Ko, D. J. Frost, C.-T. Ho, R. D. Ludescher, and B. P. Wasserman: Inhibition of yeast (1, 3)-β-glucan synthase by phospholipase A_2 and its reaction products. *Biochim. Biophys. Acta*, **1193**, 31–40 (1994).
22) P. A. Heasman and R. A. Seymour: Pharmacological control of periodontal disease. I. Antiplaque agents. *J. Dent.*, **22**, 323–335 (1994).
23) H. Tsuchiya, M. Sato, M. Iinuma, J. Yokoyama, M. Ohyama, T. Tanaka, I. Takase, and I. Namikawa: Inhibition of the growth of cariogenic bacteria *in vitro* by plant flavanones. *Experientia*, **50**, 846–849 (1994).
24) S. Hada, M. Hattori, and T. Namba: Dental caries prevention by traditional medicine. XII. Effect of components of *Ganoderma lucidum* on glucosyltransferase from *Streptococcus mutans*. *J. Med. Pharm. Soc. WAKAN-YAKU*, **6**, 100–107 (1989).