Identification of a Methioninase Inhibitor, Myrsinoic Acid B, from Myrsine seguinii Lév., and Its Inhibitory Activities

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A methioninase inhibitor from Myrsine seguinii was purified and identified as myrsinoic acid B. Its inhibitory activities as to crude methioninase from periodontal bacteria such as Fusobacterium nucleatum, Porphyromonas gingivalis, and Treponema denticola were determined. The IC\textsubscript{50} values were 10.5, 82.4, and 30.3 \(\mu\)M respectively.

Key words: methyl mercaptan; methioninase; myrsinoic acid B; Myrsine seguinii; taimin-tachibana

The principal factor in oral malodor is volatile sulfur compounds, such as methyl mercaptan.\textsuperscript{1,11} Methyl mercaptan is produced by methioninase (\(\text{L-methionine-\gamma-lyase}\)) in periodontal bacteria.\textsuperscript{2–4} To find a material to suppress oral malodor, we evaluated the effects of hundreds of plants on the methioninase activity of \(F.\ nucleatum\) JCM8532 crude enzyme. An extract of \(M.\ seguinii\) showed the strongest inhibitory effect. This paper describes the purification and the identification of the active component in \(M.\ seguinii\), and its inhibitory activities.

Dried leaves (500 g) of \(M.\ seguinii\) were ground to a fine powder and extracted with 5 liters of ethanol for 2 h at 70 °C, and resulting extract was evaporated to give an oily extract (45 g). The extract was suspended in water and successively partitioned with hexane/water. The hexane-soluble fraction (9.0 g) was subsequently chromatographed on a silica gel column with 0–100% ethyl acetate/hexane by a 10% stepwise elution method to give 11 fractions. Methioninase inhibitory activity was found in the fractions (1.8 g) eluted with 20% and 30% ethyl acetate/hexane. These fractions were further fractionated by HPLC with a diode array detector (SPD-M10A, Shimadzu, Kyoto, Japan) isocratically eluted 85% methanol/water (with 0.1% acetic acid), on an ODS column (PEGASIL-ODS: 20 \(\times\) 250 mm, Senshu Scientific, Tokyo) at an 8 ml/min flow rate for 30 min, monitored at 270 nm. The fraction eluted at \(tR = 22–23\) min was collected, yielding 191 mg of the active compound. The \(^1\text{H-NMR}\) and \(^{13}\text{C-NMR}\) data for the active compound indicated the presence of a carboxyl group (\(\delta_C 172.0\)), a benzene ring (\(\delta_H 7.75, \delta_C 162.4, 131.4, 127.1, 125.0, 123.1, 121.8\)), and terpene moieties (methyl: \(\delta_H 1.74, 1.73, 1.69, 1.63, 1.30, \delta_C 25.7, 25.6, 22.6, 17.8, 17.6\); methylene: \(\delta_H 3.29, 3.18, 2.17, 2.10, 1.55, \delta_C 37.1, 29.9, 28.3, 22.0\); methine: \(\delta_H 4.73, \delta_C 89.6\); olefin: \(\delta_H 5.29, 5.12, \delta_C 133.1, 132.2, 124.0, 121.3\); quaternary carbon: \(\delta_C 73.7\). LC-ESI-MS analysis of the active compound gave \(m/z\) 357 (M-H).

The specific rotation of the active compound (12 mg/ml methanol) was \(-45^\circ\), indicated by sodium D lines at 29 °C. These data suggest that the active compound was one of myrsinoic acids, which were isolated from \(M.\ seguinii\) as anti-inflammatory compounds. Comparing the spectral data of the active compound with the reference data, the active compound was identified as myrsinoic acid B (\(C_{22}H_{30}O_4\)) (Fig. 1).\textsuperscript{5}

Myrsinoic acid B inhibited \(\alpha\)-ketobutyrate production by \(F.\ nucleatum\) JCM8532, \(P.\ gingivalis\) W83, and \(T.\ denticola\) ATCC35405 in a dose-dependent manner (Fig. 2). \(\alpha\)-Ketobutyrate production from \(F.\ nucleatum\) was strongly inhibited by a lower concentration of myrsinoic acid B, the IC\textsubscript{50} values being obtained with...
m, than of zinc chloride, which is known as a methioninase inhibitor.\textsuperscript{6} \(\alpha\)-Ketobutyrate production from \textit{P. gingivalis} was also affected by myrsinoic acid B, the IC\textsubscript{50} of which was 82.4 \textmu M against 216 \textmu M for zinc chloride. \(\alpha\)-Ketobutyrate production from \textit{T. denticola} was also sensitive to myrsinoic acid B. The IC\textsubscript{50} was 30.3 \textmu M against 48.0 \textmu M for zinc chloride.

In all the bacterial crude enzymes tested, the inhibitory effects due to myrsinoic acid B were higher than those due to zinc chloride. The differences in effects among the three bacterial crude enzymes might have been related to the mechanisms for producing \(\alpha\)-ketobutyrate. Different pathways for producing \(\alpha\)-ketobutyrate have been identified.\textsuperscript{7,8} One of them is to produce \(\alpha\)-ketobutyrate together with methyl mercaptan directly from \textit{l}-methionine. Another known pathway is from \textit{l}-methionine to \(\alpha\)-keto-\(\gamma\)-thio-methylbutyrate, and further from \(\alpha\)-keto-\(\gamma\)-thio-methylbutyrate to \(\alpha\)-ketobutyrate and methyl mercaptan. Another distinct mechanism involves converting \textit{l}-methionine to homocysteine, and homocysteine to \(\alpha\)-ketobutyrate and hydrogen sulfide. Now we are investigating the effects of myrsinoic acid B on production of \(\alpha\)-ketobutyrate, pyruvate, and hydrogen sulfide, and we will report the results later.

In methyl mercaptan production from a bacterial suspension of \textit{F. nucleatum}, the IC\textsubscript{50} value of myrsinoic acid B was found to be 0.389 \textmu M, while that of zinc chloride was 10.7 \textmu M (Fig. 3). Growth of \textit{F. nucleatum} was not affected under 100 \textmu M of myrsinoic acid B. Therefore, the inhibitory activity on methyl mercaptan production was not due to antibacterial activity.

Fig. 2. Effects of Myrsinoic Acid B on \(\alpha\)-Ketobutyrate Production of Crude Enzymes from \textit{F. nucleatum}, \textit{P. gingivalis}, and \textit{T. denticola}.

The methioninase activities in \textit{F. nucleatum} JCM8532 (A), \textit{P. gingivalis} W83 (B), and \textit{T. denticola} ATCC35405 (C) were assayed as described in “Experimental.” ○, zinc chloride; ●, myrsinoic acid B. Values are expressed as the mean (n = 2).
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Methyl mercaptan was analyzed as described in “Experimental” (n = 3). 1) Yoshimura, M., Nakano, Y., Kamachi, H., and Koga, T., 3-Chloro- DL-alanine resistance by L-methionine- \( \alpha \)-deamino-\( \gamma \)-mercaptopetathiane-lyase activity. FEBS Lett. 3, 119–122 (2002).

It has been reported that four myrsinoic acid B related compounds were isolated. 5,8,10 By assay-guided fractionation, we isolated and identified myrsinoic acid B as the active compound, while we did not succeed in isolating the other related compounds. In conclusion, this paper reports for the first time inhibitory effects on production of \( \alpha \)-ketobutyrate and methyl mercaptan due to myrsinoic acid B.

Experimental

Plant material. M. sequini was collected in the summer of 2005, and was supplied by the Kochi Prefectural Makino Botanical Garden (Kochi, Japan).

The isolated active compound: Yellow amorphous solid, \([\alpha]_D^{20} = -45^\circ\) (c 1.2, methanol); LC-ESI-MS \( m/z: 357\) (M-H); \( ^1\)H-NMR (400 MHz, CDCl\( _3\)) \( \delta_H: 7.75\) (2H, s, H-6, H-4), 5.29 (1H, t, \( J = 7.2\) Hz, H-2'), 5.12 (1H, t, \( J = 6.0\) Hz, H-4'), 4.73 (1H, t, \( J = 8.8\) Hz, H-2), 3.29 (2H, m, H-1'), 3.18 (2H, dd, \( J = 8.8, 18.0\) Hz, H-3), 2.17 (1H, m, H-3'), 2.10 (1H, m, H-3'), 1.74 (3H, s, H-4'), 1.73 (3H, s, H-5'), 1.69 (3H, s, H-6'), 1.63 (3H, s, H-8'), 1.55 (2H, m, H-2'), 1.30 (3H, s, H-7'). \( ^13\)C-NMR (10 MHz, CDCl\( _3\)) \( \delta_C: 172.0\) (C=O), 162.4 (C-7a), 133.1 (C-3'), 132.2 (C-5'), 131.4 (C-4'), 127.1 (C-7'), 125.0 (C-6), 124.0 (C-4'), 123.1 (C-3a), 121.8 (C-5), 121.3 (C-2'), 89.6 (C-2), 73.7 (C-1'), 37.1 (C-2'), 29.9 (C-3), 28.3 (C-1'), 25.7 (C-4'), 25.6 (C-6'), 22.6 (C-7'), 22.0 (C-3'), 17.8 (C-5'), 17.6 (C-8').

Methioninase activity in bacterial extracts. F. nucleatum and P. gingivalis were grown anaerobically (10% CO\( _2\), 10% H\( _2\), 80% N\( _2\)) in 800 ml of trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md, USA) containing yeast extract (3.0 g/l), hemin (5 \( \mu \)g/ml), and menadione (0.5 \( \mu \)g/ml) at 37°C for 2 d. T. denticola was grown in 800 ml of TYGVS medium for 8 d. The crude extracts were prepared according to a previously described procedure.11 \( \alpha \)-Ketobutyrate production was assayed as described by Yoshimura et al.,2,3 with several modifications. The assay was carried out in 1 ml of 50 mM Tris–HCl buffer (pH 7.5), containing 30 mM of l-methionine, and 50 \( \mu \)M of pyridoxal 5'-phosphate with 0.3 mg of protein/ml of bacterial cell-free extracts at 37°C for 1 h.

Determination of methyl mercaptan production by bacterial suspension. Methyl mercaptan production was assayed as described by Yoshimura et al.,2,3 with several modifications. The bacteria were suspended in Tris-buffered saline [50 mM Tris–HCl buffer, pH 7.5, and 50 mM sodium chloride]. The headspace gas above the reaction mixture in the tube was analyzed by gas chromatography (GC-9A equipped with a flame photometric detector, 20% DOP on a Chromosorb W, AW DMCS, 5 × 3 mm I.D column, Shimadzu, Kyoto, Japan). The temperature of the column was 90°C, the injection port was 150°C, and the detector was 200°C, with nitrogen as the carrier gas (45 ml/min).

Evaluation of antimicrobial activity. F. nucleatum was grown in 50 ml of Trypticase soy broth. The bacterial suspensions were prepared in a double concentration of broth mixed with 10% of pre-cultured bacterial broth. A hundred \( \mu \)l from the dilution series of the extracts and 100 \( \mu \)l from the bacterial suspensions were added to 96-well microplates. The plates were incubated anaerobically for 72 h at 37°C. Minimum inhibitory concentration was determined in the wells free from turbidity.

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References


