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Note

Inhibitory Effect of Methyl Methanethiosulfinate on β-Glucuronidase Activity

Yuko WATANABE,[1] Ryoko MUROI,[2] Haruna TSUCHIYA,[2] Yasushi UDA,[2] and Kei HASHIMOTO[2,†]

[1]*United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan*
[2]*Department of Applied Biological Chemistry, Utsunomiya University, Utsunomiya, Tochigi 321-8505, Japan*

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Minced or cut-up leaves of Chinese chive (*Allium tuberosum* Rottler) contain thiosulfinates and their disproportionate reaction products. Among these organosulfur compounds, methyl methanethiosulfinate was found to be an uncompetitive inhibitor of β-glucuronidase. Approximately 80% of the enzyme activity was inhibited by methyl methanethiosulfinate at 50 μM, the IC_{50} value being comparable to 3.6 μM.

Key words: Chinese chive; *Allium*; methyl methanethiosulfinate; β-glucuronidase

Carcinogens taken into the body are mostly metabolized for detoxification by phase II enzyme-mediated conjugation, including glucuronidation in the liver, and are then excreted *via* the urine. A portion of the formed conjugates can escape excretion and undergo degradation by bacterial β-glucuronidase (EC 3.2.1.31), an exoglycosidase enzyme that catalyzes the cleavage of glucuronosyl-*O* bonds,[1)] in the intestinal tract. Such deconjugation liberates potentially carcinogenic compounds.[2,3)] The reduction of β-glucuronidase activity in the intestinal tract could therefore play an important role in preventing colonic carcinogenesis. The application of β-glucuronidase inhibitors has been investigated as a means to reduce the β-glucuronidase activity; for example, feeding such β-glucuronidase inhibitors as D-glucaric acid and its metabolite to carcinogen-treated rats reduced the fecal β-glucuronidase activity and the number of chemically induced colon tumors.[4,5)]

Many organosulfur compounds found in such *Allium* vegetables as garlic, onion, and leek are known to play a preventive role in the development of human pathologies, including cancer,[6,7)] cardiovascular diseases,[8,9)] and inflammatory diseases.[10–12)]

Chinese chive (*A. tuberosum* Rottler), an *Allium* vegetable, is cultivated and used as a flavorful food, particularly in southeastern Asia (Vietnam, Thailand, Indonesia, Malaysia, and the Philippines) and in eastern Asia (Mongolia, China, the Republic of Korea, and Japan). When the tissues of Chinese chive are damaged by a procedure like cutting or chopping, *S*-alk(en)yl cysteine sulfoxides are quickly cleaved by the action of the enzyme, alliinase (EC 4.4.1.4), to form their corresponding alk(en)yl sulfenic acids which are subsequently converted by condensation to thiosulfinates with the corresponding alkyl or alkenyl groups. The resulting thiosulfinates are continuously decomposed through a disproportionate reaction to form such compounds as mono-, di-, tri-, and tetra-sulfides.[13)] Block *et al.*[14)] have reported that, compared with allyl group-containing thiosulfinates, a higher proportion of methyl group-containing thiosulfinates like methyl methanethiosulfinate (MMTS) was found in Chinese chive. Yabuki *et al.*[15)] have recently investigated the relative amounts of eight major sulfur-containing constituents of 16 varieties of Chinese chive grown on the same farm. Among these, sulfides with methyl groups predominated over those with allyl groups.

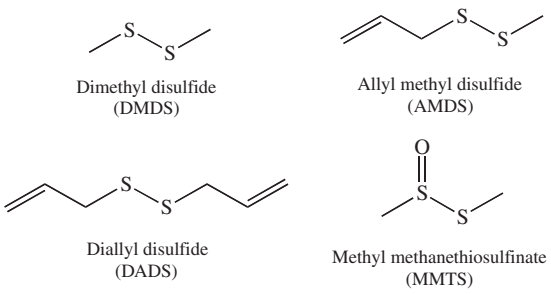

Fig. 1. Structures of the Analyzed Compounds.

The effects of MMTS and three disulfides, dimethyl disulfide (DMDS), allyl methyl disulfide (AMDS), and diallyl disulfide (DADS) (Fig. 1), on the β-glucuronidase activity were investigated in this study.

MMTS was prepared by oxidizing DMDS (Tokyo Chemical Industry, Tokyo, Japan) with perbenzoic acid in chloroform according to the method of Small *et al.*[16)] High-performance liquid chromatography (HPLC) was performed by using a Jasco HPLC System (Tokyo, Japan) equipped with a reversed phase YMC-Pack ODS-A column (150 × 4.6 mm I.D.; YMC, Tokyo, Japan). The analysis was performed under isocratic conditions, using 40% acetonitrile/water at a flow rate of 0.8 mL/min, an injection volume of 2 μL, and UV detection at 220 nm. Under these conditions, MMTS eluted after 3.9 min with a purity of 92.5%.

The effect of DMDS, AMDS (Wako Pure Chemical Industries, Osaka, Japan), DADS (Wako Pure Chemical Industries, Osaka, Japan), and MMTS on the β-glucuronidase activity was investigated. The β-glucuronidase

† To whom correspondence should be addressed. Fax: +81-28-649-5469; E-mail: keih@cc.utsunomiya-u.ac.jp

Abbreviations: AMDS, allyl methyl disulfide; DADS, diallyl disulfide; DMDS, dimethyl disulfide; HPLC, high-performance liquid chromatography; MMTS, methyl methanethiosulfinate; PNPG, *p*-nitrophenyl-β-D-glucuronide

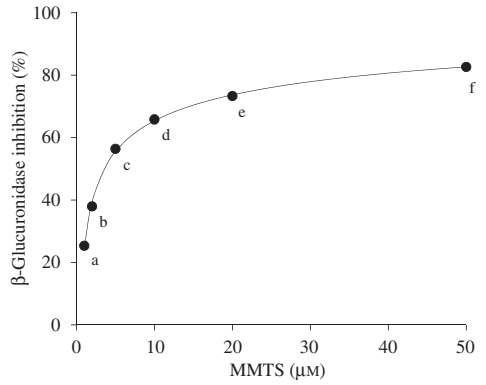

Fig. 2. Inhibition of the β-Glucuronidase Activity by MMTS.
The results are presented by bars as the mean ± SD of quadruplicate measurements, unless the error bar is smaller than the symbol. The means were compared by Tukey's test ($p < 0.05$). Significant differences are indicated by different letters.

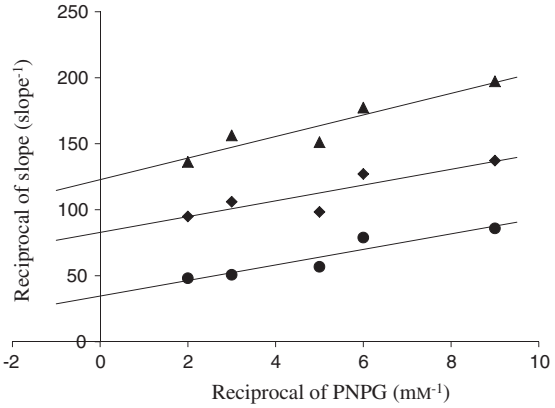

Fig. 3. Lineweaver-Burk Plots of β-Glucuronidase in the Presence of MMTS.
● Control; ◆ 1 μM MMTS; ▲ 2 μM MMTS. Results are presented as the mean of quadruplicate measurements.

activity was assayed by monitoring the *p*-nitrophenol produced from *p*-nitrophenyl-β-D-glucuronide (PNPG; Sigma-Aldrich Japan, Tokyo, Japan) according to the method of Gudiel-Urbano and Goñi[17] with a minor modification. In brief, 50 μL of each tested compound in a 20-mM phosphate buffer (pH 7.0), and 50 μL of β-glucuronidase from *Escherichia coli* (12 units/mL; Sigma-Aldrich Japan, Tokyo, Japan) were added to a 96-well microplate and incubated at 37 °C for 10 min. A 50-μL amount of 0.6 mM PNPG was then added to each well to initiate the reaction. The microplate was read with a Sunrise™ microplate reader (Tecan Japan, Kanagawa, Japan) at 405 nm and 37 °C for 5 min. The enzyme activity is expressed as the change in absorbance at 405 nm per min (slope) calculated by using Magellan™ software (Tecan Japan, Kanagawa, Japan).

We found that DMDS showed no significant inhibition (5 μM; 1.4 ± 1.5%, mean ± SD, n = 4). AMDS and DADS showed slight but significant inhibition of β-glucuronidase activity (5 μM; 8.5 ± 3.3% for AMDS and 8.5 ± 2.0% for DADS, mean ± SD, n = 4, $p < 0.05$). In contrast, MMTS exhibited relatively strong inhibitory activity at 5 μM (56.4 ± 0.7%, mean ± SD, n = 4, $p < 0.05$). There have been few studies in which the biochemical or physiological functions of thiosulfinates were compared with those of sulfides. Interestingly, Merhi *et al.*[18] have reported that MMTS, but not DMDS or DADS, inhibited cell proliferation, differentiation, and secretion of leukemic cell lines. Thiosulfinates could have potential bioactivities which their related sulfides did not have.

The inhibitory effects of MMTS at different concentrations are shown in Fig. 2. MMTS significantly inhibited the β-glucuronidase activity, even at 1 μM. This inhibitory activity increased with increasing concentration of MMTS up to 5 μM and gradually leveled off as the concentration was further increased. Approximately 80% of the enzyme activity was inhibited at 50 μM, the IC_{50} value being 3.6 μM. The inhibition kinetics of MMTS toward β-glucuronidase were analyzed by using Lineweaver–Burk plots, as shown in Fig. 3. The results show that the plots gave a family of parallel straight lines. Accompanying the increase in MMTS concentration, the values for both K_m and V_m increased, although the ratio of K_m/V_m remained unchanged. This pattern indicates that MMTS was an uncompetitive inhibitor of β-glucuronidase. This behavior indicates that the inhibitor bound at a site distinct from the substrate and combined with the enzyme-substrate complex, but not with the free enzyme. Thiosulfinates, including MMTS, inhibit platelet aggregation by inhibiting calpain activation.[19] Badol *et al.*[20] have reported that thiosulfinates rapidly interacted with sulfhydryls on calpain and inhibited its activity. A similar mechanism for β-glucuronidase inhibition by MMTS could be involved. Further investigations are required to identify the inhibitory mechanism.

Thiosulfinates are known in *Allium* species as heat-labile intermediates generated *via* the enzymatically initiated degradation of *S*-alk(en)yl-L-cysteine sulfoxides.[13] Garlic (*A. sativum* L.) has long been used as a representative vegetable possessing marked pharmacological potential. This potential has been assumed to be due to allyl 2-propenylthiosulfinate. However, its instability[21,22] has been one of the drawbacks for its utilization. Fresh Chinese chive has been shown to contain up to 9% methyl 2-propene-1-thiosulfinate, 5% 1-propenyl methanethiosulfinate, 13% allyl methylthiosulfinate, and 73% MMTS.[14] However, Seo *et al.*[23] have reported that only MMTS and allyl methylthiosulfinate were isolated from a Chinese chive extract. MMTS therefore appeared to be relatively stable compared with other thiosulfinates. Shen *et al.*[24] have reported that the half-life values of MMTS at an acidic or neutral pH at 40 °C ranged from 1.3 d to 32.9 d. We therefore consider that MMTS in the processed Chinese chive could retain its inhibitory activity against β-glucuronidase of microbiota in the intestines even after ingestion, although this needs to be confirmed by further investigations.

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