

Simultaneous speciation of selenium and sulfur species in selenized odorless garlic (*Allium sativum* L. Shiro) and shallot (*Allium ascalonicum*) by HPLC–inductively coupled plasma–(octopole reaction system)–mass spectrometry and electrospray ionization–tandem mass spectrometry

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Abstract

The simultaneous speciation of selenium and sulfur in selenized odorless garlic (*Allium sativum* L. Shiro) and a weakly odorous Allium plant, shallot (*Allium ascalonicum*), was performed by means of a hyphenated technique, a HPLC coupled with an inductively coupled plasma–mass spectrometry (HPLC–ICP–MS) equipped with an octopole reaction system (ORS). The aqueous extracts of them contained the common seleno compound that was identified as γ -glutamylmethylselenocysteine by an electrospray ionization–tandem mass spectrometry (ESI–MS/MS). Normal garlic contains alliin as the major sulfur-containing compound, which is the biological precursor of the garlic odorant, allicin. Alliin, however, was not detected in the extracts of the selenized odorless garlic. At least, four unidentified sulfur-containing compounds were detected in odorless garlic and shallot. Moreover, these Allium plants showed chemopreventive effects against human leukemia cells. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Selenium (Se) is an ultra-trace essential element in mammals, and at least 15 different selenoproteins and/or selenoenzymes are known to date [1]. Se-enriched (selenized) yeast, mushrooms and Allium plants, such as garlic, onion and leek, are used as dietary supplements owing to their anti-oxidative and anti-tumor effects [2,3]. Selenized garlic is the most popular and well-researched Allium plant that is known to accumulate Se as selenoamino acid derivatives, including Se-methyl-L-selenocysteine (MeSeCys) and γ -glutamylmethylselenocysteine (GluMeSeCys) [4–7]. These selenoamino acids are effective against mammary and prostate tumors, as well as leukemia [8–10]. How-

ever, normal garlic has a characteristic pungent smell, and its odorants are released through one's breath and/or sweat after ingestion. Moreover, gastrointestinal discomfort and nausea have been frequently reported as the adverse effects of garlic [11]. To avoid these problems, odorless garlic is used instead of normal garlic.

Odorless garlic (*A. sativum* L. Shiro) is a variant of normal garlic, and has no specific smell of garlic. However, there are few scientific papers on the odorless garlic, and it is still unclear why the odorless garlic is defective in producing the characteristic pungent smell. The major odorant of garlic is allicin, which is biotransformed from the sulfur (S)-containing amino acid derivative, allylcysteine, via alliin [12]. Thus, the speciation of S-containing compounds is important to reveal the biosynthetic mechanism underlying the characteristic smell of garlic.

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The hyphenated technique, HPLC coupled with ICP-MS, is an effective method for the speciation of Se [13–15]. Recently, ICP-MS equipped with an octopole reaction system (ORS) has been developed and shown to be useful for the speciation of not only metals and metalloids but also certain elements, such as S, phosphorus and halogens [16,17]. Therefore, the ICP-(ORS)-MS is suitable for the simultaneous detection of Se and S in such garlic samples. Moreover, electrospray ionization tandem mass spectrometry (ESI-MS/MS) is also effective for the identification of unknown seleno compounds because it provides the molecular information of the compounds [18–22].

In this study, first, odorless garlic and the low-odor *Allium* plant, shallot, were cultivated under the high Se condition. Then, the selenized *Allium* plants were evaluated by Se and S speciation to determine whether they are useful as Se supplements instead of normal odorous garlic. Finally, we examined why odorless garlic lacked the characteristic pungent smell.

2. Experimental

2.1. Reagents

Seleno compounds, namely, sodium selenite, sodium selenate, barium selenate and L-selenomethionine (SeMet), were purchased from Wako (Osaka, Japan), and barium selenite and Se-methyl-L-selenocysteine (MeSeCys) were purchased from Aldrich (St. Louis, MO, USA) and Acros Organics (Geel, Belgium), respectively. S-containing amino acid derivatives, such as allylcysteine (AlCys), methylcysteine (MeCys), alliin (AlIn), methiin (MeIn), γ -glutamylallylcysteine (GluAlCys) and γ -glutamylmethylcysteine (GluMeCys), were kindly provided by Wakunaga (Osaka, Japan). Proteinous amino acids, such as cystine (Cys₂) and methionine (Met), were purchased from Wako. The standard compounds of seleno- and thioamino acids used in this study are summarized in Table 1. Nitric acid of analytical grade and ammonium acetate were purchased from Wako. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (Celltiter 96) was obtained from Promega, Madison, WI, USA.

2.2. Cultivation of selenized odorless garlic, shallot and normal garlic

Odorless garlic cloves and shallot seeds were purchased from Fukutane (Fukui, Japan). The experimental farm at the Phytoselenium Research Laboratories (Kumamoto, Japan) was compartmented with concrete (10 m in length $\times$ 15 m in width $\times$ 1 m in depth) to prevent contamination by Se from sewage [23]. Gravel, sand and black clay were piled from the bottom of the compartment, and ridges (90 cm in width $\times$ 15 cm in height) were made in the compartment. Ten kilograms of odorless garlic cloves was seeded on four lines of the ridges and these were enriched once with Se by sprinkling barium selenate and barium selenite mixed with

a commercially available fertilizer at the concentration of 500 mg/m² each. Then, the ridges were mulched with black film. Forty kilograms of selenized odorless garlic was harvested 8 months after seeding. Selenized shallot and normal garlic were also cultivated by the same methods as those mentioned above. The concentrations of Se in the selenized odorless garlic, shallot and normal garlic determined by ICP-MS were 136.0, 226.8 and 146.6 μ g/g wet weight, respectively.

2.3. Sample preparation

A 6 g portion of the selenized odorless garlic clove was grated and mixed with four times its weight of deionized water. The mixture was transferred to a 15 ml polypropylene tube and homogenized by a Polytron homogenizer (Kinematica, Luzerne, Switzerland) under nitrogen atmosphere. The homogenate was incubated on a boiling-water bath for 20 min, and then ultracentrifuged at 105,000 $\times$ g for 60 min to obtain the extract.

2.4. Apparatus

An Agilent7500cs inductively coupled argon plasma mass spectrometer (ICP-MS; Yokogawa Analytical Systems, Hachioji, Japan) equipped with an octopole reaction system and an API3000 triple quadrupole mass spectrometer (ESI-MS/MS; Applied Biosystems, Tokyo) equipped with a Turbo Ion Spray ion source were used. The operating conditions of the ICP-MS are summarized in Table 2. The ESI-MS/MS was operated in the positive ion mode under the following conditions: ionspray voltage, 5000 V, turbo gas temperature, 450 °C; nebulizer gas, 8 U; curtain gas, 8 U; declustering potential, 20 V; focusing potential, 200 V; entrance potential, –10 V.

2.5. HPLC–ICP-MS

A 20 μ l aliquot of the extracts of the selenized *Allium* plants or the standard compounds was applied to an HPLC coupled with an ICP-MS to analyze the distributions of Se and S. The HPLC system consisted of an on-line degasser, an HPLC pump (PU610; GL Science, Tokyo), a Rheodyne six-port injector and a multi-mode gel filtration column (Shodex Asahipak GS-320 HQ, 300 mm $\times$ 7.5 mm I.D. with a guard column, 75 mm $\times$ 7.5 mm I.D.; Showa Denko, Tokyo). The column was eluted with 50 mM ammonium acetate, pH 6.5, at a flow rate of 0.5 ml/min, and the eluate was introduced directly into the nebulizer of the ICP-MS to detect Se at m/z 77, 80 and 82, and S at m/z 34.

2.6. Partial purification of major seleno compound in selenized odorless garlic for ESI-MS/MS analysis

A 2 ml aliquot of the extract of the selenized odorless garlic was applied to a preparative multi-mode gel filtration column (Shodex Asahipak GS-520P, 500 mm $\times$ 21.5 mm I.D.; Showa Denko). The column was eluted with 50 mM ammo-

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