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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.

Keywords: Selenium; Sulfur; Allium plant; Garlic; ICP-MS; Speciation

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]. However, 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 (Cys_2) 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 comparted with concrete (10 m in length × 15 m in width × 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 × 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/m^2 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 μg/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 × g for 60 min to obtain the extract.

2.4. Apparatus

An Agilent7500cs inductively coupled argon plasma mass spectrometer (ICP-MS; Yokogawa Analytical Systems, Hachiouji, 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.

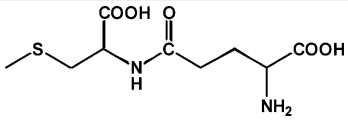
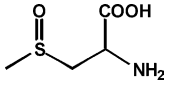
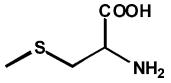
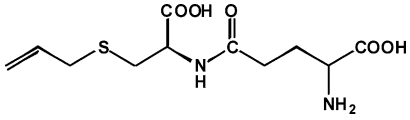
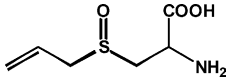
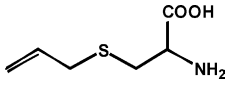
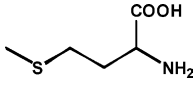
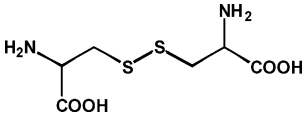
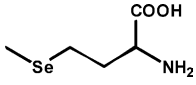
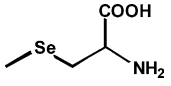
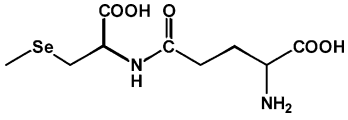
2.5. HPLC–ICP-MS

A 20 μl 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 × 7.5 mm I.D. with a guard column, 75 mm × 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 × 21.5 mm I.D.; Showa Denko). The column was eluted with 50 mM ammo-

Table 1
List of acronyms and structures of S- and Se-amino acids analyzed in this study

Species	Acronym	Structure
γ-Glutamylmethylcysteine	GluMeCys	
Methiin	MeIn	
Methylcysteine	MeCys	
γ-Glutamylallylcysteine	GluAlCys	
Alliin	AlIn	
Allylcysteine	AlCys	
Methionine	Met	
Cystine	Cys_2	
L-Selenomethionine	SeMet	
Se-methyl-L-selenocysteine	MeSeCys	
γ-Glutamylmethylselenocysteine	GluMeSeCys	

nium acetate, pH 6.5, at a flow rate of 3.0 ml/min, and an aliquot of the eluate was collected every 30 s by monitoring the absorbance at 254 nm. The Se concentration in each fraction was determined by ICP-MS. The Se-containing fractions were concentrated to approximately one-fifth of their original volumes by evaporation. Then, a 10 μl aliquot of the concentrate (46.2 μg Se/g) was infused into the ESI-MS/MS to identify the chemical species of the major seleno compound in the selenized odorless garlic.

2.7. Cell culture and measurement of cell viability under treatment with selenized Allium plants

Human promyelocytic leukemia cells, HL60, were obtained from the RIKEN Cell Bank (Tsukuba, Japan). The cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Biotrace International; Bridgend UK) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Corporation; Carlsbad, CA, USA) at 37 °C under an atmosphere containing 5% CO_2.

The cells were suspended in a 96-well plastic plate at a concentration of 1.0×10^5 cells/well, and cultured for 24 h. Then, the cells were treated with the extracts of the Allium plants at Se concentrations of 0, 0.08, 0.16, 0.31, 0.63, 1.3, 2.5 and 5.0 μg/ml for 24 h. The MTT assay was performed according to the manufacturer's instructions. Cell viability was measured in terms of the absorbance at 490 nm with a microplate reader (Molecular Device; Sunnyvale, CA, USA).

Table 2
Operating conditions of ICP-MS for speciation of Se and S

Plasma setting	
RF power (W)	1500
Nebulizer type	Babington
Nebulizer gas flow (l/min)	1.05
Make-up gas flow (l/min)	0.25
Auxiliary gas flow (l/min)	1.15
Plasma gas flow (l/min)	15.0
Collision cell	
H_2 gas flow (ml/min)	4.5
Data acquisition	
m/z monitored	34, 77, 80 and 82
Dwell time (ms)	100 for Se isotopes and 300 for S
Point per peak	1

3. Results

3.1. Separation of thioamino acids on a multi-mode gel filtration HPLC column

Separation of the candidate thioamino acids in Allium plants was conducted on a multi-mode gel filtration column (GS-320 HQ). The column shows the multiple characteristics for separation depending on the elution conditions, i.e., the separation principle is based on not only molecular size exclusion but also ion exchange and/or reverse-phase property.

The methyl derivatives of thioamino acids, such as GluMeCys, MeIn and MeCys, were separated into distinct peaks, and their retention times were 18.7, 20.7 and 22.3 min, respectively by HPLC–ICP-MS (Fig. 1a). GluMeCys was eluted more rapidly than the other two methyl derivatives because it is a dipeptide. As MeIn is an S-oxide of MeCys, the difference in molecular mass between them ($\Delta M = 16$) is relatively small compared to the exclusion size of the column (>40,000). However, these two were well separated on the column due to characteristics other than the size exclusion property.

Under the same conditions as those for the methyl derivatives, the allyl derivatives of thioamino acids, such as GluAlCys, AlIn and AlCys, were also separated into distinct peaks, and their retention times were 19.2, 21.5 and 24.1 min, respectively (Fig. 1b). A higher resolution was realized for the separation of the allyl derivatives than for the methyl derivatives, suggesting that the reverse-phase mode was more effective than the size exclusion mode for the separation of the allyl derivatives.

Third, the thioamino acids, Cys_2 and Met, were also well separated into two peaks, and their retention times were 20.6 and 22.9 min, respectively (Fig. 1c). As selenoamino acids, MeSeCys and SeMet, were separated under the same conditions in our previous report [24], MeCys (22.3 min) and Met (22.9 min) were separated on this column despite their having similar molecular masses (135 for MeCys and 149 for Met) and pK_a values (2.07 and 8.84 for MeCys, and 2.23 and 9.26 for Met). Thus, this separation was assumed to be based on the reverse-phase mode rather than the size exclusion or the ion exchange mode.

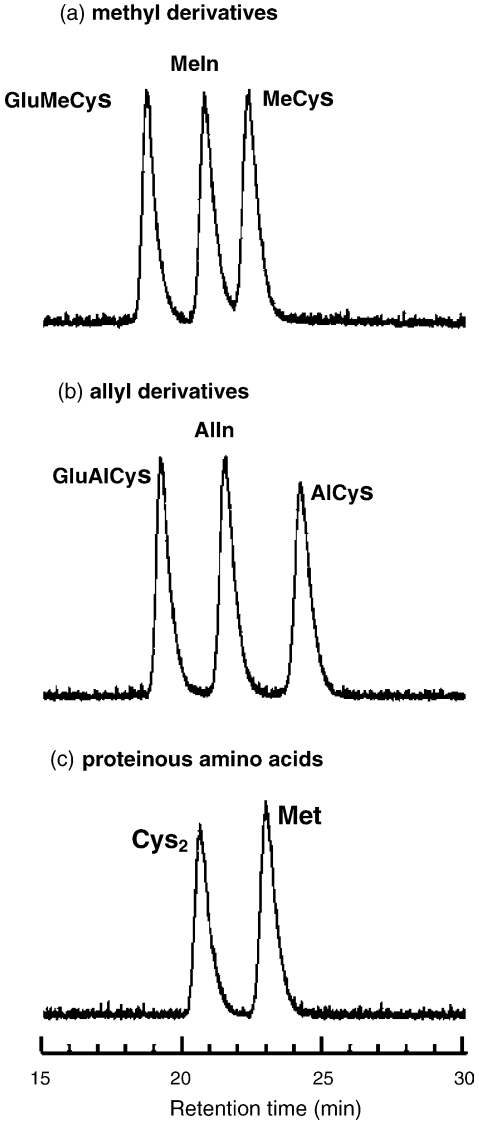


Fig. 1. Elution profiles of S in thioamino acid derivatives as determined by multi-mode gel filtration HPLC–ICP-(ORS)-MS. A 20 μl aliquot of each standard at 100 μg/ml was applied to a GS-320 HQ column and the eluate was monitored by ICP-MS equipped with an octopole reaction system at *m/z* 34.

3.2. Speciation of Se and S compounds in selenized odorless and normal garlic

Ninety percent of the Se in the extract of the odorless garlic was eluted at the retention time of 18.9 min (Fig. 2a), and this retention time did not correspond to those of any commercially available seleno compounds. Therefore, this seleno compound was subjected to ESI-MS/MS analysis after partial purification, as described later. The 8.3% Se that was eluted at 17.5 min was assigned to selenate by matching its retention time with that of an authentic standard. The minor

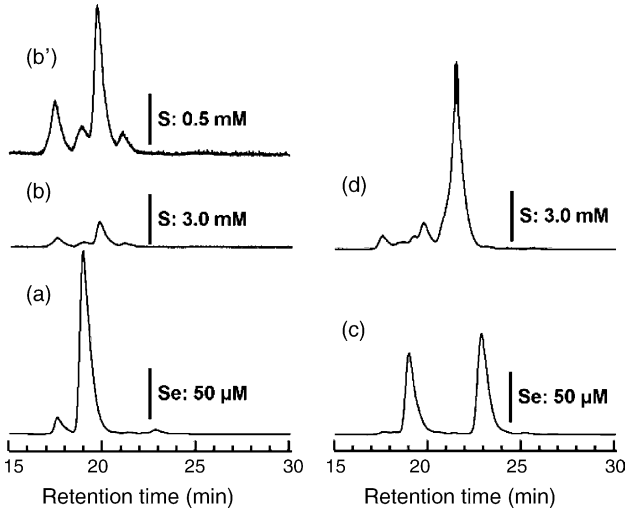


Fig. 2. Elution profiles of Se and S in extracts of selenized odorless and normal garlic. A 20 μl aliquot of the extract of selenized odorless (panels a, b and b′) or normal (panels c and d) garlic was applied to a GS-320 HQ column and the eluate was monitored by ICP-MS equipped with an octopole reaction system. The elution profiles of Se (panels a and c) and S (panels b, b′ and d) were determined based on data at *m*/*z* 77 and 34, respectively. Vertical bars indicate the detection levels for each element.

peak (1.5%) detected at 22.8 min was assigned to MeSeCys by comparison with an authentic standard.

Four distinct peaks of S were detected at the retention times of 17.4, 18.9, 19.7 and 21.1 min (Fig. 2b′). However, none of them matched the retention times of the authentic thioamino acid derivatives shown in Fig. 1. Thus, the thio-compounds in the odorless garlic could not be identified.

On the other hand, two major seleno compounds were observed in the extract of selenized normal garlic (Fig. 2c). The compound that was eluted at 18.9 min corresponded to the major compound in the odorless garlic, and that eluted at 22.8 min corresponded to MeSeCys.

The concentration of S in the normal garlic was approximately six times higher than that in the odorless garlic based on the peak area in Fig. 2d. The major portion of S was eluted at 21.5 min and assigned to AlIn. These results seemed reasonable because AlIn is a water-soluble precursor of the volatile and garlic-specific odorant, allicin, i.e., Alln is converted into allicin via alliinase.

3.3. Speciation of Se and S compounds in selenized shallot

The selenized shallot contained three seleno compounds with the retention times of 17.5, 18.9 and 22.8 min (Fig. 3a). Although the ratios of the Se peak intensities of the three compounds in the shallot were quite different from those in the odorless and normal garlic, the peaks were assignable to the same compounds, namely, selenate, an unidentified seleno compound and MeSeCys at retention times of 17.5, 18.9 and 22.8 min, respectively. In particular, the ratio of selenate was relatively higher (28%) than those of the other compounds

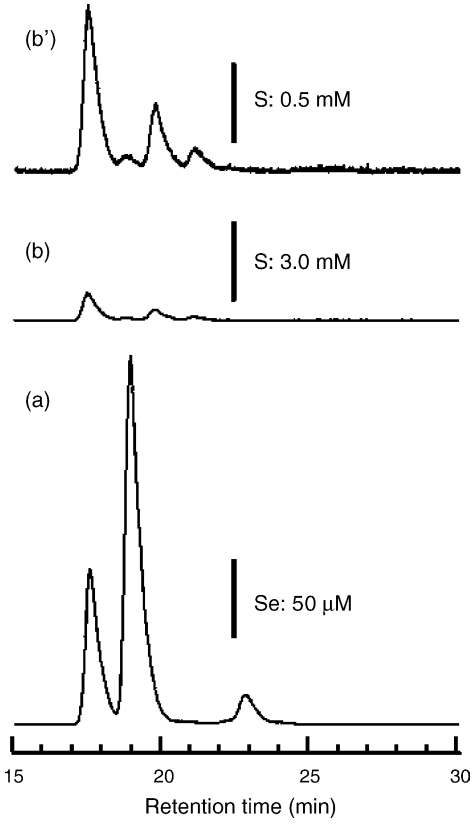


Fig. 3. Elution profiles of Se and S in extract of selenized shallot. A 20 μl aliquot of the extract of selenized shallot was applied to a GS-320 HQ column and the eluate was monitored by ICP-MS equipped with an octopole reaction system. The elution profiles of Se (panel a) and S (panels b and b′) were determined based on data at *m*/*z* 77 and 34, respectively. Vertical bars indicate the detection levels for each element.

(66% for the unidentified compound and 5.4% MeSeCys), suggesting that Se in the form of selenate in the shallot is less effectively transformed into other chemical compounds such as MeSeCys, compared to garlic.

The chemical compounds that contained S in the shallot were identical to those in the odorless garlic despite the fact that the ratios were quite different between them (Fig. 3b′). The amount of S in the shallot as estimated from the peak area was smaller than that in the odorless garlic although the shallot incorporated more Se than the garlic. This indicates that the metabolism of Se may be quite different from that of S in the Allium plants.

3.4. Identification of major seleno compound in odorless garlic by ESI-MS/MS

Se consists of six isotopes, i.e., ^{74}Se (0.89%), ^{76}Se (9.36%), ^{77}Se (7.63%), ^{78}Se (23.8%), ^{80}Se (49.6%) and ^{82}Se (8.73%). The signal showing the isotope pattern of Se was observed at around *m*/*z* 313, suggesting that the most intense peak at 313 was the ^{80}Se-containing molecular ion, $[M + H]^+$, although copious peaks of matrices were detected in the partially purified extract of the odorless garlic (Fig. 4). The

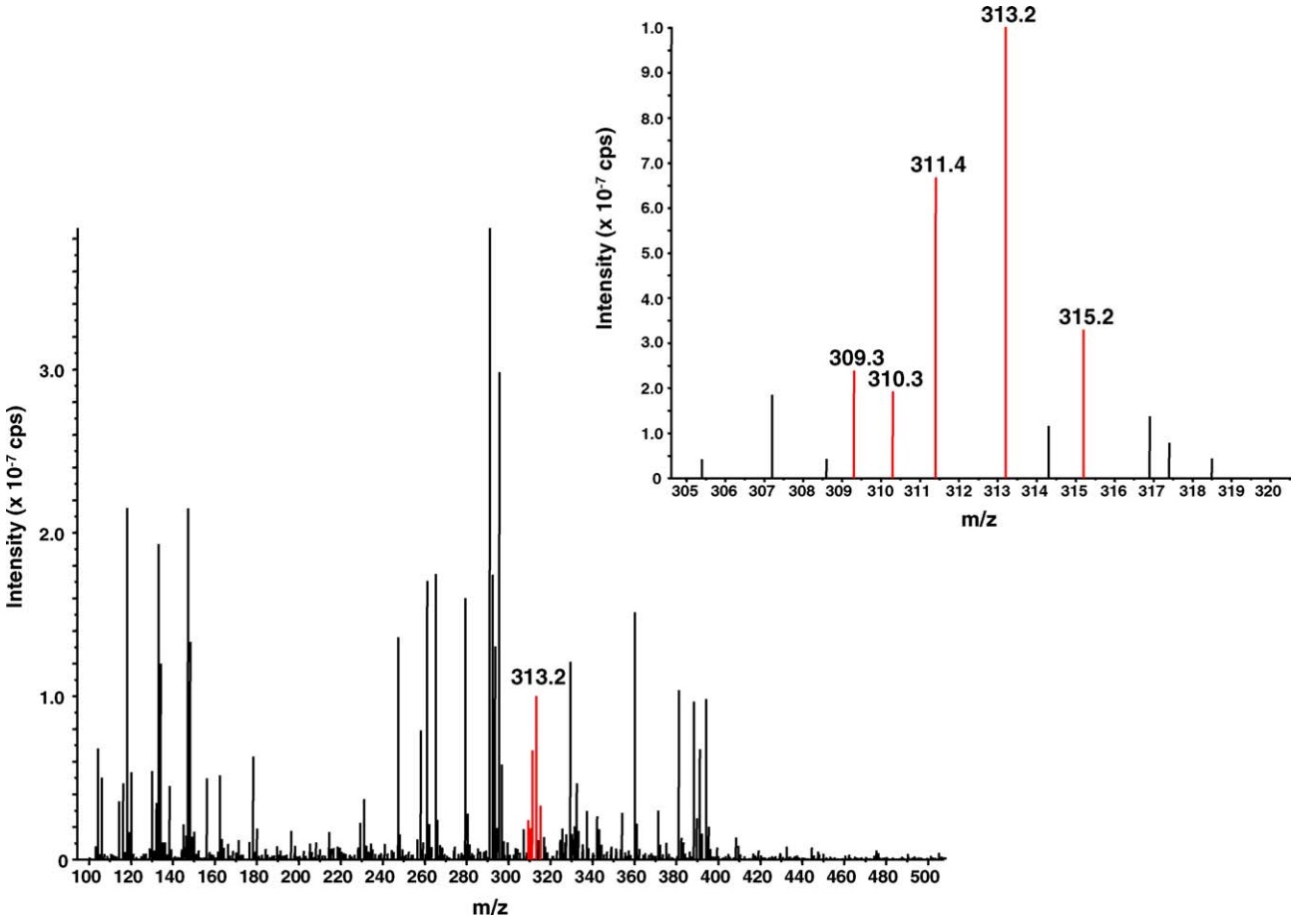


Fig. 4. Mass spectrum of partially purified extract of selenized odorless garlic. The part of the spectrum containing the characteristic isotope pattern of Se is enlarged in the inset.

precursor ions, *m*/*z* 311, 313 and 315, corresponding to ^{78}Se, ^{80}Se and ^{82}Se, respectively, were extracted and subjected to the MS/MS analysis. All the peaks observed were assignable to the fragments of GluMeSeCys, as depicted in Fig. 5d, and were well consistent with those obtained in the other Allium plants [7]. Thus, it was concluded that the primary seleno compound in the odorless garlic was GluMeSeCys, and the major Se species in the shallot was also this compound.

3.5. Cytotoxic effect of selenized odorless garlic and shallot against human leukemia cells

The extracts of the selenized odorless garlic and the shallot decreased the viability of HL-60 cells in a concentration-dependent manner. The 50% inhibitory concentration of the extracts was established based on the Se concentration in each extract in this assay. The concentrations at which the extracts showed 50% inhibition of cell viability were 3.24 and 5.39 μg Se/ml for the odorless garlic and the shallot, respectively (Fig. 6). As has been reported elsewhere, GluMeSeCys exhibits anti-tumor activity [3,5,8]. Thus, the selenized odorless garlic and the shallot seem to be potent food materials with chemopreventive effects.

4. Discussion

GluMeSeCys is found commonly in various kinds of selenized Allium plants, such as garlic (*A. sativum*), onion bulb (*A. cepa*), green onion (*A. fistulosum*) and leek (*A. tuberosum*) [2,5,7,8]. This common seleno compound was also detected as the primary metabolite in odorless garlic (*A. sativum* L. Shiro) and shallot (*A. ascalonicum*) in the present study. GluMeSeCys is recognized as the stored form of MeSeCys, which is a potential anti-tumor agent, and exerts the same pharmacological activity as MeSeCys on mammalian cells [8]. Indeed, the extracts of the selenized odorless garlic and the shallot showed cytotoxic effects on HL-60 cells that are sensitive to MeSeCys. The chemopreventive effect of the selenized shallot was slightly weaker than that of the odorless garlic owing to its lower GluMeSeCys content (66%) compared to that of the odorless garlic (90%). The percentage of selenate used as Se source was relatively high (28%) and that of MeSeCys was low (5.4%) in the shallot. This suggests that the incorporation rate of selenate into the plant exceeds the capacity of the enzyme(s) to catalyze the formation of MeSeCys, although the transformation from MeSeCys into GluMeSeCys may

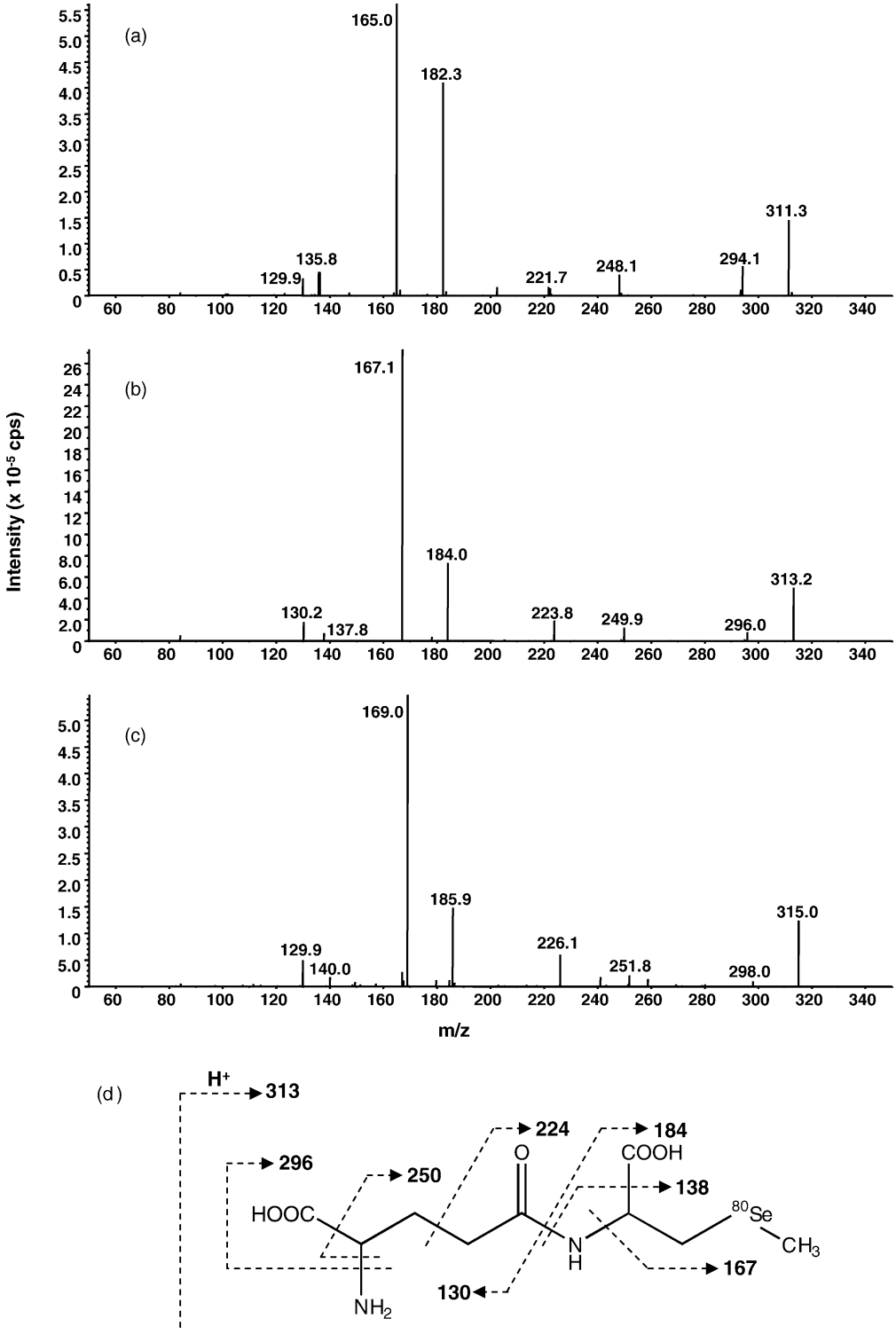


Fig. 5. Collision-induced dissociation mass spectra (ESI-MS/MS) of Se-containing molecular ions in partially purified extract of selenized odorless garlic. The dissociation of each Se-containing molecular ion, i.e., 311 (^{78}Se, panel a), 313 (^{80}Se, panel b) and 315 (^{82}Se, panel c), was induced with 20 eV collision energy, and each product ion was detected with the second mass spectrometer. The assignments of the fragments obtained are shown in panel d.

not be saturated. Moreover, inorganic Se compounds, such as selenate and selenite, are less bioavailable and more toxic than selenoamino acid derivatives [1,25,26]. Thus, the selenized shallot is not suitable for ingestion in its present form, and it is necessary to reduce the content of selenate in it.

The selenized normal garlic contained large amounts of S compounds mainly in the form of AlIn, which is the aque-

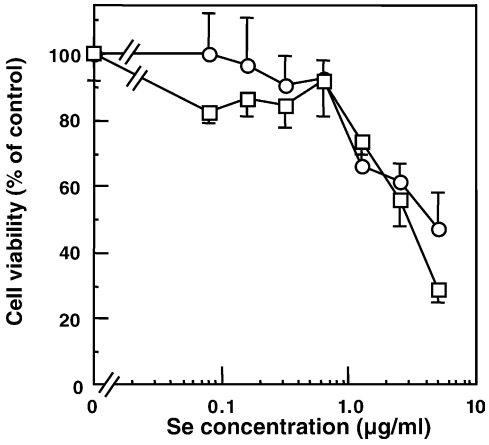


Fig. 6. Dose-dependent cytotoxicity of extracts of selenized Allium plants against human promyelocytic leukemia cells, HL60. Cells were treated with various concentrations of the extracts of selenized odorless garlic (squares) and shallot (circles) for 24 h, and cell viability was assayed with MTT reagent. Points and bars indicate mean ± SD for four assays.

ous precursor of the volatile odorant, allicin. On the other hand, AlIn was not detected in the odorless garlic, suggesting that the odorless garlic is intrinsically defective in the biosynthesis of AlIn or its precursors, although it has been speculated to be lacking in the enzyme for converting AlIn into allicin (alliinase). Four S-containing compounds were detected commonly in the odorless garlic and the shallot. However, none of them matched the authentic standards used in this study. In onion, the major thioamino acid derivative is isoalliin, and *S*-propylcysteine sulfoxide (propiin) was also detected instead of AlIn [27]. These thiocompounds may be the candidate compounds for the Allium plants in this study, further study of the speciation of S-containing compounds is needed.

Recently, one of the Se-containing allyl derivatives, i.e., selenoalliin (SeAlIn), was speculated in garlic [28]. This seleno compound is of interest because AlCys and AlIn have anti-carcinogenic and anti-cardiovascular effects [29]. Although the retention times of the Se-substituted compounds were similar to those of the corresponding thiocompounds under the elution conditions used in this study, no Se signals were detected at the possible retention times, suggesting that these Allium plants did not contain the novel selenoamino acid derivatives.

In conclusion, the selenized odorless garlic and the shallot contained GluMeSeCys and lacked the precursor of the specific odorant of garlic, AlIn. These selenized Allium plants showed anti-tumor activities similar to the selenized normal garlic. Taken together, these results indicate that the new selenized Allium plants, particularly the odorless garlic, have pharmacological and nutritional effects comparable to those of selenized normal garlic.

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