Allium Chemistry: Identification of Selenoamino Acids in Ordinary and Selenium-Enriched Garlic, Onion, and Broccoli Using Gas Chromatography with Atomic Emission Detection

Keywords: *Garlic; onion; broccoli; Allium sativum; Allium cepa; Brassica oleracea botrytis; gas chromatography–atomic emission detection; organoselenium compounds; selenoamino acids; selenocysteine; Se-methyl selenocysteine*

INTRODUCTION

Selenium (Se), an essential micronutrient whose absence causes skeletal and cardiac muscle dysfunction (Young, 1981; Sathe et al., 1992), is required for the proper function of the immune system and for cellular defense against oxidative damage and thus may play a role in the prevention of cancer and premature aging (Axley et al., 1991). Selenocysteine (**1**, $HSeCH_2CH(NH_2)$-COOH; Cys-SeH), whose incorporation into proteins is directed by a UGA codon and which has been called the 21st amino acid essential for ribosome-directed protein synthesis (Söll, 1988), is present at the active sites of glutathione peroxidase, 5′-deiodinase and selenoprotein P (Burk and Hill, 1994). Because animal Se deficiencies are typically treated by dietary supplementation with sulfur-containing amino acids and because *Allium* and *Brassica* spp. are rich sources of such amino acids, we sought to determine whether **1** and related selenoamino acids are also found in garlic (*Allium sativum*) and broccoli (*Brassica oleracea botrytis*). Garlic is claimed to contain selenoproteins (Wang et al., 1989) and a selenopolysaccharide (Yang et al., 1992), while broccoli is said to accumulate high levels of unknown forms of Se (Bañuelos et al., 1993). Cabbage (*Brassica oleracea capitata*) grown with $H_2{}^{75}SeO_3$ contained various selenoamino acids, -peptides, and -proteins (Hamilton, 1975). Development of a simple analytical procedure for selenoamino acid determination would also be useful in the examination of plants grown in high soil Se areas, e.g., regions of central California (Bañuelos et al., 1993). Since the natural S:Se ratio in garlic is ca. 1.2×10^4:1 and since the properties of S- and Se-containing amino acids are similar, leading to coelution problems (Tschursin et al., 1994), we employed the highly sensitive, element-specific technique of gas chromatography with atomic emission detection (GC–AED; Cai et al., 1994a,b) in the analysis of $ClCO_2Et$-derivatized amino acids. We have identified for the first time Se-containing amino acids in unenriched as well as Se-enriched garlic and broccoli and have also identified selenoamino acids in Se-enriched onion, obtaining different results than previously reported (Spåre and Virtanen, 1964). In the accompanying paper we identify organoselenium compounds in human breath following consumption of fresh garlic (Cai et al., 1995), suggesting that they are derived from degradation of the naturally present selenoamino acids. Finally, we propose that reduction in incidence of mouse breast tumors in carcinogen-treated mice fed Se-enriched garlic (Ip et al., 1992) is due to the enhanced levels of **1** and *Se*-methyl selenocysteine (**2**; Cys-Se-Me) in these plants.

EXPERIMENTAL PROCEDURES

Instrumentation. The GC–AED is described in the accompanying paper (Cai, et al., 1995). The injection port (split; ratio 5:1) was maintained at 250 °C; the GC oven was programmed from 120 °C (initial temperature) to 290 °C at 20 °C/min, holding at 290 °C for 3.5 min. A HP-5 30 m × 0.53 mm × 1.5 μm (film thickness) column was used. A HP 5972 MSD (Hewlett-Packard Co.) interfaced with the GC was employed for GC/MS analysis, using a HP-5 30 m × 0.25 mm × 1.5 μm (film thickness) column, the above temperature program, and an EPC-regulated He carrier gas pressure of 7.8 psi (0.7 mL/min flow at 120 °C).

Reagents. Seleno-D,L-cystine (**3**, $(Cys\text{-}Se)_2$), seleno-D,L-methionine (**4**, Se-Met), seleno-D,L-ethionine (**5**, Se-Eth), methionine (**7**), and cysteine (**8**) were purchased from Sigma (St. Louis, MO). Dilute HCl solutions of *Se*-methylselenocysteine (**2**, 9.4 mM) and *Se*-allylselenocysteine (**6**, 5.4 mM; Cys-Se-All) were provided by H. Ganther (University of Wisconsin). *S*-Allylcysteine (**9**), *S*-methylcysteine (**10**), *S*-methylcysteine *S*-oxide (**11**), and *S*-allylcysteine *S*-oxide (**12**) were prepared from **8** according to published procedures (Thomas and Parkin, 1994); $NaBH_4$ was used as a 100 mg/mL solution in 0.2 M NaOH.

Derivatization of Standard Selenoamino Acids. A water (300 μL) and $NaBH_4$ solution (100 μL) was placed in the first 1 mL vial, **3** ($(Cys\text{-}Se)_2$; 0.6 mg) was added, and the mixture was heated for 10 min at 90 °C, forming a homogeneous solution of the sodium salt of **1** (Cys-SeNa). To the second and third 1 mL vials were added 9.4 mM **2** (300 μL) and 5.4 mM **6** (300 μL), respectively. To the fourth and fifth 1 mL vials were added **4** (0.4 mg) and **5** (0.4 mg), each dissolved in 300 μL of 0.1 N HCl, respectively. To each of the five vials was added EtOH (150 μL), pyridine (50 μL), and finally $ClCO_2Et$ (50 μL), briefly shaking the vials to mix the contents. After CO_2 evolution ceased, $CHCl_3$ (500 μL) was added to each vial and the derivatives were extracted into the organic phase. The $CHCl_3$ layer remained clear during the process, while the aqueous phase became opaque. Aliquots (100 μL) of the organic phase from each vial were mixed in a new vial as "mixed standard Se-amino acids solution", and 1 μL of the latter solution was injected into the GC–AED or GC–MSD.

Plant Sample Preparation Procedure. Finely powdered, lyophilized samples of normal or Se-enriched garlic, onion, and broccoli of known total Se content were provided by C. Ip (garlic, onion) and P. Whanger (broccoli). Garlic (0.1 g), onion (0.1 g), and broccoli (0.03 g) samples were each suspended in 1 mL of 0.1 N HCl using a vortex mixer and were then each centrifuged for 8 min. To 300 μL of each supernatant in separate 1 mL vials was added, sequentially, EtOH (150 μL), pyridine (50 μL), and $ClCO_2Et$ (50 μL); mixing was achieved by briefly shaking the vials. After gas evolution had ceased, $CHCl_3$ (500 μL) was added, the derivatives were extracted into the organic phase, and 1 μL of the latter was injected into the GC–AED or GC–MSD.

RESULTS AND DISCUSSION

Selenoamino acids **1**, **2**, and **4**–**6** were volatilized by derivatization as the *N*(*O*,*Se*)-ethoxycarbonyl ethyl esters with $ClCO_2Et$ (Husek, 1991; also see Janák et al., 1994; Wang et al., 1994), separated by capillary GC, and characterized by GC–AED/MSD (Figures 1 and 2). Lyophilized normal garlic (0.02 ppm Se) or moderately Se-enriched garlic (68 ppm Se) on $ClCO_2Et$ derivatization followed by GC–AED analysis showed a single peak in the Se channel, identified as **1** by comparison with the retention time of an authentic sample (Figure 3). In 1355 ppm Se garlic, Cys-Se-Me (**2**) became the major selenoamino acid (identified by GC–MS), with minor amounts of **1** and Se-Met (**4**) (Figure 3). Compound **6**

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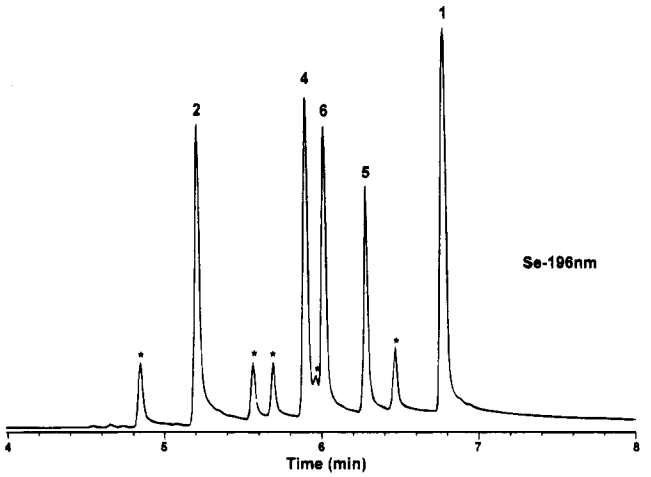

Figure 1. GC–AED analysis (Se channel, monitored at 196.1 nm) of $ClCO_2Et$-derivatized selenocysteine (**1**, $HSeCH_2CH(NH_2)COOH$; Cys-SeH), *Se*-methylselenocysteine (**2**, $MeSeCH_2CH(NH_2)COOH$; Cys-Se-Me), seleno-D,L-methionine (**4**, $MeSeCH_2CH_2CH(NH_2)COOH$; Se-Met), seleno-D,L-ethionine (**5**, $EtSeCH_2CH_2CH(NH_2)COOH$; Se-Eth), and *Se*-allylselenocysteine (**6**, $CH_2{=}CHCH_2SeCH_2CH(NH_2)COOH$; Cys-Se-All). Peaks labeled with an asterisk are byproducts associated with the derivatization procedure.

was not detected in any of the garlic samples. The S channel in the garlic GC–AED analyses (Figure 4) showed 2:1 *S*-allylcysteine (**9**) and *S*-allylcysteine *S*-oxide (**12**), significantly different from the ca. 1:10 ratio found for lyophilized garlic by LC methods (Lawson, 1993), suggesting problems in quantitating volatile sulfoxides using the $ClCO_2Et$ technique. Minor amounts of methionine (**7**) and *S*-methylcysteine (**10**) were also detected. All peak identifications were confirmed by GC–MS. In contrast to the situation with the selenoamino acids, there were only minor changes in the relative ratios of the S amino acid as the level of Se was varied from 0.02 to 1355 ppm (not shown).

Analysis of the headspace (HS) above Se-enriched garlic by HS–GC–AED showed a very similar profile of Se compounds (MeSeMe, $MeSeC_3H_5$, MeSeSMe, MeSeSeMe, MeSSeSMe, $MeSSeSC_3H_5$) (Cai et al., 1994) whether or not synthetic **2** was added, although all peak abundances are enhanced by the addition of the synthetic selenoamino acid. On the other hand, addition of synthetic Cys-Se-All (**6**) to Se-enriched garlic followed by HS–GC–AED showed an Se profile quite different from that of Se-enriched garlic, with the major peaks being All_2Se (major peak; not seen in normal garlic) and AllSSeSAll (or isomer; small peak in Se-enriched garlic). On the basis of the above observations, we conclude that **6** is not present in the lyophilized garlic samples. Further work is necessary to establish whether or not **6** is synthesized to any extent in garlic cloves. Allyl selenides are known to be particularly sensitive to thiols (Musorin et al., 1993). It appears that synthetic **6** is cleaved by garlic homogenates, probably forming $CH_2{=}CHCH_2SeH$ which is oxidized to thermally unstable AllSeSeAll, which in turn loses Se, affording the observed AllSeAll (Musorin et al., 1993). We suggest that when garlic is presented with high levels of inorganic Se fertilizer, the excess **1** formed is Se-methylated to give the major selenoamino acid, **2**. Similar analysis of Se-enriched onion (96 ppm Se) and broccoli (345 ppm Se) showed for the former equal amounts of **1** and **2** in the Se channel and for the latter ca. 2:1 **2**:**1** along with minor amounts of **4** in the Se channel and *S*-methylcysteine (**10**) and lower amounts of methionine (**7**) in the S channel (*S*-methylcysteine *S*-oxide (**11**) has been previously reported (Marks et al., 1992)). The presence of **2** and **10** in broccoli was confirmed by GC–MS. The headspace above chopped broccoli, analyzed by GC–AED, showed MeSeMe, MeSeSMe, MeSeSeMe, $MeSeSC_3H_5$, $MeSSeSC_3H_5$, $C_3H_5SSeSC_3H_5$, together with six thus far unidentified peaks, in the Se channel and MeSSMe (major), MeSSSMe, $C_3H_5SSC_3H_5$, and $C_3H_5SSSC_3H_5$ in the S channel.

Our results present as many questions as answers. In *Allium* spp., various *S*-alk(en)ylglutathione and γ-glutamyl cysteine derivatives and their *S*-oxides are involved as storage compounds and as precursors of

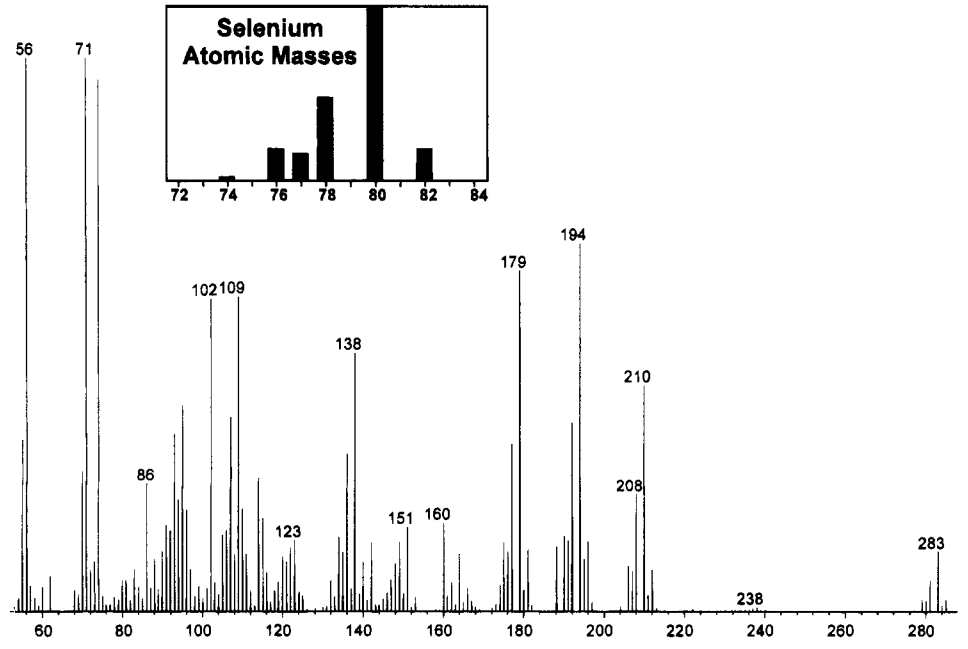

Figure 2. Mass spectrum of $ClCO_2Et$-derivatized *Se*-methylselenocysteine (**2**, Cys-Se-Me), $MeSeCH_2CH(NHCO_2Et)CO_2Et$ (M^+ 283), showing ions of $m/e \geq 53$. Major fragments are seen at m/e 210 ($M^+ - CO_2Et$), 194 ($M^+ - NHCO_2Et, H^+$), 179 ($M^+ - NHCO_2Et, Me, H^+$), 138 ($MeSeCH_2C{\equiv}NH^+$), 109 ($MeSeCH_2^+$), and 74 ($HCO_2Et^{\cdot+}$). The selenium stable natural abundance isotopic ratios are shown in the inset.

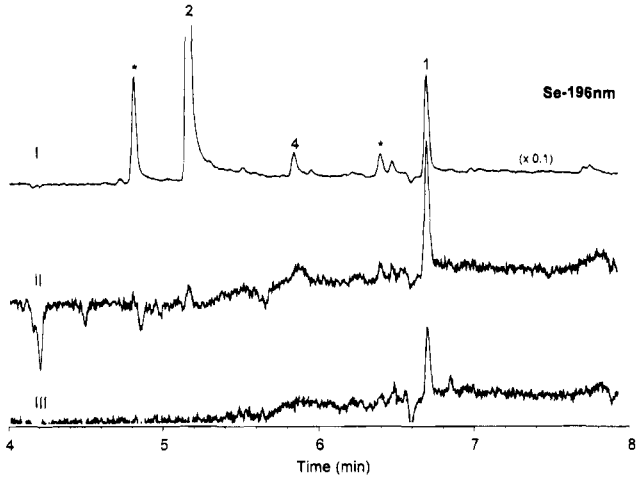

Figure 3. GC–AED analysis (Se channel, monitored at 196.1 nm) of $ClCO_2Et$-derivatized amino acids from lyophilized Se-enriched (I, 1355 ppm Se; II, 68 ppm Se) and unenriched (III, 0.02 ppm Se) garlic. Peaks labeled with an asterisk are byproducts associated with the derivatization procedure.

S-alk(en)ylcysteine *S*-oxides (Block, 1992). Are analogous Se compounds, including selenoxides (Spåre and Virtanen, 1964; Bottino et al., 1984), also present in these plants? Clearly, the only selenoamino acids seen in the present work are those easily hydrolyzed and efficiently volatilized after derivatization. Identification of selenoamino acid γ-glutamyl derivatives (Nigan and McConnell, 1965) and *Se*-oxides in *Allium* spp. and broccoli (Spåre and Virtanen, 1964; Hamilton, 1975), as well as isoselenocyanates (Bertelsen et al., 1988) in Se-enriched broccoli, analogous to compounds such as sulfurophane (Zhang et al., 1992), must await application of element-specific chromatographic methods more suited for nonvolatiles. In our hands, attempts to volatilize selenocystine with $ClCO_2Et$ were unsuccessful, leading instead to a small, long-retention time GC–AED peak thought to be elemental Se, since there was no corresponding signal on the (amplified) carbon channel. In most of our plant sample GC–AED analyses, the small "Se" peak was seen, suggesting that $(Cys\text{-}Se)_2$ (**3**) may also be present. It is possible that the **3** reported in onion (Spåre and Virtanen, 1964) may result from oxidation of **1** under sample preparation/analysis conditions. Clearly, careful control of sample preparation conditions is necessary to determine the **1**:**3** ratio in plants.

Are the Se compounds in *Allium* spp. present simply as the result of the inability of the plant to distinguish Se from S or is there a unique role for Se in these plants different from that for S, as already established for various selenoproteins? In seeking answers to these questions, caution must be used in drawing conclusions based upon studies involving fertilization with elevated concentrations of SeO_3^{2-} or SeO_4^{2-} since, as we describe herein, these ions may substitute for SO_4^{2-} in a manner not seen at lower Se levels (Bertelsen et al., 1988). Furthermore, Se compounds so produced may be further transformed to lessen any harmful or undesirable properties, e.g., by methylation (Walter et al., 1972), and to facilitate detoxification–elimination, e.g., as Me_2Se (Bañuelos et al., 1993). It is noteworthy that **1** and **2** are the major selenoamino acids found in *Allium* spp. and broccoli, in contrast to the predominance of **4** in wheat and other grains (Beilstein and Whanger, 1986; Tschursin et al., 1994), which are major sources of dietary Se in the United States (Olson et al., 1970), and

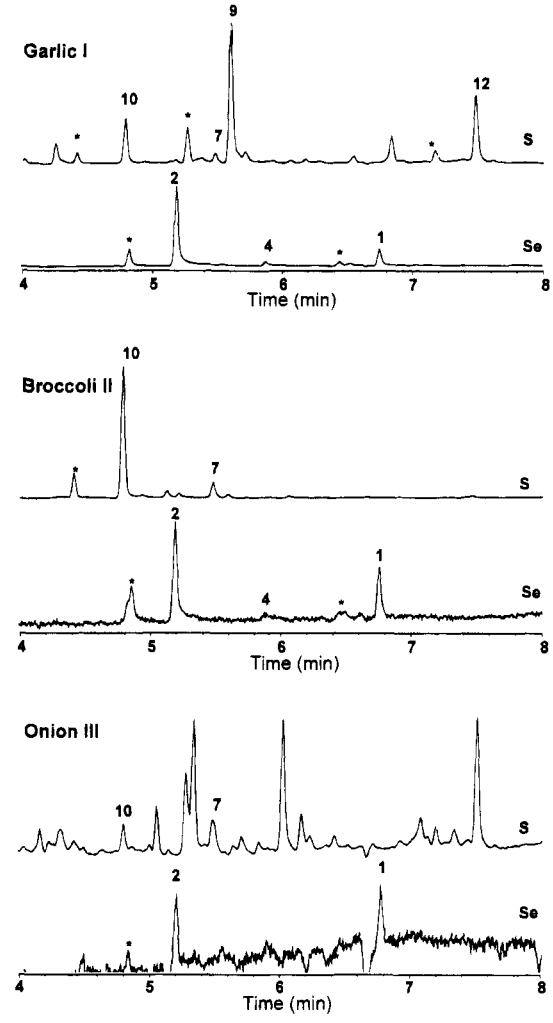

Figure 4. GC–AED analysis (upper, S channel monitored at 180.7 nm; lower, Se channel monitored at 196.1 nm) of $ClCO_2$-Et-derivatized amino acids from lyophilized Se-enriched garlic (I, 1355 ppm Se; S:Se scale 1:10), broccoli (II, 345 ppm Se; S:Se scale 1:20), and onion (III, 96 ppm Se; S:Se scale 1:10). Identification of $ClCO_2Et$ derivatives: **1**, selenocysteine (Cys-SeH); **2**, *Se*-methylselenocysteine (Cys-Se-Me); **4**, selenomethionine (Se-Met); **7**, methionine; **9**, *S*-allylcysteine; **10**, *S*-methyl cysteine; **12**, *S*-allylcysteine *S*-oxide. Peaks labeled with an asterisk are byproducts associated with the derivatization procedure.

in soybean (Yasumoto et al., 1988; Sathe et al., 1992). This difference is significant because animal-derived food was previously considered to be the major dietary source of **1** (Burk, 1989). The dietary form of Se may be relevant to the antioxidant and anticancer protection it can afford.

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Xiao-Jia Cai,[†] Eric Block,*[,‡] Peter C. Uden,*[,†] Xing Zhang,[‡] Bruce D. Quimby,[§] and James J. Sullivan[§]

Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003, Department of Chemistry, SUNY—Albany, Albany, New York 12222, and Hewlett-Packard Company, Little Falls Site, 2850 Centerville Road, Wilmington, Delaware 19808

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[†] University of Massachusetts.
[‡] SUNY—Albany.
[§] Hewlett-Packard Co.