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High-performance liquid chromatographic–inductively coupled plasma mass spectrometric evidence for Se-"alliins" in garlic and onion grown in Se-rich soil

J. Auger[a,*], W. Yang[a], I. Arnault[a], F. Pannier[b], M. Potin-Gautier[b]

[a] *I.R.B.I, UMR 6035 CNRS, Université F. Rabelais, Parc de Grandmont, 37200 Tours, France*
[b] *L.C.A.B.I.E, UMR 5034 CNRS, Université de Pau, Avenue de l'Université, 64000 Pau, France*

Abstract

Garlic and onion, are well known for their medical value, especially in against cancer and anticardiovacular diseases. "Alliins" (*S*-alk(en)yl-L-cysteine sulphoxides) are sources of major active compounds in *Allium* plants. Se incorporation into garlic significantly increases activities of garlic in cancer prevention and inhibition. Selenomethionine, selenocysteine and Se-methylselenocysteine have been identified in garlic and onion. Previously we identified γ-glutamyl-Se-methyl-L-selenocysteine, in extracts of garlic cultivated in Se-rich soil [Med. Res. Rev. 16 (1) (1996) 111], suggesting the possible existence of Se-alk(en)yl-L-cysteine selenoxides (Se-"alliins") in garlic. Several comparative experiments were carried out to demonstrate the existence of Se-"alliins" in Se-enriched garlic and onion. We found that there was one similar time-dependent Se signal in HPLC–inductively coupled plasma MS chromatograms of cold-water extracts of freeze-dried garlic powder and fresh garlic. This signal was lost when the extracts of garlic powder and fresh garlic were stored for 1 day at >4 °C, but remained in fresh onion extract at the same storage conditions. These phenomena and possible mechanisms are discussed. An additional experiment showed that *Allium* species cultivated in Se-rich soil might contain two different Se-"alliins".

Keywords: Allium spp.; Food analysis; Organoselenium compounds; Selenium; Selenium-"alliins"

1. Introduction

Allium plants, especially garlic (*Allium sativum*) and onion (*Allium cepa*), are reputed for their medical value, especially in anticancer and anticardiovacular diseases [1]. "Alliins" (*S*-alk(en)yl-L-cysteine sulphoxides) are the sources of major active compounds and flavours in *Allium* plants. In garlic, the major component is *S*-allyl-L-cysteine sulphoxide (alliin); *S*-methyl-L-cysteine sulphoxide (methiin) and *S*-propenyl-L-cysteine sulphoxide (isoalliin) are also detected [2]. In onion, isoalliin is the major component, alliin is absent and *S*-propyl-L-cysteine sulphoxide (propiin) appears. "Alliins" are located in the cytoplasm of the plant cells.

When *Allium* tissues are cut, crushed or chewed, alliinase is released from the vacuoles into cytoplasm and catalyses "alliins" to form mainly *S*-alk(en)yl thiosulfinates (Ti). Ti are unstable and for instance allicin (diallyl thiosulfinate) further decomposes to produce a series of degraded compounds, ajoenes, dithiins and sulfides, dependent on solvents used and extraction conditions. Alliinase activity in garlic is specifically inhibited by hydroxylamine [3]. γ-Glutamyl-alk(en)yl-L-cysteines, the biosynthetic intermediates of "alliins" [4], are stable storage forms of sulfur in garlic cloves and other *Allium* plants and are not affected by alliinase [5].

In garlic *S*-allyl-L-cysteine (deoxyalliin), allicin and their degraded compounds are known to be effective in inhibition of carcinogenesis and/or cardiovascular diseases in vitro or in vivo [1]. Several other Ti and sulfides from onion have similar health benefits [6] but γ-glutamyl-*S*-alk(en)yl-L-cysteines have not been shown to possess significant bioactivities [7]. Bioactivities of water-extracts of garlic decrease rapidly with time at room temperature during transformation of sulfur compounds.

Selenium incorporation into garlic significantly increases activities of garlic in cancer prevention and inhibition. Selenomethionine, selenocysteine and Se-methylseleno-

* Corresponding author. Tel.: +33-2-47-36-70-52; fax: +33-2-47-36-73-89.
E-mail address: auger@univ-tours.fr (J. Auger).

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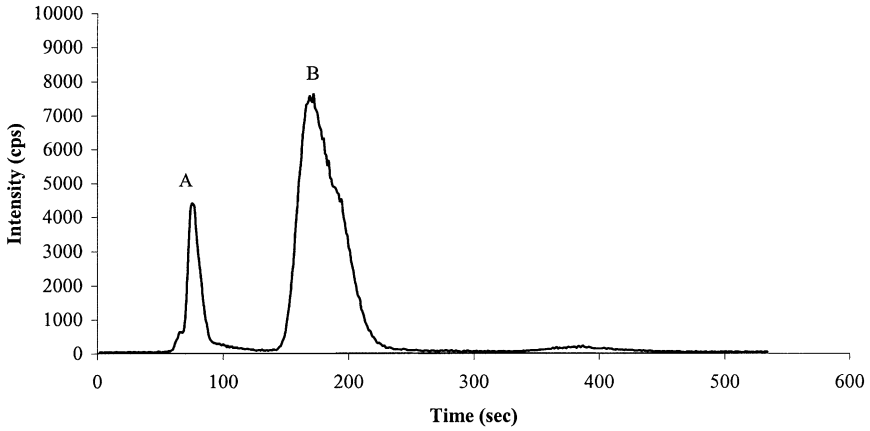

Fig. 1. RP-HPLC–ICP-MS chromatogram of cold-water extract of garlic powder.

cysteine have been identified in garlic and onion [8,9]. Many selenides, the possible decomposition compounds of selenium-compounds (Se compounds) analogous to "alliins" (Se-"alliins"), are also detected in selenium-enriched garlic by gas chromatography–atomic emission detection (GC–AED) analysis and in human breath after garlic was ingested [10]. The data were not sufficient to show existence of Se-"alliins" and Ti analogues [8] even though Se-methylselenocysteine selenoxide and Se-propenylselenocysteine selenoxide were tentatively identified by two-dimensional paper chromatography in ^{75}Se enriched onion [11]. Furthermore the only oxidation products of synthetic Se-methyl selenocysteine were claimed to be methaneseleninic acid and dimethyl selenide [12,13] and not the Se compound analoguous of methiin, Se-methiin. Se-Amino acids can also decompose to release selenides at the high temperature of GC (180 °C) and by enzymes, such as cysteine lyase, found in the human digestive system and bacteria [14]. Recently we identified γ-glutamyl-Se-methyl-L-selenocysteine (peak B in Fig. 1), in extracts of natural selenium rich garlic [15], and this provides a greatest possibility of the existence of Se-"alliins" in garlic.

If they exist in *Allium* plants, Se-"alliins" may be catalysed by alliinase through the similar chemical and biochemical pathways of Se compounds and sulfur analogues. In our previous work, we could not observe the change of Se-"alliins" during storage because alliinase protein is irreversibly denatured during extraction for 1 h at 80–90 °C. This study is designed to provide more reliable evidence for existence of Se-"alliins". We investigated by HPLC–inductively coupled plasma mass spectrometry (HPLC–ICP-MS) and compared the changes of selenium components of hot and cold-water extracts, cold-water extracts stored at 4 °C and 0 °C, cold-water extracts of garlic powder, and fresh garlic and fresh onion with and without the inhibitor alliinase. Furthermore, an unknown selenium-containing fraction deduced to be one or several Se-"alliins" was collected by reversed phase (RP)-HPLC and isolated again by size-exclusion chromatography (SEC) to obtain information on the exact number of Se-"alliins" present in this interesting fraction.

2. Experimental

2.1. Fresh garlic and onion

Garlic (7.0 ppm Se) and onion (1.5 ppm Se) were harvested in naturally seleniferous soils in Enshu, China. The total selenium was measured by ICP-MS following extraction procedure described by Casiot [16].

2.2. Garlic powder preparation

Ten cloves of garlic from Enshu in China were pooled, peeled, cleaned with distilled water, sliced, freeze-dried, finely ground and sieved (0.45 μm). The powder used for analysis contained 72 ppm Se measured by ICP-MS.

2.3. Sampling

2.3.1. Hot-water extraction

A 0.2 g amount of the garlic powder was extracted by 5 ml deionised water with stirring for 1 h at 85–90 °C, centrifuged (4000 rps, 30 min) and filtered (0.45 μm).

2.3.2. Cold-water extraction

A 0.2 g amount of the garlic powder was extracted at room temperature by 5 ml deionised water, centrifuged (4000 rps, 30 min) and filtered (0.45 μm).

2.3.3. Cold-water with inhibitor extraction

A 0.2 g amount of the garlic powder was extracted by 0.005% hydroxylamine (NH_2OH/HCl) at room temperature.

A 0.2 g amount of the garlic powder was extracted by 0.01% hydroxylamine (NH_2OH/HCl) at room temperature.

2.3.4. Fresh garlic and onion extraction

Ten bulbs of fresh garlic and fresh onion were peeled, cleaned, blended and extracted by deionised water and fil-

tered. The filtrates were stored in glass jars for 1 day at 0 °C and room temperature, respectively.

2.4. RP-HPLC–ICP-MS procedure

The filtrates were analysed by RP-HPLC using a Spherisorb column (250 mm × 4.6 mm, 5 μm) (Supelco, Bellefonte, PA, USA) and selenium was detected on-line by an Elan 6000 ICP-MS instrument (PE-Sciex, Ontario, Canada), under the conditions described by McSheehy et al. [15], on the same day of preparation and at selective days during storage. Acetic acid of analytical grade (Sigma–Aldrich, St. Quentin, France) at pH 3.0 was used as mobile phase with 1.0 ml/min flow rate following the previous studies [15].

2.5. HPSEC–ICP-MS procedure

Selenium-containing components in peak A in RP-HPLC–ICP-MS chromatogram of hot-water extract of garlic powder was collected using an automatic Dynamax fraction collector FC-2 (Houston, TX, USA), freeze-dried, isolated again and analysed on-line by HPSEC–ICP-MS. For SEC, a 160 mm × 4 mm column, filled with G-75 Sephadex Gel (Pharmacia, Uppsala, Sweden) was used. The sample and mobile phase (deionised water, with flow rate 1.0 ml/min) was pumped with a peristaltic pump (Minipuls3, Gilson, Villers le Bel, France). Selenium in the effluent was monitored using the PE Elan 6000 ICP-MS fitted with a cross-flow nebuliser. Injections were carried out using a model 7725 injection valve (Rheodyne, CA, USA) with a 100 μl injection loop. A HP model 1100 (Hewlett-Packard, Waldbronn, Germany) HPLC pump was used. The software used to present on-line HPLC–ICP-MS data was Turbochrom4 (PE-Sciex).

Water was purified to 18.2 MΩ cm^{-1} resistivity using a Milli-Q system (Millipore, Bedford, MA, USA). Acetic acid, methanol and hydrochloric acid (Sigma–Aldrich, St. Quentin, France) were of analytical grade. The chromatographic mobile phases were degassed using a Branson model 1210 ultrasonic cleaner (Danbury, CT, USA).

3. Results and discussion

All RP-HPLC–ICP-MS chromatograms obtained present two peaks, A and B (Fig. 1), with variable relative intensity (Table 1).

3.1. RP-HPLC–ICP-MS chromatograms of hot-water and cold-water extracts of garlic powder

HPLC detection by ICP-MS indicated the presence of Se compounds in cold-water extracts of garlic. Signals of Se compounds in peak A for the cold-water extract analysis (Fig. 1) were somewhat smaller than those in hot-water one (Table 1, samples 1 and 2). This difference was probably due to lower extraction capacity of cold water compared with hot water. A great loss of the signals for peak A is observed after storage for 1 day at 4 °C (Table 1, sample 3). It is possible that these Se compounds might be catalytically decomposed by an enzyme (e.g. alliinase), or other degradation mechanism.

Enzymatic activities change with temperature from 0 to 35 °C, generally having the highest value between 25 and 30 °C. Zero and 4 °C were chosen here only because they are the normal temperatures for food storage.

The effects of storage conditions of garlic powder on relative peak intensity were also investigated (Table 1, samples 4–7). Compared with the chromatogram obtained at the day of extraction (Fig. 1), the loss of Se compounds in peak A

Table 1
Relative intensity of peaks A and B in *Allium* extracts RP-HPLC–ICP-MS chromatograms

Samples		Peak A	Peak B
1	Cold-water extract of garlic powder	[a]	[b]
2	Hot-water extract of garlic powder	[b]	[b]
3	Cold-water extract of garlic powder stored at 4 °C for 1 day	[c]	[b]
4	Cold-water extract of garlic powder stored at 4 °C for 1 week	[c]	[b]
5	Cold-water extract of garlic powder stored at 0 °C for 1 week	[a]	[b]
6	Cold-water extract of garlic powder stored at 4 °C for 1 month	[c]	[b]
7	Cold-water extract of garlic powder stored at 0 °C for 1 month	[a]	[b]
8	Cold-water extract of garlic powder without hydroxylamine	[c]	[a]
9	Cold-water extract of garlic powder with 0.005% hydroxylamine	[c]	[a]
10	Cold-water extract of garlic powder with 0.01% hydroxylamine	[a]	[a]
11	Cold-water extract of garlic powder with 0.005% hydroxylamine stored for 1 day at 4 °C	[c]	[a]
12	Cold-water extract of fresh onion stored for 1 day at room temperature	[c]	[d]
13	Cold-water extract of fresh garlic stored for 1 day at room temperature	[d]	[d]

[a] Moderate level.
[b] High level.
[c] Low level.
[d] Absence.

was inhibited by low temperature (0 °C) (Table 1, samples 5 and 7) during storage even during 1 month. Storage at 4 °C did not inhibit the loss (Table 1, samples 4 and 5). To avoid the loss of selenium active compounds, garlic products such as uncooked selenium-enriched garlic juice should be stored at 0 °C.

3.2. RP-HPLC–ICP-MS chromatograms of cold-water extracts of garlic powder with inhibitor

The above experiments could not rule out the possibility that loss of Se compounds was caused by microbial degradation at 4 °C instead of enzymatic reaction. Different amounts of specific inhibitor of alliinase, hydroxylamine, were added to investigate the change of Se compounds of cold-water extracts during extraction (Table 1, samples 8–10) and storage at 4 °C for 1 day (Table 1, sample 11). Hydroxylamine (0.01%) remarkably prevented the loss of the signals of peak A during extraction (Table 1, sample 10).

So the loss of Se compounds present in peak A may be due to alliinase activity.

This activity can be observed not only working on Se-“alliins” but also on several other possible Se compounds. It was reported that L-cysteine and its alk(en)yl derivatives were competitive inhibitors of alliinase through formation of a non volatile thiazolidine from the amino-acid and the enzyme-bound pyridoxal phosphate [3], whereas the degradation of “alliins” by the alliinase gives volatile compounds that cannot be identified by RP-HPLC under the condition used there. Therefore, if peak A was of Se-cysteine derivatives different from “alliins”, a new peak should occur with the loss of peak A because of formation of Se-thiazolidines. We have not found this possible “new peak”, therefore, the possibility that the compound in peak A can belong to Se-“alliins” is more likely than to belong to other Se-cysteine derivatives.

3.3. HPSEC–ICP-MS chromatogram of collected Se compounds in peak A

Some compounds with great structural similarities may not be well separated by RP chromatography in the conditions used for ICP-MS detection as discussed by Casiot [16]. Garlic contains various “alliins” with similar chromatographic properties and therefore not well separated [17]. To ascertain the exact number of Se compounds present in peak A of RP-HPLC, SEC was used to analyse the collected eluates from peak A. In result, two selenium signals, with similar intensity were detected by ICP-MS [15]. They perhaps stand for two different Se-“alliins”.

We previously identified γ-glutamyl-Se-methylselenocysteine that is probably the biosynthetic intermediate of putative Se-methylselenocysteine selenoxide by analogy of the fact that γ-glutamyl-*S*-methylcysteine is the intermediate of *S*-methyl-L-cysteine sulphoxide. This result seems to be consistent with previous reported results [10] indicating suggestion that selenium, contrary to sulfur, has preferential combination with methyl compared to other alk(en)yl groups. Our experiments did not show which selenium-substituted sulphoxide the two compounds were, respectively. Further structural analysis like electrospray tandem mass spectrometry for them needs to be done.

As present conclusions, we think that selenium can incorporate into “alliins” by substituting sulfur in garlic. Two Se-“alliins” in selenium-enriched garlic seem to be catalysed to decompose by alliinase. Freeze-drying process does not destroy the catalysed properties of alliinase to Se-“alliins”.

3.4. RP-HPLC–ICP-MS chromatograms of fresh garlic and onion

Peak A is not lost in fresh onion extract stored for 1 day at room temperature (Table 1, sample 12) whereas it is lost in fresh garlic (Table 1, sample 13). May be the Se compound of peak A in onion extract is different from that in garlic extract. Are there other factors inhibiting alliinase activity to Se-“alliins” in onion bearing in mind that this alliinase is not exactly the same as in garlic?

4. Conclusion

Although this work supports existence of two Se-“alliins” in garlic, we have not given any structural identification. Other possibilities of the disappearing Se compounds identity, for example Se al(ken)ylselenocysteine cannot be excluded. Se-methylselenocysteine was detected to be one of the main Se compounds in selenium-enriched garlic [8] but Block et al. [13] proved that non-enzymatic oxidation of these compounds did not lead to Se-methiin. If peak A is Se-methylselenocysteine or its non oxidised analogues, there should exist alk(en)yl-cysteine lyase responsible for the loss of the compounds we observed in garlic. There is no literature report of the existence of such lyase in garlic. To obtain any identification of these compounds, further and direct evidences are still needed using more sensitive methods and equipments.

All the results give indications that Se-analogues of “alliins” are present in *Allium* species.

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