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THE SEARCH FOR ANTICARCINOGENIC ORGANOSELENIUM COMPOUNDS FROM NATURAL SOURCES

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ABSTRACT: Consumption of Se-enriched plants or yeast-based nutritional supplements is reported to reduce the risk of cancer. Separation and identification of natural organoselenium compounds in these plants is essential to understand the basis for their biological activity. Earlier work suggests that plants convert inorganic selenium in the soil or growth medium into organoselenium compounds, such as selenoamino acids, following a route similar to the sulfur assimilatory pathway. To separate and detect selenoamino acids in plant extracts, we employ ion pair LC with an inductively coupled plasma mass spectrometer (ICP-MS) detector and capillary GC with an atomic emission detector (AED), for underivatized and derivatized compounds, respectively. Volatile selenium compounds, such as those found in human garlic breath, have been analyzed using GC-AED. Results involving Se-enriched garlic and yeast-based nutritional supplements are presented.

KEY WORDS: Selenoamino acids, ICP-MS, GC-AED, garlic (*Allium sativum*), yeast

INTRODUCTION

Selenium, once considered an element whose compounds were toxic and of no biochemical significance, is now recognized as an essential micronutrient whose absence causes skeletal and cardiac muscle dysfunction.[1,2] It 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.[3] The dramatic change in the way the element and its compounds are viewed stems from discoveries that selenium prevents liver necrosis in rats, that selenocysteine ($HSeCH_2CH(NH_2)COOH$; Cys-SeH) is present at the active site of glutathione peroxidase, 5'-deiodinase, selenoprotein P and various other essential selenoproteins,[4] that in animal studies supernutritional levels of selenium (ca. 3 mg Se per gram of diet, which is 30 times the required level of 0.1 mg per gram of diet) inhibited or retarded carcinogenesis, and that in human trials enhanced dietary levels of selenium (as a selenized yeast supplement containing 200 mg Se, given daily) reduced the incidence of colorectal, lung or prostate cancers.[5] The level of 200 mg Se per day is well below the level of ca. 2000 mg Se per day at which point toxic effects of Se begin (fatigue, "garlic breath", hair loss, immune system impairment, weakened fingernails, etc.). It has also been established that garlic enriched with selenium shows enhanced cancer preventitive properties compared to normal garlic.[6-8] Significantly, synthetic *Se*-alk(en)yl selenoamino acids, postulated to be present in normal and Se-enriched garlic, also show anticarcinogenic activity.[6-8]

Garlic is one of several vegetables containing elevated levels of selenium.[9] The bioavailability of selenium in food products of vegetable origin is high (ca. 60% of total content), although little is known about the form in which selenium is found. Selenoproteins[10] and a selenopolysaccharide,[11] are said to be present in garlic. Broccoli is said to accumulate high levels of unknown forms of Se.[12] Cabbage grown with $H_2{}^{75}SeO_3$ is reported to contain various seleno-amino acids, -peptides, and -proteins.[13] In 1964, Virtanen reported on the basis of radioisotope studies that onion contained the selenoamino acids selenocystine ($(HOOCCH(NH_2)CH_2Se)_2$) and selenomethionine ($HOOCCH(NH_2)CH_2CH_2SeMe$) and tentatively identified selenoamino acid selenoxides.[14] Selenomethionine selenoxide[15] and *Se*-methyl selenocysteine selenoxide (and selenone)[16] are reported to be present in marine phytoplankton and algae, respectively. Virtanen's discovery suggested that there might be a selenium-based flavor chemistry in ***Allium*** spp. parallel to that based on sulfur, e.g. originating from soil selenate (SeO_4^{-2}) or selenite (SeO_3^{-2}). In selenized yeast supplements as well in Se-enriched garlic, Se is believed to be present in an organic form. However the low levels makes characterization difficult. While the natural abundance of sulfur in garlic and onion is typically more than 10,000 times higher than that of selenium, Se-enrichment could occur in crops grown in regions containing higher than average levels of Se in the soil, such as parts of central California. In our work we seek to develope new methods for determining the speciation of Se at very low (e.g. "natural") concentrations and establish the manner in which Se exerts its anticancer action.

THE BIOLOGICAL ROLE OF SELENIUM

One way in which selenium compounds may exert their protective effects is by intercepting so-called "reactive oxygen species", which otherwise would damage lipids or cleave DNA. In the course of normal respiration, ca. 1-4% of oxygen escapes complete 4-electron reduction to water after accepting the initial electron, forming superoxide anion radical ($O_2^{\bullet -}$). While superoxide can serve a useful function in destroying pathogens, it can also be converted to hydrogen peroxide and then, via a trace-metal-dependant Fenton reaction, to hydroxyl radicals (HO•) which can cleave DNA and damage cell membranes. Protonation of superoxide gives the hydroperoxyl radical (HOO•).[17] Damage to cell membranes can occur when these radicals initiate peroxidation of polyunsaturated fats (and lipoproteins) giving lipid hydroperoxides. Inside cells, the latter are destroyed by antioxidant enzymes such as glutathione peroxidase; superoxide dismutases and catalase destroy superoxide and hydrogen peroxide, respectively. The antioxidants acting within cells complement small-molecule antioxidants, such as lipid-soluble vitamin E and carotenoids which act in cell membranes, and water soluble vitamin C as well as certain selenoproteins, active in blood and extracellular fluids. In glutathione peroxidase, selenium (as Cys-SeH) removes an O atom from peroxides; in a catalytic cycle the resultant Cys-SeOH is then reduced back to Cys-SeH by glutathione. Because the pK_a of free Cys-SeH is 5.2, compared with the >8 value for free cysteine, and because Se^- is more nucleophilic than S^- but at the same time bonds to Se are weaker than those to S, Cys-SeH can be considered as a "super-active" cysteine when

involved in catalytic processes. Because the incorporation of selenocysteine into proteins is directed by a UGA codon, it has been called the 21st amino acid essential for ribosome-directed protein synthesis.[18]

ANALYTICAL METHODS AND RESULTS

Separation and identification of the trace organoselenium compounds found in Se-enriched yeast, garlic and other plants is critical to understand the biological activity involved. Based on precedence from the literature, we assume that plants convert inorganic selenium in the soil or growth medium into organoselenium compounds, such as selenoamino acids, following a route similar to the sulfur assimilatory pathway. Two different analytical approaches are under investigation for determination of trace selenoamino acids, ion pair LC with an inductively coupled plasma mass spectrometer (ICP-MS) detector and capillary GC with an atomic emission detector (AED), for underivatized and derivatized compounds, respectively. The quality of the chromatography for LC ICP-MS is such that excellent separation is obtained for cis-trans (E,Z) isomers of *Se*-1-propenylselenocysteine. LC conditions are sufficiently mild that they can be applied to thermally unstable selenoamino acid selenoxides.[19] Alternatively, treatment of selenoamino acids with ethyl chloroformate affords stable volatile derivatives which can be characterized by GC-MS and GC-AED. In the analysis of plant material, to establish a selenium mass balance, total selenium is also determined by microwave digestion of the sample using concentrated HNO_3 followed by ICP-MS measurements. This method works well for levels higher than 2 mg Se/g.[19]

Using the technique of GC-AED, the Se emission line at 196 nm is monitored to identify organoselenium species while concurrently monitoring S and C by lines at 181 and 193 nm, respectively; assignments are confirmed by GC-MS. Analysis of the headspace above chopped garlic using GC-AED shows MeS_nMe, MeS_nAll, and $AllS_nAll$ (n = 1-3, All = allyl) in the S channel. The Se channel shows seven peaks: dimethyl selenide (MeSeMe), methanesulfenoselenoic acid methyl ester (MeSeSMe), dimethyl diselenide (MeSeSeMe), bis(methylthio)selenide ($(MeS)_2Se$), allyl methyl selenide (MeSeAll), 2-propenesulfenoselenoic acid methyl ester (MeSeSAll), and (allylthio)(methylthio)selenide (MeSSeSAll).[20,21] Structures were established by GC-MS using synthetic standards. This same technique when used to analyze human garlic breath (the subject consumed, with brief chewing, 3 g of fresh garlic with small pieces of white bread, followed by 50 mL of cold water) showed in the Se channel dimethyl selenide (MeSeMe) as the major Se component along with one-tenth to one-fortieth the amount of $MeSeC_3H_5$, MeSeSMe and $MeSeSC_3H_5$; the S channel showed AllSH, MeSAll and AllSSAll with lesser amounts of MeSSMe, $MeSSC_3H_5$, an isomer of AllSSAll (presumably MeCH=CHSSAll), $C_3H_5SC_3H_5$ and $C_3H_5SSSC_3H_5$.[22,23] In this same study we also examined the composition of the Se and S compounds in garlic breath as a function of time. After four hours, the levels of MeSeMe, AllSSAll, AllSAll and MeSSMe were reduced by 75% from the initial levels of 0.45 ng/L (MeSeMe), 45 ng/L (AllSSAll), 6.5 ng/mL (AllSAll), and 1.8 ng/L (MeSSMe). The AllSH could only be found in breath immediately after ingestion of garlic. In view of the very low threshhold detection level for low molecular weight organoselenium

compounds,[24] it is likely that compounds such as MeSeMe contribute to the overall odor associated with garlic breath. It has been previously reported that MeSeMe, which has a garlic-like odor, is found in the breath air of animals fed inorganic Se compounds[25] and humans who have accidentally ingested Se compounds.[26] Studies involving consumption of larger quantities of garlic (38 g) indicate persistence of levels of sulfur compounds as high as 900 ppb in the subject's breath for more than 32 hours.[27]

Lyophilized normal garlic (0.02 ppm Se) or moderately Se-enriched (68 ppm Se) garlic was derivatized with ethyl chloroformate to volatize the selenoamino acids, likely precursors of the headspace Se compounds. Analysis by GC-AED showed selenocysteine, identified by comparison with the mass spectral fragmentation and the retention time of an authentic standard. In garlic, more heavily Se-enriched (1355 ppm Se), *Se*-methylselenocysteine was the major selenoamino acid found along with minor amounts of selenocysteine and traces of *Se*-methionine; the S channel showed 2:1 allyl-cysteine and allyl-cysteine *S*-oxide along with minor amounts of methionine.[28] There were only minor changes in the ratios of the sulfur amino acids as the level of Se was varied from 0.02 to 1355 ppm. Similar analysis of Se-enriched onion (96 ppm Se) revealed the presence of equal amounts of *Se*-methylselenocysteine and selenocysteine in the Se channel.

Water extracts of Se-enriched plants were also analyzed by HPLC-ICP-MS using C8 or C18 columns with 98/2 water/methanol and 0.1% trifluoroacetic acid (ion pair conditions).[29,30] These analyses show that *Se*-methylselenocysteine is the major component along with lesser

amounts of *Se*-methionine, selenocystine, and selenate and selenite salts in high selenium garlic. However a considerable number of interesting unknown selenium-containing components remain to be identified. More than 20 selenium species were found to be present in Se-enriched yeast. Selenium speciation in these samples was found to be sample dependent. Thus, the chromatographic profile of Se-enriched garlic changes with the level of selenium present.[19] Se-enriched yeasts from different sources also show distinctly different chromatographic profiles.[19] The basis for these differences remains to be established.

Oxidation of the selenoamino acid standards with excess hydrogen peroxide was investigated.[19] For each selenoamino acid oxidized, a new Se-containing peak was produced. No original selenoamino acid peaks were found, indicating that all of the reactants were consumed. All of the selenoamino acid oxidation products eluted within the first six minutes. The earlier elution times are presumed to result from the increased polarity of the species arising from the addition of oxygen. When selenomethionine (LC peak retention time 7.2 minutes) was oxidized with hydrogen peroxide the presumed selenoxide (retention time 2.2 minutes) could be completely reduced back to selenomethionine with thiosulfate solution, as previously reported.[31] The oxidation products of *Se*-1-propenylselenocysteine could be separated on a 25 cm C18 column, indicating that the geometrical isomerism persisted unchanged during oxidation. Work is in progress to further characterize the various *Se*-alk(en)yl selenoamino acid selenoxides.

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