

A study of Se-Hg antagonism in *Glycine max* (soybean) roots by size exclusion and reversed phase HPLC–ICPMS

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Abstract An attempt was made to study selenium (Se) and mercury (Hg) interactions in plants, specifically soybean (*Glycine max*), by inductively coupled plasma mass spectrometric detection. Greenhouse-cultivated plants were subjected to treatment with different regimens of Se and Hg and analyzed for their metabolized species in roots, stems, leaves, pods and beans. Most of the water-soluble Hg was found to be localized in the roots in association with Se in a high molecular weight entity, as identified by size exclusion chromatography. This entity was also extracted in protein specific isolate, but it resisted enzymatic breakdown. Complete breakdown of this high molecular weight species was accomplished by acid hydrolysis. Optimization of the conditions for acid hydrolysis is discussed. Hg and Se species found in root extract were studied by ion-pairing chromatography. In a sub-study, the Se distribution pattern was found to be unaffected by the presence of Hg, but the amount of Se assimilated was found to be higher in plants coexposed to Hg.

Keywords Ion chromatography · Ion exchange · Mass spectrometry · ICP–MS · Metals · Heavy metals · Se and Hg · Acid hydrolysis

Introduction

The toxicity of mercury (Hg) is known to be alleviated by the presence of selenium (Se) in biological systems. This antag-

onism of Se and Hg was first observed by Parizek et al. in 1967 [1], where Se co-administration prevented renal necrosis and mortality in rats that were injected with mercuric chloride. Although this protective action of Se has been known for a long time, studies of the interactions of Se and Hg at the molecular level have been rare. Bacterial [2, 3], fish-based [4, 5], avian [6, 7] and mammalian [8, 9] studies have shed some light on this issue. A significant contribution along these lines by Yoneda and Suzuki identified a possible role of the human plasma protein Selenoprotein P in the binding of Hg [10].

Before we can sequester Hg in the environment at the lower levels of the food chain and thus prevent it from entering higher organisms, a detailed understanding of the molecular intricacies of how Hg is transformed in plants in the presence of Se is required. Investigations of Se–Hg interactions in terrestrial plants have appeared recently [11] and attempts are now being made to understand their chemistry [12]. With a complete knowledge of Hg–Se interactions in plants, appropriate genetic modifications of plants can be made that should reduce the levels of Hg in soil or water through phytoremediation.

Currently, a limited number of plant studies have discussed the ratio of the total amount of Se to that of Hg in different plant compartments as well as morphological differences in plants that arise due to variations in Se and Hg supplementation levels [11, 13]. These initial studies have elucidated the relative distributions of Hg in the presence and absence of Se in various plant compartments. However, they have not provided insights into the Se and/or Hg species present in the plants. The only recent report that has ventured into this territory, using size exclusion chromatography (SEC), revealed that the Se and Hg complex in hydroponically grown *Brassica juncea* plants in our laboratory was possibly proteinaceous in nature [12].

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Moreover, it was observed that higher amounts of Hg were absorbed when plants were coadministered Hg and Se. However, the suspected “proteinaceous” complex resisted enzymatic breakdown under various conditions, limiting the insights that could be gained into the nature of the Hg association. An ability to dissociate this complex should aid the analyses of the Se/Hg compound(s) in plants that help to sequester Hg through their roots.

To fulfill these shortcomings, and owing to the tentatively proteinaceous nature of the Se–Hg entity, *Glycine max* (soybean) plants were chosen for the current study. Since a higher amount of Hg was absorbed when Se was fed to *Brassica* plants along with the Hg [12], the soybean plants were treated with Se and Hg. The effects of the presence of Hg on Se absorption were studied by treating some plants with just Se and others with both Se and Hg and then comparing the distributions of Se among the various plant compartments. The distribution of Hg among the various plant compartments was also determined. An attempt to identify the nature of the possible molecular complex that binds with Se and Hg and dissociation of the complex was made using inductively coupled plasma mass spectrometry (ICP–MS) in order to detect chromatographically resolved molecular fragments of interest.

Experiments

Reagents and standards

All commercial standards were used without further purification. All reagents and standard solutions were prepared in doubly deionized (DDI) water and used within two weeks. Selenomethionine, selenocystine, methylselenocysteine, heptafluorobutyric acid, TRIS and 2-mercaptoethanol were purchased from Sigma Chemicals (Milwaukee, WI, USA). Sodium selenite was from ICN Biomedicals Inc. (Aurora, OH, USA).

Acetone, methanol, HCl and HNO₃ were from Pharmaco (Hartford, CT, USA); ammonium acetate and sodium hydroxide were from Fisher Scientific (Fairlawn, NJ, USA); mercuric chloride was from Aldrich Chemicals (Milwaukee, WI, USA); and the yttrium internal standard was obtained from Spex Certiprep (Metuchen, NJ, USA).

Instrumentation

All chromatographic analyses were performed on an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, degasser, autosampler, column compartment and a diode array detector. The analytes were nebulized into an Agilent 7500 ce (Agilent Technologies, Tokyo, Japan) after passing

through the diode array detector. The instrumental parameters, along with the chromatographic parameters, the run conditions and the mobile phases, are listed in Table 1.

In all, two types of chromatographic columns under the influence of four different mobile phases were utilized. Size exclusion studies were performed on a Superdex 200 10/300 GL (Amersham, Uppsala, Sweden). Calibration of the column was performed using a mixture of standard proteins (thyroglobulin $M=669$ kDa, ferritin $M=440$ kDa, albumin $M=66$ kDa, myoglobin $M=17.6$ kDa, aprotinin $M=6.5$ kDa, cobalamin $M=1.3$ kDa) under the run conditions given in Table 1 along with 280-nm UV detection. Ion pairing reversed phase chromatography was achieved on a C-8 column (Alltech, Deerfield, IL, USA).

Sample preparation

Plant growth and supplementation

Soybean seeds were obtained from a local farm. Seeds were sown in the greenhouse in thirty 2-gallon containers. Two different sowing media, soil and a 50:50 mix of soil and ProMix[®] used in the greenhouse, were used and they were watered daily. The seeds were sown in late spring and germination of the seeds was observed within five days. During growth, plants were exposed to a 14-h photoperiod.

Table 1 Instrumental parameters

Chromatographic conditions	
Chromatography A	
Pump	Agilent 1100 series
Column	Superdex-200 (Amersham)
Column dimension	10×300 mm, 10 μm particle size
Mobile phase	30 mM TRIS-HCl buffer, pH 7.5 (0.1% 2-mercaptoethanol where specified)
Flow rate	0.7 mL/min
Injection volume	100 μL
Chromatography B	
Pump	Agilent 1100 series
Column	C-8 Alltima (Hamilton)
Column dimension	4.6×250 mm, 5 μm particle size
Mobile phase (Se analysis)	0.1% HFBA with 3% MeOH, pH 2.5
Mobile phase (Hg analysis)	60 mM ammonium acetate + 0.1% 2-mercaptoethanol pH 6.5
Flow rate	0.9 mL/min
Injection volume	50 μL
ICP-MS Conditions	
System	Agilent 7500 ce
Forward power	1500 W
Collision gas flow	4 mL min ⁻¹ H ₂
Isotopes	⁷⁷ Se, ⁷⁸ Se, ⁸⁰ Se, ⁸² Se, ²⁰⁰ Hg, ²⁰² Hg
Dwell time	0.1 s

Miracle Grow[®] solution was given once every week to all plants as the nutrient base. Three types of supplementation were given to the plants after four weeks of growth, while one set was left untreated as the control (Table 2). Three levels of Se treatment using aqueous Na₂SeO₃ at 60, 120 and 360 mM were applied to the plants. A 45 μM Hg treatment was applied to plants treated with 60 mM Se using aqueous HgCl₂. After supplementation, the plants were carefully watered to prevent any run-off. Overall, the plants grown in the 50:50 mix were healthier than the soil-grown plants. Twelve weeks after sowing, fully grown plants were harvested and washed several times with deionized water. The washed plants were then separated into root, stem, leaf, pods and beans and were frozen immediately in liquid nitrogen and ground into fine powder by mortar and pestle. Plant compartments from plants grown in the same medium and under similar supplementation conditions were combined, lyophilized and stored at -21 °C until analysis.

Analysis of total Se and Hg

A CEM Explorer-Discover microwave system (Matthews, NC, USA) was used to determine the total Se and Hg concentrations in the lyophilized plant compartments. About 0.05 g of ground portions of plant compartments were placed in a glass vial to which 1 mL of 68% HNO₃ was added, and this was allowed to sit in a clean air hood overnight. Vials were closed with septum-sealed caps and subjected to digestion at 150 °C for 10 min by maintaining a pressure below 170 psi. The digested solution was cooled and diluted with deionized water, so that the resulting concentration of HNO₃ was 2%. Yttrium was added as internal standard to each of the extracts analyzed by standard addition with ICP-MS under the conditions indicated in Table 1.

Extraction from plant compartments

About 50 mg of the plant sample were placed in glass vials along with 2 mL of 30 mM TRIS, and this mixture was sonicated overnight in a Fisher Scientific FS30 sonicator (Pittsburgh, PA, USA). To extract low molecular weight compounds, 0.1 M HCl was used as the extractant. The resulting heterogeneous mixtures were centrifuged at 20 °C at a speed of 5,000 rpm in a Sorvall RC-5B from DuPont Instruments (Newtown, CT, USA). The supernatants thus obtained were filtered through 0.25 μm PVDF filters from Agilent Technologies (Hamburg, Germany) and the filtrates were stored at -21 °C until analysis. All samples were used within a fortnight after the extraction.

Isolation of water-soluble proteins was accomplished by adding acetone to the TRIS extract so that the resultant solution was 80% acetone. Total precipitation of suspected proteins was achieved by storing this solution at -21 °C overnight. The resulting pellet was segregated by centrifuging the acetone precipitate at 10,000 rpm. The pellet was redissolved in an appropriate amount of TRIS to maintain a protein concentration similar to its parent solution. This solution was used as a protein isolate.

Microwave-assisted acid hydrolysis of root extract

Protein isolate of Se and Hg treated roots was subjected to microwave-assisted acid hydrolysis using a CEM Explorer-Discover unit. During digestion, the microwave was operated under maximum power while the pressure was maintained at 200 psi. A given amount of root extract was treated with varying concentrations of HCl (0.001–1.5 M) and digested in pressure-sealed microwave vials. Different temperatures were applied for various hold times to the samples to facilitate the disintegration of the Se-Hg entity

Table 2 Total amounts of Se and Hg in various plant compartments of samples grown under different treatment conditions (% RSD for *n*=3 are given)

Amount of supplementation	Amounts found in different plant compartments				
	Root [mM] (% RSD)	Stem [mM] (% RSD)	Leaf [mM] (% RSD)	Pods [mM] (% RSD)	Beans [mM] (% RSD)
Se in control plants	0.028 (5.8)	0.011 (4.7)	0.005 (6.6)	0.010 (3.4)	0.011 (3.8)
60 mM Se	3.962 (0.7)	0.887 (1.2)	2.000 (0.9)	0.262 (3.9)	0.055 (2.2)
45 μM Hg*	1.65 (0.4)	0.135 (1.7)	0.215 (1.0)	0.006 (5.3)	0.002 (9.5)
120 mM Se	4.200 (1.4)	1.412 (1.6)	2.250 (1.5)	0.312 (0.2)	0.525 (2.9)
360 mM Se	13.75 (4.5)	4.537 (1.0)	9.212 (2.5)	0.537 (2.8)	1.812 (2.5)

*Hg was administered only to these plants; the concentrations of Hg were below the method detection limit for all other supplementation conditions

observed in size exclusion studies at the dead volume. All acid-hydrolyzed samples were neutralized before analysis.

Results and discussion

Total metal analysis

All of the compartments from plant samples that were grown in both soil and 50/50 mix were analyzed for Se and Hg uptake. The plant compartments of root, stem, leaf, pod and soybean seed were evaluated for total amounts of Se and Hg (Table 2) accumulated under different supplementation conditions. The amount of natural Se present in the control plant samples was found to be at least 3.5×10^3 times higher than the LOD of Se (1.4×10^{-6} mM). When plants were treated with Na_2SeO_3 there was a significant accumulation of Se in different parts of the plant. This amount accumulated was in direct proportion to the concentration of Se supplemented. In the plants, the maximum amount of Se was found to occur in the roots. Lower Se concentrations were found in the aerial parts of the plant; among these parts, the highest amount of Se was found to occur in the leaf, followed by the stem, the beans and the pods. Overall, based on the total amount of Se present in all aerial parts of the plant, the soybean plant can be categorized as a selenium accumulator, since it accumulates over 100 mg of Se/kg dry weight of the plant [14].

The Hg concentrations in plants that were not supplemented with Hg were below the method's detection limit (LOD: 3.7×10^{-7} mM). It was noted that plants that were treated with Hg and Se exhibited Hg distribution profiles that followed the Se profile, except in the beans (Table 2). Unlike Se, the relative amount of Hg was higher in the pods than in the seed. Consequently, the highest concentration of Hg was found in the roots, followed by the leaf, the stem, the pods and the seeds (Table 2). Moreover, the ratio of Hg transported to aerial parts of the plant was much smaller than for Se. Relative to the amount of supplementation, the amount of Hg accumulated in the root was found to be higher than the amount of Se accumulated. This indicated that most of the Hg was sequestered in the roots, which could be due to a protective mechanism in the plant. Likewise, the amount of Se that was present in the roots, relative to the amount supplemented, was higher than for other cases of supplementation, indicating a possible role of Hg in such behavior. In plants supplemented with 60 mM Se along with Hg, the amount of Se accumulated was almost the same as 120 mM supplemented plants. This is contrary to the previous observation where a direct proportionality was seen between the amount of Se accumulated and that supplemented when plants were

treated with Se alone. All of these observations indicate the presence of antagonistic/synergistic effects between Se and Hg. To further explore Se/Hg interactions at the molecular level, chromatographic studies on extracts of various plant compartments were conducted, with greater focus on the roots of Se/Hg-treated plants.

Chromatographic studies

SEC–UV–ICP–MS

TRIS–HCl extracts of various plant compartments grown under all supplementation conditions were subjected to SEC in order to investigate the translocation of the supplemented elements within the plants. Simultaneous on-line UV detection at 280 nm and ICP–MS with Hg and Se monitoring was performed to gain insights to the molecular nature of the plant components that incorporate the supplemented elements. Elution of the plant extracts under the influence of 30 mM TRIS at pH 7.5 revealed that the control plant compartments do not present any significant Se or Hg species.

The roots of the plants enriched with Se and Hg showed both elements appearing as high-MW entities; this MW was either the same as the column's upper limit (660 kDa) or greater. This suggests that they associate with a compound heavier than 660 kDa, while an overlap at 280 nm suggests that this high molecular weight species may be associated with some protein, although the species itself is not simply a protein because its MW is too high (Fig. 1a). Some Se was also found in the molecular weight region between 6 and 1.3 kDa. For the stems, leaves and pods (Fig. 1b–d, respectively), it was noticed that a minor amount of Se was seen at the void volume, while the majority of the Se was associated with compounds of molecular weight <6 kDa, suggesting that these may be peptides, amino acids and/or inorganic forms. However, the Se was distributed across the entire molecular weight range in the beans (Fig. 1e). These observations suggest the translocation of water-soluble Se species from the roots to the aerial parts of the plant. In contrast, no significant amounts of Hg were found in the water-soluble fractions of any other plant compartments, except for the high molecular weight region of the root extract.

A feeble peak from Hg was observed in the dead volume with simultaneous elution of Se in the stems, leaves, pods and beans (Fig. 1b–e). However, since all of the Hg eluted was in the dead volume, attempts to break down the Hg complex in order to investigate its association with Se were performed. Since the root extract had the highest concentration of Hg, it was used to investigate these interactions. However, it is important to note that the extraction efficiency of the TRIS extract of the Se and Hg treated

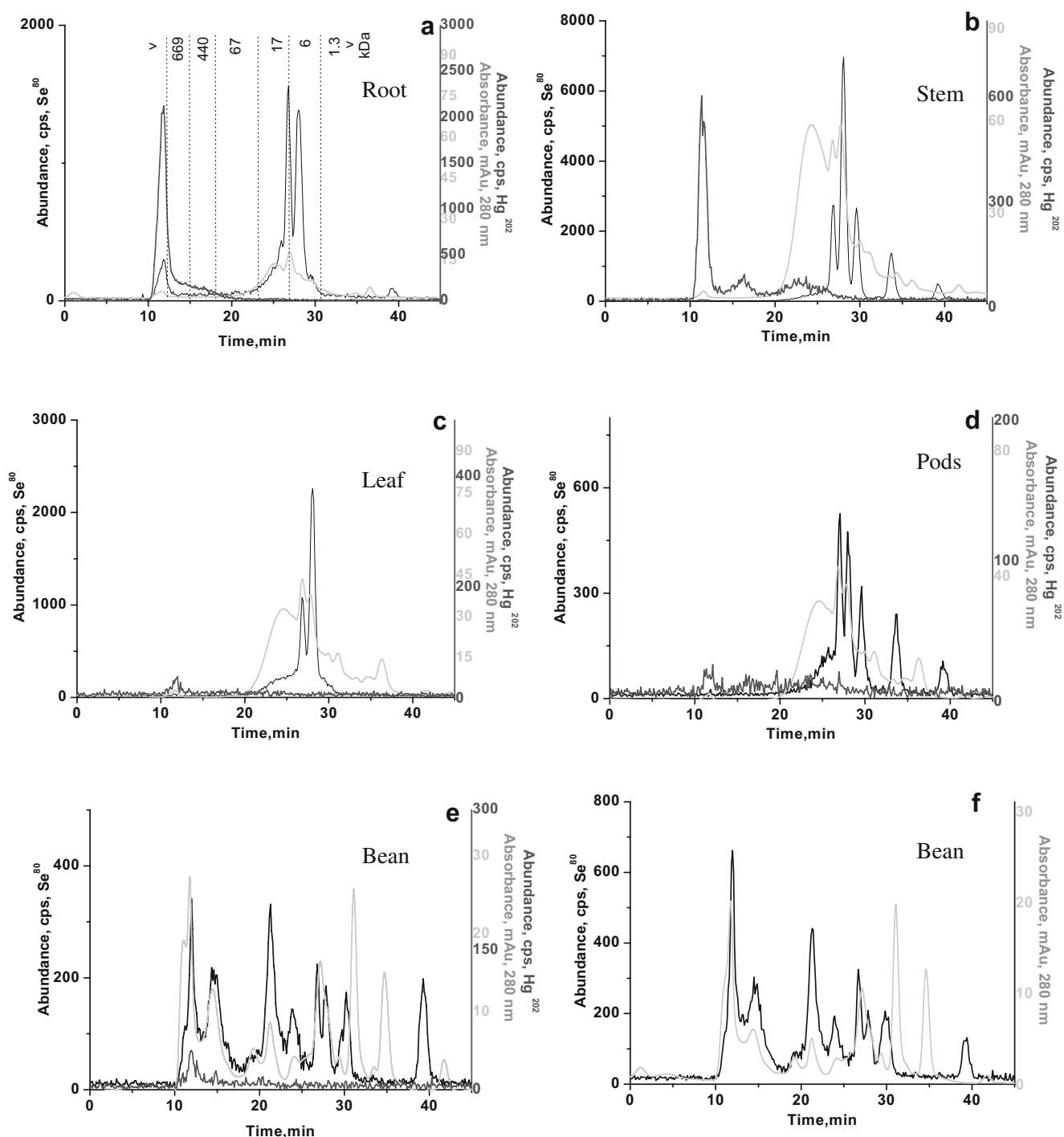


Fig. 1 SEC-ICP-MS chromatograms of plant compartments of *Glycine max* enriched with 60mM Se (IV) and 45 μM Hg(II) (a–e), and of 360 mM Se(IV)-supplemented bean extract (f). The molecular weight markers shown in a apply to all other SEC figures. Data

markers: black lines, ^{80}Se ; dark gray lines, ^{202}Hg ; light gray lines, UV absorbance at 280 nm. For run conditions see *Chromatography A* in Table 1

roots was 40% for Se and 33% for Hg. Such low extraction efficiency values indicate the association of the majority of the Se and Hg with water-insoluble components of the root.

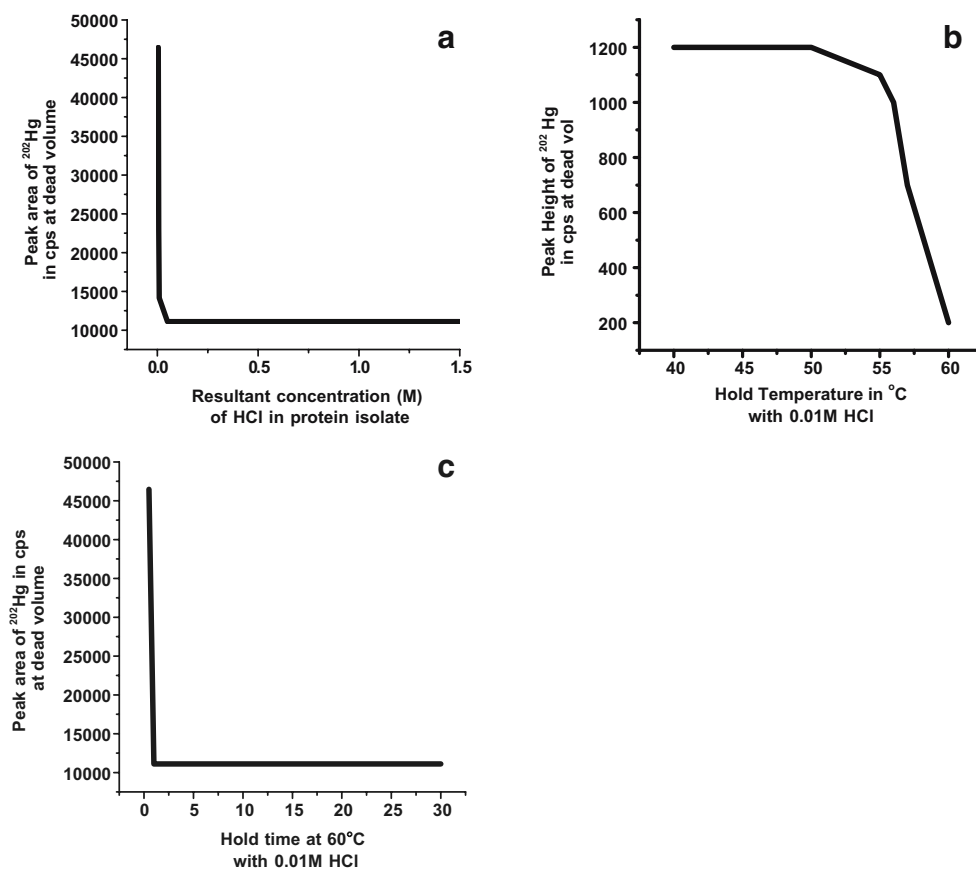
The distribution of Se in the roots, stems, leaves, pods and beans of Se-treated plants was found to be similar to that of the Se in the Se and Hg treated plants. An example

of this can be seen in the Se profile for beans from 360 mM Se-supplemented plants (Fig. 1f). Moreover, the UV absorbance at 280 nm in this extract indicates a possible association of Se with proteins. It would be interesting to extend this study to assess whether the association involves specific or nonspecific binding of Se with soybean proteins.

Hydrolysis of the Se–Hg complex

The coelution of Hg and Se in the void volume of the root extract (Fig. 1a) does not help us to identify their molecular forms and/or to explore their coexistence in a macromolecule. Since Hg in animals has been suggested [10] (but not proven) to associate with Se-containing proteins, the proteinaceous components were isolated from the root extract. The protein isolate showed the presence of Se along with Hg in the void volume during SEC runs, as seen in the whole root extract (Fig. 1a). The presence of UV absorbance at 280 nm leads us to further speculate that a protein might be involved in the large Se/Hg entity (Fig. 3a). To evaluate whether both of the elements were associated with the same molecule, enzymatic digestion was performed on the protein isolate. Trypsin and proteinase K were used separately to perform proteolysis, following a procedure similar to that used in our earlier study [12]. However, neither enzyme could break down the high MW species at void volume sufficiently to allow any conclusions to be drawn about the coexistence of Se and Hg in a molecule (data not included). The reason for the inactivity of the enzymes toward the complex can be explained based on previous observations which suggest that thio amino acids at the active sites of enzymes are inactivated by inorganic Hg [15, 16] or that the protein portion is not a significant component of the overall entity.

Fig. 2 Optimization of microwave conditions for acid hydrolysis of Se and Hg complex with respect to acid concentration (a), hold temperature (b), and hold time (c). Neutralized samples were analyzed by *Chromatography A* approach (see Table 1)



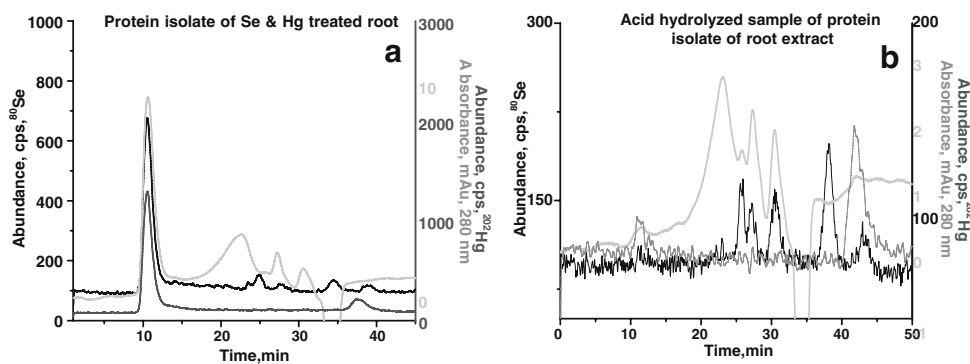
Due to the shortcomings of the enzymatic digestion of the Hg-containing species, protein breakdown via mild, nonspecific methods were explored. Amino acid analysis of proteins by acid hydrolysis with 6 M HCl is a widely known technique [17]. Acid hydrolysis at a quarter of this concentration of HCl was used as a starting point for the digestion of the protein isolate under study. In order to obtain effective control over the temperature and faster performance, microwave-assisted hydrolysis was performed. Different parameters were varied to ascertain the mildest possible conditions for hydrolysis of the Se and Hg peak at void volume. This was assessed by monitoring the peak areas or heights of Hg in the void volumes of the SEC chromatograms of the hydrolyzed samples. All of the acid-hydrolyzed samples were neutralized before being introduced for SEC analysis, and the resulting dilution effects were taken into account.

Optimization of variables for acid hydrolysis

Concentration of acid

One of the most important parameters that needs to be monitored is the amount of acid used for the hydrolysis. HCl at various concentrations in the range of 0.001 to 1.5 M HCl was applied to the protein extract and the mixture was then

Fig. 3 **a** Protein isolate of soybean root extract. **b** Microwave-assisted acid-hydrolyzed extract obtained under ideal hydrolysis conditions. *Chromatography A* (see Table 1) with 0.1% 2-mercaptoethanol in the mobile phase was employed



subjected to microwave digestion at 100 °C for 30 min at maximum power. It was noticed that 0.01 M HCl was the minimum concentration required to cause the dissociation of the high molecular weight species to which the Hg was bound (Fig. 2a). A similar trend was also observed in the case of Se and also for the UV peak (280 nm), which showed a decrease in intensity in the void volume, suggesting that the high MW species was breaking down. Therefore, 0.01 M HCl was chosen as the concentration of acid required for hydrolysis.

Temperature of hydrolysis

The ideal temperature for microwave-assisted digestion with 0.01 M HCl was studied by varying the temperature used during hydrolysis between 40–100 °C, at increments of 10 °C except between 50–60 °C, where 2 °C increments were employed (Fig. 2b). This study showed that at a temperature of 60 °C results in the complete disappearance of the Hg, Se and UV peaks in the dead volume. As the

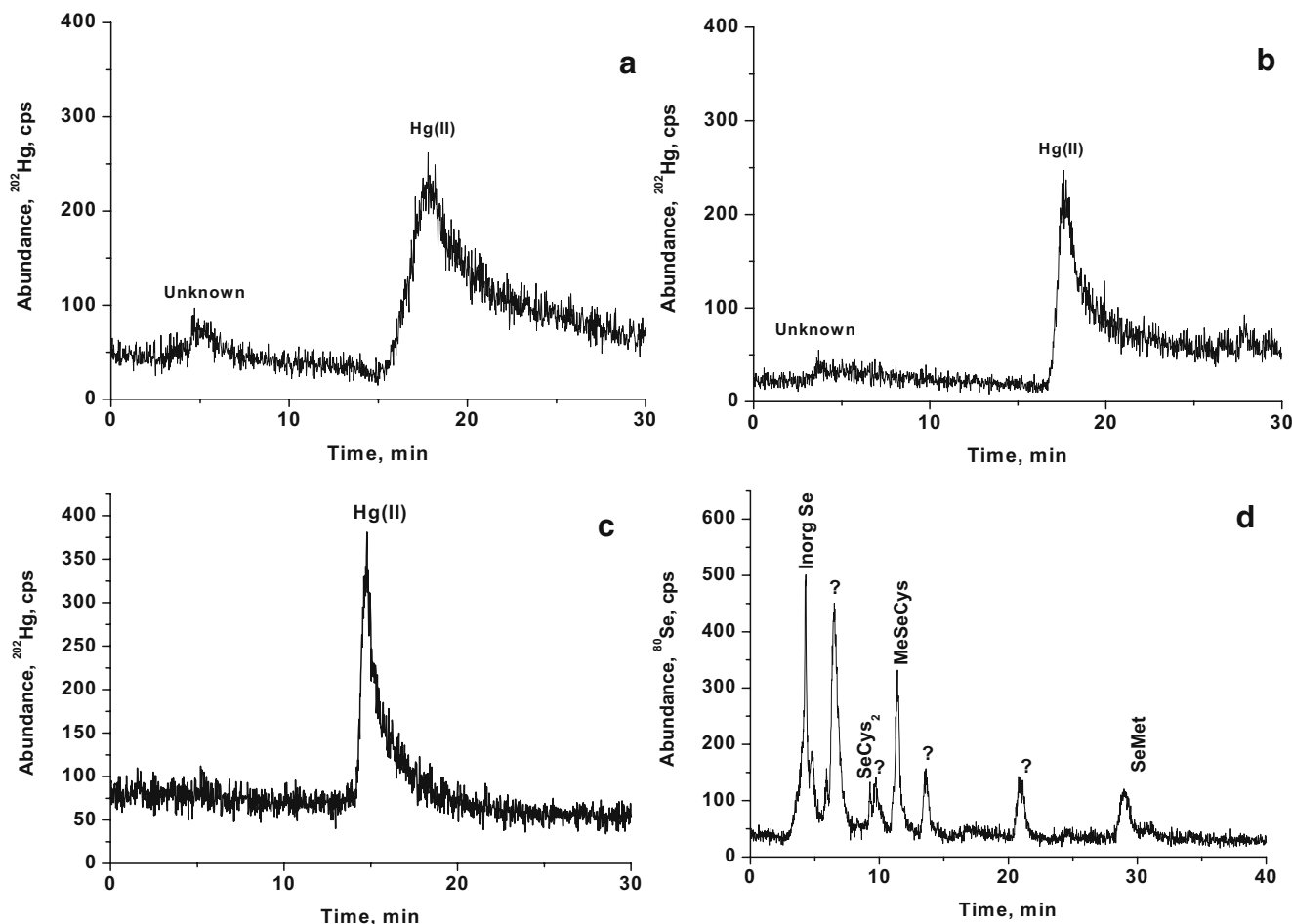


Fig. 4 IP-RP-LC-ICPMS of **a** the TRIS extract of the roots, **b** the protein isolate of the roots, **c** the acid-hydrolyzed protein digest, and **d** the HCl extract of the Se- and Hg-supplemented root. Chromatographic conditions were as listed in Table 1 for *Chromatography B*

temperature dropped below 60 °C, partial breakdown of the high MW species was observed, which decreased in proportion to the temperature until 52 °C was reached. Below this temperature, the entity remained virtually intact. A minimum temperature of 60 °C therefore seems to be essential for the successful hydrolysis of the high molecular weight entity.

Hydrolysis time

The time required for hydrolysis was optimized between a hold time of 30 min and 0 min, while the ramp time was held constant at 1 min. A ramp time of 1 min with no hold time was found to be sufficient to hydrolyze the complex with 0.01 M HCl at 60 °C (Fig. 2c). Lower ramp times were not investigated.

In two different experiments, the protein isolate of the root extract was treated with either acid or microwave digestion alone. Interestingly, in both cases the complex stayed intact, indicating the importance of using both of these factors during the hydrolysis of the complex.

SEC of the hydrolyzed samples

Acid hydrolysis of protein isolate was thus performed using 0.01 M HCl at 60 °C with a ramp time of 1 min. TRIS elution of acid-hydrolyzed samples resulted in the disappearance of the Hg and Se peaks in the void volume (Fig. 3b). Indeed, no Hg peak was observed at all, across all the molecular weight regions. Flow injection analysis of the acid-hydrolyzed extract showed the presence of the same amount of Hg in the acid-hydrolyzed sample as seen for the protein extract, implying that TRIS is incapable of eluting the hydrolyzed Hg species. It was therefore necessary to use a new mobile phase capable of complexing with Hg. A 0.1% v/v 2-mercaptoethanol was added to the 30 mM TRIS mobile phase. In this case, the acid-hydrolyzed sample showed the elution of Hg in the low molecular weight region and no other Hg peak (Fig. 3b). Re-runs of the protein isolate (Fig. 3a) from the root then showed the presence of a small amount of Hg in the low molecular weight region along with the peak at dead volume under the influence of 2-mercaptoethanol. The retention time (t_r) of the Hg(II) standard matched that of the Hg peak in the low molecular weight region of the root extracts. Comparison of the normalized peak areas for the Hg in the protein isolate and the hydrolyzed samples revealed that the Hg peak in the hydrolyzed sample exhibited poor chromatographic recovery (<10%). This implies that the breakdown product of the Hg complex in the void volume may be too hydrophobic to be eluted. It must be noted that this kind of heavy metal behavior, especially from Hg, is not unusual [18, 19]. Other chromatographic methods might be more useful for analyzing the hydrolyzed Hg species of the root extract.

IP–RP–ICP–MS

Further analysis of the protein isolate and its hydrolysate was performed by chromatography using 60 mM ammonium acetate and 0.1% v/v 2-mercaptoethanol in 5% methanol. Under these conditions, t_r of the Hg(II) standard was 16 min. When the whole root extract was analyzed, an unidentified minor mercury peak was observed in the void volume and another peak that matched the t_r of Hg(II) was observed (Fig. 4a). No other Hg peaks were observed under the influence of this mobile phase, even after an hour. The protein isolate showed the presence of a very small Hg peak in the void volume and another peak corresponding to Hg (II) (Fig. 4b). Finally, the acid hydrolysate showed only one peak corresponding to t_r of Hg(II) (Fig. 3c). These observations suggest that the Hg species eluting in the void volume of SEC and that observed in the void volume during IP–RP could be the same. Considering its behavior on the reverse phase column, it can be inferred that the Hg complex is uncharged or that the charge is not sufficiently accessible for any retention mechanism to be displayed by this complex. However, upon comparing the peak intensities of Hg in the SEC and IP chromatograms, it can be concluded that only ~20% recovery of Hg was obtained using IP–RP–LC (compare Fig. 3a and 4b). This suggests that IP–RP–LC, under the given conditions, may not be the correct choice for eluting the Hg complex in the root extract. Se species were also not well-resolved by this mode of separation.

The extension of the reversed phase studies to small Se species in the plant extracts aids our understanding of the biochemical pathway of Se. Generally, C-8 columns are employed with perfluorinated ion-pair reagents like trifluoroacetic acid or heptafluorobutyric acid used as mobile phases in order to study small Se compounds [20]. This study employed 0.1% HFBA with 2.5% MeOH as the mobile phase. To selectively release small Se molecules from the roots, HCl extraction was performed.

Under the given experimental conditions, a set of Se standards in our laboratory were analyzed. The HCl extract from the roots was investigated by retention time matching and the standard addition method (Fig. 4d). The majority of the Se in the root extract occurred in its inorganic form. Selenomethyl selenocysteine (MeSeCys) was the next most abundant species found in the root extract. A trace amount of selenomethionine (SeMet) was found at t_r 28 min. A minor amount of selenocystine (SeCys₂) was found, which eluted close to an unknown peak at ~8 min. However, further confirmation of the presence of SeCys₂ requires the application of alternate chromatographic methods. A major Se species at 7.2 min was found, which did not match with the t_r values of any of the standards available. Some minor peaks were also observed whose identities could not be

established within the limitations of this study. The existence of small quantities of SeMet indicate that it may be converted to other species like MeSeMet, which is the precursor of volatile forms of Se. SeCys₂ is the dimerized product of selenocysteine (SeCys) that contains Se–Se linkages, which are usually involved in the formation of selenoproteins [21]. Since the presence of selenium in protein was seen in the protein isolate of the root extract, it is possible that SeCys linked by amide bonds is present in these proteins. The presence of high amounts of MeSeCys could be due to the volatilization of Se from root extract. This compound can be cleaved to form volatile Se species such as dimethylselenide and dimethyldiselenide. No Hg species were found in the above chromatograms. Flow injection analysis, however, reveals the presence of Hg, which implies an inability of the mercury species to elute under the influence of this mobile phase.

Conclusions

Hg is bound to a macromolecule and is sequestered in the roots of soybean plants, as observed in Indian mustard (*B. juncea*) in a previous study. The macromolecule binding to Hg might include bonding through a protein and it seems to be specific to the root. Enzymatic digestion did not prove to be a fruitful approach to dissociating this macromolecule. Microwave-assisted acid hydrolysis was successfully implemented for the complete breakdown of the macromolecule that binds with Se and Hg. Although this study is the first to report total dissociation of the Se and/or Hg complex in plants, further analysis is required before the macromolecule can be successfully characterized, which can help to illuminate the specific influence of Se in the binding with Hg. Addition of 2-mercaptoethanol was necessary to complex Hg(II) during all aqueous chromatography. However, the Hg in the hydrolyzed sample could not be eluted by either SEC or RP chromatographic methods, suggesting that alternative, probably hydrophobic, chromatographic methods should be attempted. Se was transported to all parts of the plant; moreover, the distribution pattern and its molecular transformation did not seem to be affected by the presence of Hg. However, a higher amount of Se was assimilated in plants that were treated with Hg

compared to plants treated purely with Se, indicating a possible protective response of plant to Hg. Most of the Se in the root extract was found in the low molecular weight region, which was later found to include inorganic Se using ion-pair chromatographic studies. A few Se species like SeMet and MeSeCys, along with some unknown compounds, were also found in the root extract.

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