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Exclusion of Selenium from Proteins of Selenium-Tolerant *Astragalus* Species[1]

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ABSTRACT

Protein fractions from three selenium-tolerant and three selenium-sensitive *Astragalus* species, grown in the presence of [^{75}Se]selenate, were analyzed for their selenium content. Though tolerant species are known to accumulate considerably more selenium than do sensitive plants, protein fractions from the three selenium accumulators were found to contain significantly less selenium (0.46 to 0.57 picomoles selenium per milligram protein) than did protein fractions from the three nonaccumulators (4.17 to 5.02 picomoles selenium per milligram protein). Under similar conditions, seedlings of *Vigna radiata* (L.) Wilczek had taken up selenium (6.31 picomoles selenium per milligram protein) at levels comparable to those observed in the proteins of the nonaccumulator *Astragali*. These results establish that the ability to tolerate and to circumvent the toxic effects of selenium, characteristic of the accumulator species of *Astragalus*, is associated with a reduced incorporation of this element into protein.

Selenium tolerance, characteristic of several plant species, is associated with the accumulation of high levels of this element without toxic effects (for review see ref. 11). Tolerance in these accumulator plants is due, conceivably, to exclusion of selenium from proteins, inasmuch as species sensitive to low levels of the element are known to synthesize and incorporate both selenomethionine (4, 8) and selenocysteine (2) into their polypeptides. Exclusion of selenium from proteins would reduce toxic effects that ordinarily result from the synthesis of selenium-containing polypeptides with their altered chemical and biological properties. This exclusion hypothesis is supported by the observation that protein-bound selenium was absent from the accumulator *Neptunia amplexicaulis*, grown in the presence of selenite (9). Exclusion, as an explanation for reduced toxic effects, was also suggested by the data from a comparative study of sensitive and tolerant plants in which less selenium was detected in the proteins of the accumulator (7).

To assess the validity of an exclusion hypothesis, selenium levels in the proteins of selenate-grown accumulator and nonaccumulator species of *Astragalus*, as well as in *Vigna radiata* (L.) Wilczek, were surveyed.

MATERIALS AND METHODS

Plant Species and Growth Conditions. The species of *Astragalus* that were studied are listed in Table I; the selenium-sensitive *Vigna radiata* (mung bean), obtained from a local health food outlet, was also included. Seeds were sterilized by soaking for 20 min in a 1:1 mixture of 3% (v/v) H_2O_2 and 95% (v/v) ethanol; *Astragalus* species were then scarified in concentrated H_2SO_4 for 20 min. After thorough washing in sterile distilled H_2O, seeds were germinated on filter paper discs in Petri dishes. The discs were soaked in a nutrient solution that consisted of (per liter of distilled H_2O): $CaNO_3 \cdot 4H_2O$, 0.95 g; KNO_3, 0.61 g; $MgSO_4 \cdot 7H_2O$, 0.30 g; $NH_4H_2PO_4$, 0.12 g; ferric tartrate, 0.005 g. Germinated seedlings were suspended so that root tips were immersed in 30 ml of nutrient solution that contained 50 μl $Na_2{}^{75}SeO_4$ (Amersham Corp.; 412.5 mCi/mmol) and were grown for an additional 4 days at room temperature under constant light (21 to 22 klux at plant tops).

Preparation of Protein Fraction. Selenium-labeled seedlings were weighed and homogenized in an Omni-Mixer (Ivan Sorvall, Inc., Newtown, CT) with 1.5 ml extraction buffer/g of plant material. The extraction buffer consisted of 100 mM Tris-HCl (pH 8.6), 20 mM $MgCl_2 \cdot 6H_2O$, 10% (w/v) glycerol, and 25 mM β-mercaptoethanol; the β-mercaptoethanol was added to the buffer immediately prior to use. Cell debris was removed by centrifugation of the homogenate at 8,000*g* for 10 min. The supernate was centrifuged at 30,000*g* for a further 10 min to pellet subcellular particles. Protein in the second supernate was precipitated by addition of 350 mg $(NH_4)_2SO_4$/ml supernate and collected by centrifugation at 15,000*g* for 10 min. This crude protein fraction was redissolved in a minimum amount of extraction buffer, reprecipitated with $(NH_4)_2SO_4$ and again collected by centrifugation at 15,000*g* for 10 min.

To ensure removal of low molecular weight material, the second protein precipitate was purified by dialysis. Breakdown of selenocysteinyl residues and loss of protein-bound ^{75}Se during this treatment was prevented by carboxymethylation of the protein fraction. Fifty mg of protein were dissolved in 3 ml of Tris-HCl (pH 8.6) to which were added 15 mg EDTA, 3.61 mg recrystallized urea, 0.1 ml β-mercaptoethanol, and 4.1 ml distilled H_2O. This denaturation-reduction mixture was adjusted to 12 ml with a solution of 8 M recrystallized urea that contained EDTA (2 mg/ml), and incubated at room temperature for 4 h under an atmosphere of N_2. The denatured, reduced protein was then carboxymethylated by addition of 1.0 ml of a solution that contained 268 mg iodoacetic acid/ml of 1 N NaOH. After incubation at room temperature for 15 min under N_2, the mixture was dialyzed at 4 C against distilled H_2O that was frequently changed over a 3-day period.

Radioactive selenium in the dialyzed extract was determined with a Packard Auto-Gamma scintillation spectrometer. The energy of the gamma ray can be counted directly without the liquid scintillation cocktail necessary for beta counting; no correction for decay was necessary. Protein was assayed by the Lowry method (6).

Deproteinization of Protein Fraction. Since ammonium sulfate precipitation is not entirely specific for protein, the possible asso-

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Table I. *Species of Astragalus Used in This Study and Place of Collection*

Accumulators[a]

A. bisculatus Gray; Laramie, WY; 1961
A. crotalariae Gray; Indio, CA; 1976
A. beathii Porter; gift from Professor S. F. Trelease, formerly of Columbia University, New York City (location of collection unknown)

Nonaccumulators[a]

A. lentiginosus Dougl.; Palm Desert, CA; 1976
A. douglasii Gray; San Bernadino, CA; 1964
A. palmeri Gray; Alpine Village, CA; 1963

[a] For a full description of these species see ref. 1.

Table II. *Incorporation of ^{75}Se Into Proteins of Selenium-Sensitive and Selenium-Tolerant Species of Astragalus*

Seedlings were grown for four days in the presence of [^{75}Se]selenate and a protein fraction prepared by ammonium sulfate precipitation as described.

Species[a]	^{75}Se Content of Protein Fraction
	pmol Se/mg protein[b]
Accumulators	
A. bisulcatus	0.57
A. crotalariae	0.47
A. beathii	0.46
Nonaccumulators	
A. lentiginosus	4.17
A. douglasii	4.65
A. palmeri	5.02

[a] *Vigna radiata,* a selenium-sensitive plant, incorporated 6.31 pmol Se/mg protein, an amount similar to that taken up by the nonaccumulator *Astragalus* species. *V. radiata* (formerly *Phaseolus aureus* [l.] Roxb) is not listed among selenium accumulating plants (10); it is often used as a representative selenium-sensitive species (2, 3, 5).

[b] Figures are the average of at least three individual determinations.

ciation of ^{75}Se with nonprotein contaminants in the dialysate was investigated. The dialysate was deproteinized by addition of an equal volume of 95% (v/v) water-saturated phenol followed by vigorous stirring for 1 h. Layers were separated by centrifugation at 12,000*g* for 20 min, and the upper aqueous (nonprotein) layer was removed and assayed for ^{75}Se.

RESULTS

To compare the amount of selenium bound to proteins of selenium-tolerant and selenium-sensitive plants, labeled seedlings were homogenized and particulate material removed by centrifugation. Between 50 and 60% of the selenium absorbed by tolerant as well as by sensitive plants was recovered in the cell-free supernate. Virtually all insoluble selenium was removed by the low-speed (8,000*g*) centrifugation.

A protein fraction was prepared from the cell-free supernate by ammonium sulfate precipitation and immediately carboxymethylated to prevent the possible loss of protein-bound ^{75}Se by a breakdown of selenocysteinyl residues. The carboxymethylated fraction was dialyzed and the ^{75}Se content determined with the gamma spectrometer (Table II). Significant differences between the amounts of selenium associated with proteins of accumulator and sensitive species were evident. Accumulator species possessed between 0.46 and 0.57 pmol Se/mg protein, whereas the selenium content of the sensitive nonaccumulator plants ranged from 4.17 to 6.31 pmol Se/mg protein.

Inasmuch as the ammonium sulfate precipitates may have contained small amounts of nucleic acids and polysaccharides, dialysates were deproteinized by phenol treatment to determine if any selenium had become bound to these nonprotein substances. In all species examined, less than 4% of the ^{75}Se in the dialysate was recoverable in the aqueous layer after phenol treatment. The major selenium-containing component of the dialysate, therefore, was protein.

DISCUSSION

Within the genus *Astragalus,* two groups are recognized with respect to selenium sensitivity. A small number of species accumulates relatively large amounts of selenium and they are insensitive to the element; growth of the other species is inhibited by even very low levels (20 μg/g) of selenium (12). In the experiments described here, the striking and consistently lower amount of selenium incorporated into proteins by accumulators provides convincing evidence that selenium-tolerant species of *Astragalus* have evolved a mechanism whereby they exclude selenium from their polypeptides.

There are three possible ways by which such an exclusion could occur. In accumulators, the initial steps of selenium metabolism may not result in synthesis of the selenoamino acids which, in sensitive plants, ordinarily would be incorporated into proteins. This possibility is supported by the identification of a variety of nonprotein selenoamino acids, such as Se-methylselenocysteine, which are synthesized by a number of the selenium-tolerant species (for review see ref. 11). Synthesis of these compounds would perhaps divert selenium from the formation of selenocysteine and selenomethionine. Also to be considered is a possible discrimination against selenium compounds during protein synthesis itself. Evidence for such a discrimination against selenocytsteine is provided by experiments which demonstrated that the cysteinyl-tRNA synthetase of the accumulator *A. bisculatus* was unable to use the selenium analog as a substrate (3). Crude polysome preparations from both accumulator and nonaccumulator species seem to incorporate selenomethionine into polypeptide chains during elongation (5). A third possibility, whereby the selenium content of accumulator proteins might be reduced, is enzymic removal of selenium from preformed proteins. Such a reaction would be comparable to the posttranslational deamination of glutamine and asparagine residues to form, respectively, glutamic and aspartic acids (for review see ref. 13).

Synthesis of proteins in which cysteine is replaced by the selenium analog, selenocysteine, may be a major cause of the harmful effect of selenium on sensitive plant species (2). Exclusion of selenium from polypeptides will, therefore, restrict the toxic effects of this element by reducing synthesis of these dysfunctional selenium-substituted proteins.

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