Identification of Selenocysteine in the Proteins of Selenate-grown

**Vigna radiata**

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**ABSTRACT**

Selenocysteine, the selenium analog of cysteine, was identified in proteins of *Vigna radiata* (L.) Wilczak grown with selenate. To stabilize selenocysteine and prevent its breakdown, the carboxymethyl derivative was synthesized by the addition of iodoacetic acid to the protein extract from **Se**-selenate-grown plants. A **Se**-labeled component of the carboxymethylated protein hydrolysed possessed chromatographic properties identical to those of a **C**-labeled carboxymethylselenocysteine standard during paper and thin layer chromatography and during gel-exclusion, anion-exchange, and cation-exchange column chromatography. Detection of selenocysteine in proteins of a selenium-sensitive plant, and the possibility that the presence of this compound alters normal functions, provides an explanation for the toxic effects of selenium.

The widespread toxic effects of selenium compounds have been attributed to the incorporation of selenocysteine and selenomethionine into polypeptides, with resultant structural alterations believed to affect protein function. Substitution of selenomethionine for methionine does not always prove detrimental; over one half of the 150 methionine residues present in the enzyme beta-galactosidase could be replaced by the selenium analog without deleterious effect on enzyme activity (5).

Cysteine, however, unlike methionine, plays a critical structural role in proteins by virtue of disulfide bridge formation between residues in adjacent stretches of the same or different polypeptide chains. Replacement of cysteine by selenocysteine could disrupt protein function through substitution of selenium for the sulfur in these disulfide bridges. Therefore, it becomes essential to determine whether selenocysteine is actually able to enter the polypeptide chain. Although synthesis of selenocysteine by pea chloroplasts has been demonstrated (9, 10), the instability of this compound has hindered efforts to detect it in polypeptides (for review see ref. 13). Several attempts to identify selenocysteine in protein hydrolysates have been unsuccessful (2, 11, 12); other reports, in which detection was claimed, have been questioned (18).

To ascertain whether selenocysteine could be identified in the proteins of a selenium-sensitive plant, experiments were initiated with *Vigna radiata*, grown in the presence of selenate. The instability of selenocysteine was circumvented by incubation of protein extracts with iodoacetic acid prior to hydrolysis, treatment that causes formation of the stable carboxymethyl derivative of this amino acid. The results establish the presence of selenocysteine in polypeptides of selenate-grown *V. radiata*; selenium toxicity may be attributed to formation of dysfunctional proteins in which cysteine has been replaced by selenocysteine.

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**MATERIALS AND METHODS**

Plant Material and Culture Conditions. Seeds of *V. radiata* (L.) Wilczak (mung bean, formerly *Phaseolus aureus* [L.] Roxb.) were germinated in Perlite and grown at 30 C under constant light (21 to 22 klx at the plant tops). The nutrient solution was a low-sulfur Hoagland medium that consisted of (per liter distilled H2O): 0.95 g Ca(NO3)2·4H2O; 0.61 g KNO3; 0.30 g MgSO4·7H2O; 0.12 g NH4H2PO4; 0.005 g ferric tartrate. When the first leaves appeared, cultures were supplemented with 25 ml of nutrient solution that contained 50 μCi sodium **Se**selenate (Amersham/Searle; specific radioactivity, 294.6 mCi/mmoll, and the seedlings then grown for an additional 3 days.

Preparation of Carboxymethylated Protein Hydrolysate. Leaves and roots of **Se**-labeled plants were excised, weighed, and homogenized in an Omni-Mixer (Ivan Sorvall, Inc., Newton, Conn.) with 1.5 ml extraction buffer/g plant material. This extraction buffer contained 100 mm Tris-HCl (pH 8.6), 20 mm MgCl2·6H2O, 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 8,000g for 10 min, and the supernatant was collected. Protein was precipitated by addition of 350 mm (NH4)2SO4/ml supernatant and pelleted by centrifugation at 15,000g for 10 min. This crude protein fraction was redissolved in a minimum amount of extraction buffer, reprecipitated with (NH4)2SO4, and collected by centrifugation at 15,000g for 10 min. Protein was denatured, reduced, and carboxymethylated by the technique of Crestfield et al. (3). Fifty mg protein were dissolved in 3 ml 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 water. This denaturation-reduction mixture was adjusted to 12 ml with a solution of 8 m recrystallized urea that contained EDTA (2 mg/ml) and was incubated at room temperature for 4 h under an atmosphere of N2.

Denatured, reduced protein was carboxymethylated with iodoacetic acid. A solution that contained 268 mg iodoacetic acid/ml of 1 n NaOH was added to the protein, and the mixture was incubated at room temperature for 15 min under N2. The reaction was stopped by dialysis at 4 C against distilled H2O that was changed frequently over a 3-day period. Protein in the dialysate was precipitated with 350 mg (NH4)2SO4/ml dialysate, collected by centrifugation, and dried at room temperature under reduced pressure. To hydrolyze protein, 5 mg were suspended in 1 ml 6 N HCl in an ignition tube, and the tube evacuated, sealed, and incubated at 110 C for 48 h.

Preparation of **14C**CMSCys Standard. Ten mg selenocysteine (Sigma) were suspended in 3 ml Tris-HCl (pH 8.6), to which was added 0.1 ml β-mercaptoethanol. Reduction to selenocysteine was carried out at room temperature for 4 h under N2. Selenocysteine

1 Abbreviation: CMSecys, Se-carboxymethylselenocysteine.
was carboxymethylated by addition of 10 μCi iod[2,14]C]acetic acid (Amersham/Searle; specific radioactivity, 54 mCi/mmol) which had been dissolved in 1 ml of 1 N NaOH, then incubated at room temperature for 15 min under N2. The carboxymethylation reaction was stopped by absorbing 1-ml aliquots of the mixture onto a 1.5-× 10-cm DEAE-cellulose (Whatman anion-exchange column equilibrated with 25 mM Na-acetate (pH 5.4). After 40 ml of this equilibration buffer had been passed through the column, the sample was eluted with a 200-ml linear acid gradient from 0 to 1 M. Ten μl of each fraction were placed in 5 ml Bray's cocktail (1), and 14C was assayed in a Packard Tri-Carb liquid scintillation spectrometer. The UV spectrum of each radioactive peak was scanned with a Beckman model 25 spectrophotometer.

Paper Chromatography. A sample of 75Se-labeled protein hydrolysate that contained 5,000 cpm radioactivity was applied with 5,000 cpm [14C]CMSecys to Whatman grade 1 paper strips (2.54 cm wide). Descending chromatography was carried out with the following solvent systems: EBFW, ethanol-1-butanol-88% (v/v) formic acid-H2O (12:4:1:3); PAM, 1-propanol-1.5% NH4OH (7:3); EAW, ethanol-glacial acetic acid-H2O (13:0.2:6.8); BADW(P), 1-butanol-citroone-diethylene-H2O (66:1.2:3); BAM, 2-butanol-4-dioxane-2-propanol-88% (v/v) NH4OH (3:1); BEW, 1-butanol-ethanol-H2O (4:1:1); BPW, 1-butanol-pyridine-H2O (1:1:1).

TLC. A sample that contained 5,000 cpm 14C and 5,000 cpm 75Se was spotted onto cellulose TLC aluminum sheets (EM Laboratories, Elmsford, N. Y.). Samples were separated into components with one of the following solvent systems: BAW, 1-butanol-glacial acetic acid-H2O (4:1:1); PW, H2O-saturated phenol; BADW(T), 1-butanol-citroone-diethylene-H2O (10:10:2.5); PFW, 2-propanol-88% (v/v) formic acid-H2O (4:2:1).

Column Chromatography. A sample that contained 5,000 cpm of both 75Se and 14C was applied to each of four separate chromatography columns.

The gel-exclusion chromatography system used was a 1.5-× 82-cm Sephade G-10 column (Sigma; particle size, 40–120 μm) equilibrated with 25 mM Na-acetate (pH 5.4). The sample was eluted with equilibration buffer.

Cation-exchange was performed with a 1.5-× 10-cm column of AG50W-X8 (Bio-Rad), 200 to 400 mesh, hydrogen form. This column was equilibrated with a citrate-phosphate buffer (pH 2). After application of the sample, 30 ml of citrate-phosphate buffer (pH 2) were passed through the column, which was then eluted with a 100-ml linear gradient (pH 2 to pH 4) of citrate-phosphate buffer.

Two anion-exchange chromatography columns were used. The first was a 1.5-× 10-cm column of DEAE-cellulose (Whatman) equilibrated with 25 mM Na-acetate (pH 5.4). After application of the sample, the column was washed with 40 ml of this buffer and then with a 200-ml linear gradient (0–0.4 M) of acetic acid.

The second anion-exchange system was a 0.9-× 15-cm column of AG1-X8 (Bio-Rad), 200 to 400 mesh, formate form. The column was equilibrated with distilled H2O, the sample applied, and 40 ml distilled H2O were passed through. The sample was then eluted with a 100-ml linear formic acid gradient (0–0.1 M).

Assay of Chromatograms. Paper chromatograms and TLC sheets were cut into 1-cm wide strips which were placed in vials and assayed for 75Se in a Packard Auto-Gamma scintillation spectrometer. Five ml Bray's cocktail (1) were then added to each vial and 14C counted with a Packard Tri-Carb liquid scintillation spectrometer. Fractions from columns were first counted for 75Se, and then 0.5 ml of each fraction was added to 5 ml of scintillant for assay of 14C. Bray's cocktail was used with fractions from Sephade G-10, DEAE-cellulose, and AG1-X8 columns; fractions from the AG50W-X8 column were added to Ready-Solv MP (Beckman). In each assay, the 14C counts were corrected for the contribution made by the β component of the 75Se.

RESULTS

Preparation of [14C]CMSecys Standard. To determine the presence of CMSecys in the protein hydrolysate, it was first necessary to prepare an authentic 14C-labeled CMSecys standard. This standard was separated from other radioactive components of the reactive mixture by DEAE-cellulose column chromatography (Fig. 1). The UV spectrum of fraction 23, characterized by strong A at 220 nm, was identical to that of unreacted ioacetic acid. In contrast, the spectrum of the peak at fraction 2, except for a weak shoulder at about 250 nm, was devoid of any UV absorbance; this spectrum is in agreement with one that has been described for CMSecys (4). Fraction 2, therefore, was used as the [14C]CMSecys standard in all chromatographic procedures.

Paper Chromatography. The authentic [14C]CMSecys standard was added to the 75Se-labeled carboxymethylated protein hydrolysate; samples were resolved by paper chromatography with seven different solvent systems. A 75Se-labeled component of the hydrolysate possessed chromatographic properties identical to the [14C]CMSecys standard in each solvent system tested. A typical example, with solvent system BADW(P), is shown in Figure 2.

TLC. The [14C]CMSecys standard co-chromatographed with a 75Se-labeled component of the protein hydrolysate in each of the four solvent systems used for one-dimensional TLC. Two-dimen-

![Fig. 1. Separation by DEAE-cellulose column chromatography of the products from reaction between selenocysteine and iod[2-14]C]acetic acid. A linear acetic acid gradient (---) 0 to 1 M, was passed through the column; 5 ml fractions were collected and assayed for 14C (•--•). The peak at fraction 2 is [14C]CMSecys.](image1)

![Fig. 2. Paper chromatography of the 75Se-labeled carboxymethylated protein hydrolysate (O--O) and the [14C]CMSecys standard (•--•) with solvent system BADW(P). A coincidence of 75Se- and 14C-labeled peaks is seen at an Rf value of 0.20. Coincidence between the two peaks was also observed with six other solvent systems. Rf values were: EBFW, 0.68; PAM, 0.25; EAW, 0.89; BAM, 0.70; BEW, 0.97; and BPW, 0.72.]
The presence of selenocysteine as an integral component of some mammalian and microbial enzymes is well documented (for review see ref. 14; also 7, 15), and the ability of plants to synthesize this selenoamino acid has been demonstrated (9, 10), its incorporation into the proteins of plants sensitive to selenium has never been established. Yet, evidence for this incorporation, with its possible effects on disulfide bridge formation, is of central importance to an understanding of selenium toxicity. The failure of several attempts to identify selenocysteine in proteins from various organisms (2, 11, 12) is perhaps attributable to breakdown of the compound during the concentrated acid treatment ordinarily used for protein hydrolysis (6). In a number of studies which do report identification of selenocysteine, the conclusions are often based on limited experimental evidence (for review see ref. 13).

Selenocysteine breakdown can be prevented by treatment of protein extracts with iodoacetic acid in order to synthesize the stable carboxymethyl derivatives of selenocysteinyl residues. Through utilization of this technique, it was possible to identify \(^{75}\)Se-labeled CMSecys in protein hydrolysates from \(V.\ radiata\) grown with \([^{75}\)Se]selenate. Conclusive evidence that selenocysteine is incorporated into the proteins of this plant is provided by the observation that a \(^{75}\)Se-labeled component of the protein hydrolysate possessed identical chromatographic properties to the authentic \([^{14}\)C]CMSecys standard in a variety of separation systems.

Selenocysteine and cysteine differ in at least two crucial respects, differences that could seriously affect protein function. The selenium atom is larger than that of sulfur, and the bond between two selenium atoms has been estimated to be approximately one-fifth longer than the equivalent disulfide bond (8). The second difference, a pK value lower for the selenol group than for the sulfhydryl radical, specifies that selenocysteine is ionized at lower pH values than is cysteine (6). These disparities between selenocysteine and cysteine may be responsible for selenium toxicity because of alterations in the relative positions of adjacent polypeptide chains normally linked by disulfide bridges. This change in conformation, associated with replacement of cysteine by selenocysteine, would be expected to have a decisive effect on the substrate-binding properties of a selenium-substituted protein.

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**Literature Cited**

11. Olson OE, EJ Novacek, EJ Whitehead, IS Palmer 1979 Investigations on
selenium in wheat. Phytochemistry 9: 1181–1188
Corrections

Page 758, second paragraph, last sentence: reference number 18 should be number 13.

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Gerhard Sandmann and Peter Böger. Copper-mediated Lipid Peroxidation Processes in Photosynthetic Membranes.
Page 798, Tables I, II, and III: change the units below the box headings from nmol/mg Chl·h to μl/g Chl·h.

Vol. 66: 973–977, 1980
Figure 7 on page 975 and Figure 8 on page 976 are incorrectly transposed.

Vol. 66: 1198–1199, 1980
Page 1198: the negative sign before the first term of equation 1 should be deleted.