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Chemical Examination of Seleniferous Cabbage *Brassica oleracea capitata*

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The water–methanol soluble extract of seleniferous cabbage leaves was separated into several ninhydrin-positive spots or bands using thin-layer chromatography (TLC). The chromatographic behavior, ninhydrin color, and $R_f \times 150$ values of the radioactive spots were compared with TLC spots of chromatographically pure known sulfur or selenium compounds. Radioactive band eluates were co-spotted on TLC plates with selected sulfur or selenium compounds, and the plates subjected to one- or two-dimensional development with different solvent systems for identification purposes. Evidence obtained strongly suggests the presence of these ^{75}Se-containing soluble compounds in seleniferous cabbage: *Se*-methylselenomethionine, selenocystathionine, *Se*-methylselenocysteine selenoxide, selenohomocystine, *Se*-methylselenocysteine, and selenomethionine. Evidence indicating the presence of selenopeptides, selenoproteins, and substituted selenium containing cysteine or cystine type compounds was obtained. The presence or absence of selenocystine in the cabbage extract could not be definitely established.

Cabbage (*Brassica oleracea capitata*) is a common family garden and commercially grown member of the Cruciferae (Mustard) family and is capable of absorbing, metabolizing, and storing in its tissues relatively large quantities of selenium. The available selenium present either as selenate, selenite, or organic compounds is readily absorbed and metabolized. This vegetable is grown in many areas and is consumed by large numbers of people. A strong possibility exists that humans consuming seleniferous cabbage may suffer harmful effects. Sulfur compounds present in cabbage have been extensively studied and many have been identified. The organic selenium containing compounds present in cabbage are of interest but have not been extensively investigated. Knowledge of the relative amounts and chemical identity of the selenium compounds present in cabbage will assist in an understanding of the metabolism of selenium by members of the *Brassica* genus. This information will assist in predicting the harmful effects of ingestion of seleniferous Cruciferae.

MATERIALS AND METHODS

Cabbage plants were grown from seed, in the greenhouse, on black loam soil with selenium added periodically as a dilute acid solution of $H_2{}^{75}SeO_3$. The plants were approximately 2 months of age when green leaves were removed for fractionation. The leaves were rinsed in distilled water, dried, and dipped successively in five portions of hexane to remove epicuticular wax. The hexane portions were combined and these as well as other extracts were reduced in volume in a rotary evaporator utilizing low temperature and reduced pressure. The hexane rinsed leaves were reduced to a finely divided slurry and successively extracted with Bligh and Dyer (1959) reagent until the residue was free of green color and the extract contained little ^{75}Se. The extracts were composited in a separatory funnel, additional water and chloroform were added, and the extract was partitioned into chloroform and water–methanol soluble phases. The amount of radioactivity in the fresh leaves, concentrated rinses or extracts, and the insoluble residues was measured.

Chromatographically pure known sulfur and selenium compounds were obtained from commercial sources or interested scientists and were chromatographed on the same plates as were the extract fractions or were co-spotted with each other or plant fractions. Bands or spots were identified by detection reagents such as ninhydrin, naphthoresorcinol, and sulfuric acid and were removed from the plates and activity measurements made. Selected active bands were removed and eluted, and the eluates chromatographed alone or co-spotted with known compounds. Using the $R_f \times 150$ values, detection reagents, band intensity, and other chromatographic characteristics a tentative identification of the selenium compound or compounds present in that particular band was made.

RESULTS AND DISCUSSION

A summary of solvent fractionation of several samples of cabbage leaves and the ^{75}Se content and percentage of radioactivity present in the components is found in Table I. The amount of radioactivity, 0.14%, present in the hexane wash of the leaf surfaces, soluble epicuticular wax, was less than expected and apparently was a result of nonstress greenhouse conditions. No efforts were made to fractionate the hexane soluble material.

The green chloroform extract contained chlorophyll, lipids, and other compounds and represented 0.38% of the total ^{75}Se. Limited TLC indicated numerous spots or bands and two carried measurable ^{75}Se.

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The water–methanol soluble fraction contained 91.18% of the plant ^{75}Se. The water insoluble plant residue contained 8.18% of the ^{75}Se in contrast to 1.97 and 14.72% of the selenium present in the insoluble residues of *Stanleya* and onion plants (unpublished data and Hamilton, 1975).

The water extract was subjected to TLC and the three solvent systems selected produced a satisfactory spacing of 17–20 bands as indicated by ninhydrin, naphthoresorcinol, or sulfuric acid detection reagents. A summary of the TLC separation of water extract components using these solvent systems is included in Table II. The $R_f \times 150$ solvent values, the indicated radioactive compound responsible for the spot or band activity, and the percentage of total ^{75}Se present in comparable bands of all solvent system chromatograms are shown.

A dense brown band that proved to be ninhydrin positive displayed little mobility in any solvent and contained 1.11% of the total ^{75}Se present in the extract. The ninhydrin positive material apparently contained water soluble selenopeptides and selenoproteins which could be eluted, rechromatographed, or hydrolyzed by enzymes with little loss of radioactivity. Peptides, proteins, and other unidentified compounds contained 13.79% of the total cabbage leaf selenium. Peterson and Butler (1962) reported that the residue after 80% ethanol and hot water extraction contained 2.5 to 6.3% of the total ^{75}Se present in young *Neptunia* plants and 61.5% in red clover foliage. Selenium containing peptides have been identified in accumulator species of *Astragalus* and *Stanleya* by Shrift and Virupaksha (1965). Selenium is incorporated into wheat grain and straw and Pronase hydrolysis of the wheat proteins indicated that selenomethionine represented 51.5% of the hydrolysate ^{75}Se (Olson et al., 1970). Detectable amounts of unidentified selenium compounds were shown to be present in wheat, one of which might have been selenocysteic acid, by Olson et al. (1970).

A ninhydrin positive band with relatively small R_f values was present in all chromatograms and accounted for 2.90% of the ^{75}Se in the extract. The R_f values were identical with those of known *S*-methylmethionine and the zone eluates were co-spotted and developed with the known compound for identification purposes. The presence of *Se*-methylselenomethionine in accumulator plants such as *Astragalus preussi* and *A. crotolariae* was reported by Shrift and Virupaksha (1965). McRorie et al. (1954) identified a heat labile compound present in cabbage juice as *S*-methylmethionine and suggested that in light of the enhanced activity of this methionine analog and its wide distribution in plants it might play an important role in the storage and transfer of methyl groups. Lewis et al. (1971) reported a similar finding for the *S*-methylmethionine occurring in plants. These workers have proposed a similar mechanism for seleniferous plants that have been shown to contain selenium analogs of this compound.

All chromatograms of the cabbage extract fractions exhibited a highly radioactive purple ninhydrin band with solvent R_f values identical with those of known L-cystathionine and selenocystathionine. Comparable solvent bands contained 19.96% of the total extract radioactivity. Co-spotting of the eluates, L-cystathionine, and selenocystathionine followed by development yielded one ninhydrin positive active spot indicating that the band radioactivity was present in selenocystathionine. Nearly all of the water-soluble cytotoxic activity of Venezuela monkey nuts was identified by Aronow and Kerdel-Vegas (1965) as selenocystathionine. Consumption of these nuts by humans resulted in abdominal discomfort, nausea, vomiting, diarrhea, and alopecia. Peterson and Butler (1971) found selenocystathionine to be the second most abundant soluble amino acid in *Morinda reticulata* representing 20% of the nitrogen and 90% of the selenium present in the plant foliage. Virupaksha and Shrift (1963) reported that selenocystathionine accounted for 10% of the Cl_3CCOOH soluble selenium in *Stanleya pinnata*. Young and Maw (1958) considered cystathionine to be the key intermediate in the metabolic conversion of methionine to cysteine and also in the synthesis of methionine from cysteine in microorganisms. Whether selenocystathionine plays a similar role in the synthesis of selenoamino acids by plants has not been established.

Table I. Solvent Fractionation of Cabbage Leaves, ^{75}Se Content, and Percentage of Total Radioactivity Present in Fractions

Procedure and fraction	^{75}Se content, cpm	Portion of radioact., %
Green leaves	80,249,800	100.00
Hexane wash	115,150	0.14
Chloroform-soluble extract	301,260	0.38
Water-soluble extract	73,179,260	91.18
Insoluble residue	6,560,500	8.18
Loss	93,630	0.12

Chromatograms carried a band that developed a purple ninhydrin color and possessed $R_f \times 150$ values equal to those of DL-homocystine and selenohomocystine. This band contained 9.22% of the total extract ^{75}Se and the radioactivity was readily eluted. Co-spotting of the eluate, DL-homocystine, and selenohomocystine followed by one- or two-dimensional development yielded one active purple ninhydrin spot. On the basis of different R_f values for homocystine and homocysteine in solvent system C and other tests the presence of homocysteine or selenohomocysteine in the extracts at the time they were chromatographed could not be demonstrated. Careful extraction and immediate TLC of the extract gave no evidence of the presence of selenohomocysteine. Excised leaves of accumulator as well as nonaccumulator *Astragalus* species actively metabolized selenomethionine and the accumulator leaves contained *Se*-methylselenocysteine, selenohomocystine, and *Se*-methylselenomethionine while the nonaccumulator species did not form significant amounts of *Se*-methylselenocysteine or selenohomocystine (Virupaksha et al., 1966).

Two high activity ninhydrin positive bands with different R_f values were present on all chromatograms. The lower band R_f values of 54, 55, and 26 with solvents A, B, and C were equal to those of known *S*-methylcysteine sulfoxide and *Se*-methylselenocysteine selenoxide. This band carried 21.51% of the extract ^{75}Se. Co-spotting of the eluate, *S*-methylcysteine sulfoxide, and its selenium analog and one- or two-dimensional development yielded one dense ninhydrin positive active spot confirming the presence of *Se*-methylselenocysteine selenoxide. The other red-pink ninhydrin positive bands had R_f values of 95, 87, and 67 in solvent A, B, and C chromatograms and these were equal to comparable R_f values of *S*-methylcysteine and *Se*-methylselenocysteine. The radioactivity measurements indicated that 26.70% of the extract ^{75}Se was present in *Se*-methylselenocysteine and this fact was verified by band elution and eluate chromatography. Members of the Cruciferae family such as turnip, cabbage, Chinese cabbage, cauliflower, kohlrabi, radish, mustard, and broccoli were examined by Morris and Thompson (1956) and the 80% ethanol extract was found to contain considerable quantities of *S*-methylcysteine and 10- to 200-fold larger amounts of the sulfoxide. They found that these two compounds contained 4 to 20% of the total soluble plant nitrogen. Excised leaves of *Stanleya* and other accumulator plants supplied selenite or selenate contained *Se*-methylselenocysteine as an abundant soluble organic selenium compound but no mention of

Table II. Indicated Selenium Compounds, Solvent System $R_f \times 150$ Values,[a] Total Extract ^{75}Se, and Percentage of Radioactivity in Comparable Solvent Bands

Indicated selenium compounds	Solvent systems $R_f \times 150$ A[b]	B[c]	C[d]	Portion of radioact., %
Total extract activity			3,636,930	100.00
Selenopeptides and proteins	5	3	3	1.11
Se-Methylselenomethionine	11	14	9	2.90
Selenocystathionine	31	32	17	19.96
Selenocystine	45	40	34	0.41
Se-Methylselenocysteine selenoxide	54	55	26	21.51
Selenohomocystine	62	48	48	9.22
Selenocysteine type compound	87	64	58	0.61
Se-Methylselenocysteine	95	87	67	26.70
Selenomethionine	109	96	85	14.62
Unknown				2.96

[a] Ascending sandwich development, 150 mm, silica gel H coated laboratory prepared glass plates. [b] 2-Methyl-1-propanol-acetic acid-chloroform-water-methanol (40:15:10:15:20), developed twice. [c] 1-Propanol-NH_4OH (70:30), single development. [d] 1-Butanol-acetic acid-water (60:15:25), single development.

the presence or absence of the selenoxide was made by Shrift and Virupaksha (1965).

A pink band, the uppermost dense ninhydrin positive band, had the same R_f values on all extract chromatograms as did DL-methionine or selenomethionine. Comparable bands, of all chromatograms reported, contained 14.62% of the total extract ^{75}Se. Chromatograms co-spotted with the eluate of these bands, methionine, and selenomethionine and subjected to one- or two-dimensional development with selected solvents carried only one active ninhydrin positive spot confirming the presence of selenomethionine. Morris and Thompson (1956) found cysteine, methionine sulfoxide, and *S*-methylcysteine to be present in considerable quantities and to contain 0.30, 0.23, and 3.7% of the total soluble nitrogen of turnip leaves. Ngo and Shargool (1972) germinated rapeseeds in contact with [^{35}S]sulfide and found that there was an immediate uptake of label with the subsequent formation of labeled cysteine, cystathionine, homocysteine, and methionine. The 80% ethanol extract of bromegrass, sprayed 7 days previously with a selenite solution, contained selenocystine and selenomethionine representing 42 and 17% of the absorbed ^{75}Se (Jenkins and Hidiroglou, 1967). Olson et al. (1970) found large amounts of selenomethionine in the protein hydrolysates and little of the free compound in the hot water extracts of wheat grain or straw and this suggests that, once formed, this amino acid is quickly incorporated into proteins and peptides.

The remaining portions of the chromatograms, approximately one-half the total area, contained ninhydrin positive as well as organic compound bands with some activity. The ^{75}Se content of the total area was 3.98% of the total extract radioactivity and the identity of the selenium compounds present was not established. A purple ninhydrin band with R_f values equal to those of DL-cystine and DL-selenocystine contained 0.41% of the extract ^{75}Se. This band may contain selenocystine or a compound or compounds with similar chromatographic behavior. The areas between the selenohomocystine and *Se*-methylselenocysteine bands of solvent A and C chromatograms and between solvent B bands of *Se*-methylselenocysteine selenoxide and *Se*-methylselenocysteine contained several ninhydrin positive bands and accounted for 0.61% of the extract ^{75}Se. On the basis of anticipated R_f values selenium analogs of compounds such as cysteine, methionine sulfoxide, methionine sulfone, substituted cystines, and substituted cysteines, if present, would be expected to be located in these areas.

Cabbage and many other plants are capable of absorbing and metabolizing selenium supplied them in a water-soluble form and their tissues contain relatively large amounts of numerous organic selenium compounds. Identification of these compounds will lead to a better understanding of the biosynthetic pathway or pathways of selenium in these plants. A definite need exists for isolation and identification of selenium compounds present in small amounts and hopefully newer techniques will make this possible. Greater knowledge of the chemical identity of the selenium compounds will assist in determining whether selenium incorporation into plant compounds involves pathways different than those for sulfur or whether such incorporation is a chance or random substitution of selenium atoms for the more abundant sulfur atoms. Knowledge of the identity of the organic selenium compounds will assist in a better understanding of their toxicity due to the intake of significant amounts of selenium by animals.

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