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Cadmium-binding protein components of flaxseed: Influence of cultivar and location ☆

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Abstract

The distribution of cadmium-(Cd)-binding components from five flaxseed cultivars grown at three locations in southern Manitoba was investigated to examine genotypic and environmental effects. Three protein fractions with different electrostatic properties, eluting at 0.10, 0.25 and 0.50 M NaCl by ion-exchange chromatography DEAE-Sephacel, represented 12%, 66% and 7% of the bound (extracted) protein, respectively, while 15% of the protein remained unbound. Cadmium and other divalent metal (zinc, copper and calcium) contents of protein fractions were strongly influenced by location. Cultivar differences in protein and cadmium contents of the protein fractions were highly significant. Cadmium and zinc accumulated similarly in the 0.10 and 0.25 M protein fractions at 51% and 40–43%, respectively. Transfer of copper occurred prominently in the 0.50 M fraction while most of the calcium (55%) remained unbound. The distribution of cadmium, zinc, copper and calcium in fractions of flaxseed proteins was strongly influenced by cultivar and location, indicating differences in their accumulation, migration and transfer.

Keywords: Flax (*Linum usitatissimum* L.); Protein fractions; Cadmium; Cultivars; Locations

1. Introduction

Cadmium (Cd) absorbed from the soil and translocated in plants can enter the food supply, thereby presenting a food safety concern. High intake of Cd (over the provisional tolerable weekly intake of 400–500 μg per individual) by humans results in excessive Cd loads that may lead to impairment of kidney function and other chronic toxicities (FAO/WHO, 1995). The toxicity of cadmium is still not completely understood and different processes are apparently involved (Romero-Puertas, Palma, Gomez, Del Rio, & Sandalio, 2002). These mechanisms include lipid peroxidation, inhibition of chlorophyll biosynthesis, oxidative stress induced by oxygen free radical production or decreased antioxidants. Cadmium produces a concentration-dependent imbalance in the antioxidant status of pea plants, resulting specifically in depressed superoxide dismutase and catalase activities that, in turn, increase lipid peroxidation rate. Cadmium treatment also impedes the cellular antioxidant defence system by reducing copper zinc superoxide dismutase (CuZn-SOD) activity by 95%, probably due to modifications of CuZn-SOD biosynthesis at translational or transcriptional level (Romero-Puertas et al., 2002). The FAO/WHO (1995) provisional guideline level of 0.1 ppm for Cd in flaxseed is currently under debate.

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On the positive side, a recent report (Waalkes & Diwan, 1999) indicates that cadmium can effectively inhibit tumor formation in the liver and lung in mice when administered at non-toxic doses and even when given well after tumor formation. Cadmium dose-dependently reduces tumor growth and metastasis of human lung carcinoma xenografts, independent of apoptosis. Therefore, cadmium-induced tumor suppression could be accomplished with non-toxic doses, which would be a positive attribute for any cancer chemotherapy. The cytotoxic impact of cadmium is often a function of the level of cellular metallothionein, a cadmium-inducible metal-binding protein encoded by the *MT* gene which sequesters cadmium and thereby mitigates its toxicity (Masters, Kelly, Quaife, Brinster, & Palmiter, 1994; Waalkes & Goering, 1990). Studies cited by Waalkes and Diwan (1999) have shown that metallothionein is poorly expressed in liver and lung tumors from mice, in human hepatocellular carcinomas and pulmonary small cell carcinoma, possibly predicting a common sensitivity to cadmium. The cytotoxic effect of cadmium is often a function of the level of cellular metallothionein; low methallothionein levels generally correspond to high cadmium-induced cytotoxicity (Waalkes & Diwan, 1999). Therefore, the hypersensitivity of liver tumors, and possibly lung tumors, may be due to poor expression of metallothionein in the tumor cells.

In flax, a high concentration of Cd, exceeding the dietary critical value or maximum level of 0.3 ppm (Marquard, Bohm, & Freidt, 1990), is known to accumulate in the seeds, even at low soil Cd level. Further, cadmium accumulation in flaxseed is genotype- and environment-dependent (Becher, Worner, & Schubert, 1997; Grant, Dribnenki, & Bailey, 2000; Li et al., 1997). However, the underlying mechanism of the genotypic difference, as well as the distribution of Cd in flaxseed is still unclear. Our previous study (Li-Chan, Sultanbawa, Losso, Oomah, & Mazza, 2002) elucidated the presence of Cd-binding components in protein extracts of dehulled and defatted flaxseed cultivar NorMan with phytochelatin-like components present in two fractions eluting at high salt concentrations of 0.45 and 0.50 M NaCl. A recent investigation (Lei, Li-Chan, Oomah, & Mazza, 2003) of the distribution of Cd in flaxseed protein of cultivar NorMan showed that about 80% and 73% of the extracted proteins and cadmium, respectively, from flaxseed bound to the ion-exchange column. The Cd-binding protein was distributed mainly in the 0.10 and 0.25 M fractions with 66% and 25% of the Cd, respectively. Distribution of zinc was similar to that of cadmium with 44%, 36% and 20% of eluted zinc in the 0.10, 0.25 M NaCl and unbound fractions, respectively. The copper contents were approximately equally distributed (~41%) in the 0.10 and 0.25 M fractions. Calcium was distributed in every protein fraction with the highest and lowest contents, 51% and 11%, in the unbound and 0.10 M NaCl fraction, respectively.

The present study is a continuation of our earlier investigations (Lei et al., 2003; Li-Chan et al., 2002) aimed at determining the genotypic difference and distribution of Cd in flaxseed. Since cadmium can affect the activity of metalloenzymes, CuZn-SOD, in particular (Romero-Puertas et al., 2002), the contents of copper, zinc and calcium in flaxseed protein fractions were also examined. Information on the genotypic and environmental effects of cadmium accumulation and distribution in flaxseed is paramount in the long-term objective of reducing the risk of Cd toxicity and ensuring the continued safety with increased flaxseed utilization in functional foods and the nutraceutical industry.

2. Materials and methods

2.1. Source of materials and sample preparations

Tris Ultrapure [Tris-(hydroxymethyl)aminomethane] was from ICN Biomedicals, Inc., Costa Mesa, CA. Sodium chloride and hydrochloric acid (ACS certified) were from Fisher Scientific, Nepean, ON, Canada. 2-Mercaptoethanol electrophoresis reagent was from Sigma–Aldrich Canada Ltd., Oakville, ON, Canada. Bicinchoninic acid (BCA) protein assay reagents A and B were from Pierce Chemical Company, Rockford, IL. The deionized distilled water (~18 MΩ) used for all experiments was produced by a Barnstead water purification system. DEAE-Sephacel was purchased from Amersham Pharmacia Biotech, Inc., Baie d'Urfe, PQ, Canada.

Five flaxseed cultivars, grown at three locations, Morden, Portage La Prairie (referred herein as Portage) and Rosebank in Southern Manitoba in 1997, were obtained from the Agriculture and Agri-Food Canada Cereal Research Center (Morden, MB, Canada). Samples were processed essentially as described previously (Lei et al., 2003) by dehulling flaxseed with a Strong Scott Barley Pearler according to Li-Chan et al. (2002) and an air aspirator (Oomah, Mazza, & Kenaschuk, 1996). The dehulled seeds were ground in a small coffee mill and defatted by two changes of hexane and one change of petroleum ether, 1 h each with magnetic stirring, using a seed-to-solvent ratio of 1:10 (w/v). The dehulled defatted meal was air-dried under a fume hood for at least 4 h.

2.2. Protein extraction, fractionation and characterization

Protein was extracted from dehulled defatted meal (3 g) according to the method of Li-Chan et al. (2002) with 16 volumes of nitrogen-purged extraction buffer (0.10 M Tris + 0.10 M NaCl + 10 mM mercaptoethanol, pH 8.6) at 4 °C for 16 h with constant magnetic stirring. The extract was centrifuged (12,000*g* for 20 min, Beckman Avanti J25, Beckman Instruments Inc., Palo Alto, CA) at 6 °C, and the supernatant was further centrifuged at 28,000*g* for 25 min.

Ion-exchange (IE) chromatography was carried out by using a modification of the procedure of Li-Chan et al. (2002) using the BioRad automated econo system

low pressure chromatography. The anion-exchange resin DEAE-Sephacel was packed in a column (5.0×30 cm) and equilibrated with nitrogen-purged buffer consisting of 0.01 M Tris + 0.01 M NaCl at pH 8.6. The protein extract from 3 g of dehulled defatted meal was diluted 10-fold with distilled and deionized water (dd water) and then filtered through a 0.45 μm nylon filter. The extract was placed over the column and the unbound proteins were removed by washing with equilibrating buffer until the absorbance at 280 nm was <0.1. Bound fractions were eluted by step gradients with increasing concentrations of NaCl from 0.10 to 1.0 M in 0.01 M Tris buffer at pH 8.6 at a flow rate of 10 ml/min Fractions (20 ml/min) were collected and monitored for conductivity (Hanna Instruments 3310 conductivity meter, Hanna Instruments, Woonsocket, RI) and absorbance at 280 and 254 nm (SpectraMax Plus^{384} Microplate Spectrophotometer, Molecular Devices Corporation, Sunnyvale, CA).

All analyses were performed on the pooled column fractions. Cadmium, zinc, copper and calcium contents in the IE fractions were measured by inductively coupled plasma mass spectrometry (ICP-MS), operated with an ultrasonic nebulizer (USN) and a pulse membrane desolvator by Elemental Research Inc. (North Vancouver, BC, Canada). The detection limit for Cd was 0.1 ppb. The metals in the buffer blank were Ca, 3.97 ± 0.91 ppm: Cu, 0.07 ± 0.02! ppm; Zn, <0.03 ppm; and Cd, <0.3 ppb. Cadmium content of the original seed samples was measured according to the procedure of Grant et al. (2000) on a Varian 300/400 atomic absorption spectrophotometer at 228.8 nm, using a graphite furnace with deuterium correction (detection limit 0.01 ng Cd/ml).

Protein contents of pooled column fractions were measured in triplicate, using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Company, Rockford, IL) with serum albumin as the protein standard. Protein ($N \times 6.25$) contents of solid samples were determined by a nitrogen combustion method (FP-428, LECO Instruments Ltd., Mississauga, ON, Canada).

Data were subjected to analysis of variance by the general linear models (GLM) procedure, means comparison by Duncan's test, and Pearson correlation according to Statistical Analysis System (SAS Institute, 1990). Data in Tables for each fraction are mean values for cultivars ($n = 3$, grown at three locations), locations ($n = 5$, for all cultivars grown at individual location) and overall ($n = 15$). Differences among samples were considered to be significant at $P < 0.05$ unless stated otherwise.

3. Results and discussion

Average protein content of flaxseed cultivars grown at three locations in southern Manitoba was 19% ($N \times 6.25$) (Table 1). Processing, particularly lipid removal (defatting), more than doubled the protein content. The amount of protein loaded on the column ranged from 472 to 609 mg and differed significantly among cultivars, AC McDuff and Flanders, in particular. The highest amount of bound protein was eluted by 0.25 M NaCl, followed by 0.10 and 0.50 M NaCl. The protein content of the major fraction (0.25 M) varied significantly between cultivars AC Linora and AC McDuff, while differences among the other three cultivars, Flanders, NorLin and CDC Normandy (referred to herein simply as Normandy) were not significant. Cultivar differences were not statistically significant for protein contents of the 0.10 and 0.50 M fractions. The 0.25, 0.10, and 0.50 M fractions constituted 66%, 12% and 7%, respectively, of the total amount of protein loaded on to the

Table 1
Protein contents (g/100 g) of seed products and ion-exchange fractions of flaxseed cultivars grown at three locations

Fraction	Cultivar					Location			Overall mean
	AC Linora	AC McDuff	Flanders	NorLin	Normandy	Morden	Portage	Rosebank	
Seeds*	18.7	19.0	19.3	18.2	19.1	19.5e	19.4e	17.7f	18.9r
Dehulled*	20.9	20.8	21.3	20.4	21.7	22.1e	21.5e	19.4f	21.0q
Meal*	45.6	45.2	46.1	43.6	47.0	48.1e	46.6e	41.9f	45.5p
Load (mg)	494.7ab	608.9a	472.3b	519.6ab	525.6ab	547.2	498.7	526.4	524.1
Ion-exchange fraction (mg)									
Unbound	68.4b	96.3a	90.7a	81.5ab	79.3ab	80.1	83.1	86.6	83.3t
0.10 M	58.4	71.1	76.3	67.4	58.2	58.5	72.4	68.0	66.3t
0.25 M	323.5b	398.2a	346.7ab	344.0ab	354.6ab	360.7	358.7	340.9	353.4s
0.50 M	39.3	56.0	34.9	33.7	26.3	40.3	45.2	28.6	38.0u
(% Load)									
Unbound	14.1b	15.5ab	16.6a	15.5ab	15.2ab	14.8f	14.9f	16.5e	15.4w
0.10 M	11.8	11.4	13.9	12.9	10.9	10.6	13.0	12.9	12.2x
0.25 M	66.9	64.0	63.2	65.2	68.8	67.3	64.2	65.4	65.6v
0.50 M	7.2	9.0	6.3	6.4	5.1	7.3	7.9	5.3	6.8y

Percentage calculations were based on the total amount eluted from the column. 'a–c' and 'e,f' Means within a row for cultivars and locations, respectively, with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test. 'p–y' Overall means within a column with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

* Protein content of seeds, dehulled seeds and dehulled defatted meal are expressed as g/100 g ($N \times 6.25$).

column. About 15% of the loaded protein was not bound by the anion-exchange resin DEAE-Sephacel (i.e., 85% bound), a result comparable to the 80% bound protein previously observed (Lei et al., 2003). Apparently, cultivars have no significant effect on the distribution of protein fractions in flaxseed.

Flaxseed cultivars grown at Rosebank had significantly lower protein contents in the seed, resulting in lower protein contents in dehulled seeds and dehulled defatted meal than those grown at Morden and Portage. Although this difference was minimized by adjusting or normalizing the column load (500–550 mg), the protein content of the 0.50 M fraction of cultivars grown in Rosebank was still lower by 30–35% (29 mg vs. 40–45 mg) than those from Morden and Portage. This difference was also reflected in the significantly elevated distribution of protein in the unbound fraction as a percentage of total column loads.

Seed cadmium content, expressed as a mean for all three locations, was highest and lowest for cultivars Flanders (range 380–1543 ppb) and Normandy (range 254–1007 ppb), respectively (Table 2). The 1.5-fold variation in average Cd accumulation in seed between these two cultivars was within the range of 1.4–2.5 reported by Marquard et al. (1990) for 16 flaxseed genotypes grown at several locations in Germany. Cultivar differences in levels of cadmium in seed were similar to those observed by Grant et al. (2000) for flaxseed grown at three sites in Manitoba over three years. Cadmium concentrations in the seed was higher than those reported previously (Marquard et al., 1990) (65–582 ppb) and (Hocking & McLaughlin, 2000) (426 ± 25) for cultivar NorLin common with our study. Similarly, seed Cd content for cultivar Flanders (range 380–1543 ppb) was higher than the 200–535 ppb range reported by Cieslinski et al. (1996).

Cadmium contents of flaxseed protein fractions differed significantly among cultivars. Thus, the load fraction of cultivar Flanders had significantly higher level of Cd than those of AC Linora, NorLin and Normandy. The Cd content of the unbound fraction of cultivar Flanders was significantly lower than those of AC Linora and NorLin. Hence, Flanders among all cultivars had the highest and lowest concentrations of Cd in the load and unbound fractions, respectively, probably a reflection of the elevated seed cadmium level. Cd distribution of the ion-exchange fractions, expressed as percent of the load, segregated the cultivars into two groups. Cultivars Flanders and AC McDuff, with the lowest cadmium percentage in the unbound fraction, showed similar trends in cadmium distribution in the 0.10, 0.25, and 0.50 M protein fractions at 45–46%, 46%, and 6.5%, respectively. The second group, cultivars AC Linora, NorLin and Normandy, with high percentages of unbound fraction, had similar cadmium distributions in the 0.10 and, 0.25 M protein fractions at 51–56% and 33–37%, respectively.

Location had a significant ($P = 0.037$) effect on cadmium content of flaxseed grown in southern Manitoba, with cultivars grown at Morden (1196 ppb) significantly higher (3–4×) than those from Portage (294 ppb) or Rosebank (355 ppb). This contrast with the results of Grant et al. (2000), indicating that flaxseed at Rosebank had two to four times higher cadmium contents than at two other Manitoba locations, Brandon and Minnedosa, not included in our study. The elevated level of cadmium in the column load, 0.10 and 0.25 M protein fractions stem from the high initial cadmium content of flaxseed from Morden, suspected to be a region with metal-rich black soil. Cadmium content of cultivars grown in Portage and Rosebank were not significantly different at ($P = 0.05$) according to Duncan means. Cadmium generally concentrated mostly in the 0.10 and 0.25 M flaxseed protein fractions at 51% and 40% of the column load, respectively.

Flaxseed cultivars NorLin and AC McDuff had the highest and lowest zinc contents of the column load, respectively (Table 3), resulting in the lowest and one of the highest concentrations of unbound fraction, respectively. This conversely indicates that the highest

Table 2
Cadmium contents of seed and ion-exchange fractions of flaxseed cultivars grown at three locations

Fraction	Cultivar					Location			Overall mean
	AC Linora	AC McDuff	Flanders	NorLin	Normandy	Morden	Portage	Rosebank	
Seeds (ppb)	542b	607ab	778a	625ab	523b	1196x	294y	355y	615
Ion-exchange fraction (μg/g)									
Load	4.6b	4.8ab	6.0a	4.5b	3.6b	8.1x	3.3y	2.7y	4.7
Unbound	1.6a	0.5ab	0.4b	1.6a	0.6ab	1.5x	0.6y	0.7xy	0.9
0.10 M	20.4	15.9	15.8	21.2	22.3	35.7x	11.0y	10.7y	19.1
0.25 M	2.4	3.0	3.1	2.3	1.9	4.2x	1.8y	1.6y	2.5
0.50 M	3.0	2.6	5.7	3.4	2.6	3.3	3.4	3.6	3.5
(% Load)									
Unbound	5.7a	2.4c	1.2c	5.2ab	3.1bc	2.9	3.3	4.3	3.5
0.10 M	50.6	45.1	46.2	56.2	54.9	52.8	42.1	50.8	50.6
0.25 M	36.2	46.0	46.2	32.8	36.8	41.1	39.8	37.9	39.6
0.50 M	7.4	6.5	6.5	5.8	5.2	3.2	8.7	7.0	6.3

Percentage calculations were based on the total amount eluted from the column. 'a–c' and 'x,y' Means within a row for cultivars and locations, respectively, with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 3
Zinc contents of ion-exchange fractions of flaxseed cultivars grown at three locations

Fraction	Cultivar					Location			Overall mean
	AC Linora	AC McDuff	Flanders	NorLin	Normandy	Morden	Portage	Rosebank	
Ion-exchange fraction (μg/g)									
Load	199	181	243	259	195	249	216	181	215
Unbound	35.1	25.9	36.8	14.7	23.8	30.0	32.2	19.6	27.3
0.10 M	1091a	520b	602b	834ab	841ab	1071x	648y	613y	778
0.25 M	128a	112ab	133a	115ab	87.3b	121	125	99.0	115
0.50 M	56.6	68.3	192	106	69.6	63.9	138	103	101
(% Load)									
Unbound	2.3ab	2.9a	3.la	1.2b	2.4ab	2.3	2.7	2.1	2.4
0.10 M	57.2a	42.2c	45.2bc	55.0ab	54.7ab	53.7	47.4	51.6	50.9
0.25 M	39.0b	50.6a	46.0ab	40.4b	40.6b	41.9	45.2	42.9	43.3
0.50 M	2.3	4.3	5.7	3.4	2.3	2.2	4.7	4.2	3.7

Percentage calculations were based on the total amount eluted from the column. 'a–c' and 'x,y' Means within a row for cultivars and locations, respectively, with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

concentrations of zinc was bound to the column and subsequently eluted for cultivar NorLin and the least for cultivars Flanders and AC Linora.

Zinc content of the 0.10 M fraction was significantly different among cultivars and locations. A twofold difference in zinc content was observed in the 0.10 M fraction of cultivars AC Linora and AC McDuff. The 0.10 M fraction of cultivars grown in Morden had a significantly higher level of zinc than those grown in Portage or Rosebank. The elevated level of zinc of the 0.10 M fraction from Morden corresponds to its high cadmium content, suggesting that parallel accumulation of cadmium and zinc for this fraction may be site-specific. Zinc content of the 0.25 M fraction was also cultivar-dependent with cultivars AC Linora and Normandy having the highest and lowest concentrations, respectively. Zinc distribution of the ion-exchange fractions, expressed as percent of the load, segregated the cultivars into two groups, similar to those observed for cadmium. Flanders and AC McDuff, with the highest zinc percentage in the unbound fraction, showed similar trends in zinc distribution in the 0.10, 0.25, and 0.50 M protein fractions at 42–45%, 46–51%, and 4–6%, respectively. The second group, cultivars AC Linora, NorLin and Normandy, with low percentage of unbound fraction, had similar zinc distributions in the 0.10 and, 0.25 M protein fractions at 55–57% and 39–41%, respectively.Generally, zinc concentrated mostly in the 0.10 and 0.25 M protein fractions at 51% and 43% of the column load, respectively. The distribution of zinc in the 0.10 and 0.25 M protein fractions was equal for Flanders (45–46%), relatively low and high for AC McDuff (42% and 51%, respectively), and conversely relative high and low for the other cultivars (55–57 and 39–41, respectively).

The high copper content of cultivar NorLin in the column load paralleled those in the 0.25 and 0.50 M protein fractions (Table 4). This resulted in enrichment of copper at 47% of the total column load of the 0.50 M fraction at the expense of other protein fractions. Conversely, copper distribution in AC McDuff occurred mostly in the 0.25 M fraction at 75% of the column load with minimal amount (8%) in the 0.50 M protein fraction. Hence, the ratio of

Table 4
Copper contents of ion-exchange fractions of flaxseed cultivars grown at three locations

Fraction	Cultivar					Location			Overall mean
	AC Linora	AC McDuff	Flanders	NorLin	Normandy	Morden	Portage	Rosebank	
Ion-exchange fraction (μg/g)									
Load	90.9	89.1	114	118	88.2	102	106	92.2	100
Unbound	57.5	20.6	52.9	41.8	32.3	48.0	31.2	43.9	41.0
0.10 M	336a	110b	212ab	275ab	312a	286	172	289	249
0.25 M	177	110	168	293	234	246	146	197	197
0.50 M	3600	87	4199	5932	6892	6360	1940	4127	4142
(% Load)									
Unbound	4.9	3.3	3.7	0.8	1.0	3.1	3.3	1.8	2.7
0.10 M	23.1	13.4	18.2	10.2	10.2	10.1	17.8	17.1	15.0
0.25 M	44.6	75.2	48.7	42.3	46.2	42.4	60.8	51.0	51.4
0.50 M	27.4	8.0	29.5	46.7	42.6	44.4	18.1	30.0	30.8

Percentage calculations were based on the total amount eluted from the column. 'a,b' Means within a row for cultivars with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

copper contents in the 0.25 and 0.50 M fractions was 9.3, compared with 0.33–0.56 for the other cultivars. Copper content of the 0.10 M fraction of cultivar AC McDuff was significantly lower than those of AC Linora and Normandy. The 0.10 M fraction of AC Linora had thrice and twice the levels of copper and zinc, respectively of AC McDuff. Cultivars NorLin and Normandy showed similar trends in copper distribution of the ion-exchange protein fractions. Flaxseed grown in Morden had a higher copper content in the column load, mostly reflected in the 0.25 and 0.50 M protein fractions, than those from Portage or Rosebank. Conversely, cultivars grown at Portage with low copper content in the column load also had low copper contents in the 0.25 and 0.50 M protein fractions. Copper concentrated mainly in the 0.25 and 0.50 M fractions with 51% and 31% of the total column load, respectively. Cultivars grown in Portage had a high percentage of the copper in the 0.25 M fraction (61%) and a low percentage in the 0.50 M fraction (18%); those from Morden had a high percentage of copper (44%) in the 0.50 M protein fraction and an equivalent amount (42%) in the 0.25 M fraction.

Cultivar AC Linora had the highest calcium content in the column load and unbound protein fractions, indicating that a proportionately low amount of calcium was bound to the column (Table 5). Cultivar Normandy, with the lowest calcium load, had a higher concentration of calcium in the 0.10 M protein fraction than did NorLin (about one-fifth the concentrations). Calcium content of protein fractions was cultivar-dependent, although differences were not statistically significant, except for the 0.25 M fraction. In this fraction, calcium content of cultivar Flanders was significantly higher (3.8-fold) than that of NorLin. Significant differences were also observed in the calcium content of the 0.25 M fraction of cultivars grown at Portage and Rosebank. The distribution of calcium in protein fractions reflected the geographic origin of flaxseed cultivars. For example, protein from flaxseed cultivars grown in Rosebank had the lowest calcium content in the 0.10, 0.25 and 0.50 M fractions, due to the significantly higher calcium content of the unbound fraction (72%) than those from Portage or Morden. Calcium distributions (as a percentage of column loads) were similar for cultivars grown at Morden and Portage. Almost 55% of the total calcium load was unbound, while 21% remained in the 0.50 M fraction (almost twice the amount found in the 0.10 M fraction). The general distribution of calcium in the unbound and 0.10 M fractions, at 55% and 10% respectively, corresponds to a previous report for cultivar NorMan (Lei et al., 2003).

Some results of this study, particularly the general distribution of zinc and calcium in the ion-exchange fractions of Flanders, relate to earlier data for cultivar NorMan (Lei et al., 2003). An overall comparison of the distribution of divalent metal in cultivars with the highest and lowest seed cadmium contents (Flanders and Normandy, respectively), reveals that high seed cadmium results in high accumulation of cadmium and zinc in the 0.25 and 0.50 M fractions but their relatively low accumulation, with elevated level of copper, in the 0.10 M fraction. Conversely, the low seed cadmium cultivar enables low accumulation of cadmium and zinc in the 0.25 and 0.50 M fractions but their relatively high accumulation, with reduced level of copper, in the 0.10 M fraction. The specific distribution of divalent metals in each of the protein fractions, based on initial cadmium seed content, suggests a very complex genotypic-protein interaction.

Cadmium, zinc and calcium accumulation in regard to protein content was low in Normandy. Therefore, this cultivar may be considered a low divalent metal accumulator in the three locations of this study. Cultivar AC McDuff consistently showed the lowest cadmium, zinc, copper and calcium contents, when expressed on per protein basis, across all fractions. Cultivar NorLin can generally be classified as a high accumulator, especially for zinc and copper.

Flaxseed cultivars grown in Morden generally had significantly higher cadmium: calcium, cadmium: zinc and cadmium: copper ratios than those from Portage or Rosebank (Table 6). These high ratios, especially of the 0.10

Table 5
Calcium contents of ion-exchange fractions of flaxseed cultivars grown at three locations

Fraction	Cultivar					Location			Overall mean
	AC Linora	AC McDuff	Flanders	NorLin	Normandy	Morden	Portage	Rosebank	
Ion-exchange fraction (μg/g)									
Load	1408	1067	1141	1305	970	1346	1023	1166	1178
Unbound	6347	3006	2942	3397	4154	3775	3818	4315	3969
0.10 M	681	1267	1122	350	2003	1379	1545	329	1084
0.25 M	253ab	258ab	502a	130b	207ab	289xy	460x	127y	280
0.50 M	3932	2206	2788	5012	3437	3530	3904	2991	3475
(% Load)									
Unbound	64.6	46.2	48.6	55.8	57.5	45.4y	45.9y	72.4x	54.6
0.10 M	5.6	15.4	10.8	4.4	14.5	14.1	11.5	4.8	10.2
0.25 M	13.9ab	16.9ab	25.7a	5.7b	11.5ab	16.8	18.6	8.8	14.7
0.50 M	15.9	21.4	14.9	34.0	16.4	23.7	23.9	13.9	20.5

Percentage calculations were based on the total amount eluted from the column. 'a–c' and 'x,y' Means within a row for cultivars and locations, respectively, with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

and 0.25 M protein fractions, emanated from the high ratio of the column load. Conversely, low column loads of the cadmium: metal ratios were reflected in the low ratios of the protein fractions. Cultivars grown in Portage had the lowest cadmium: calcium and cadmium: zinc ratios of the 0.25 M fraction, while those grown in Rosebank had the lowest cadmium: zinc and cadmium: copper ratios in the 0.50 M protein fraction.

Analysis of variance (data not presented) showed that cadmium and zinc contents of protein fractions were significantly different for location ($F = 187$ and 11.98 at $P < 0.0001$, respectively). Cultivar differences in protein and cadmium contents of the protein fractions ($F = 6.9$, $P = 0.0004$; and $F = 7.6$, $P = 0.0002$, respectively) were highly significant. Calcium, cadmium, copper and zinc contents showed strong positive association with protein content in flaxseed fractions. The Pearson correlation coefficients for calcium, cadmium, copper and zinc were 0.595, 0.646, 0.550 and 0.790 for protein contents, respectively (Table 7). This analysis also showed significant positive correlation between calcium, cadmium and zinc contents ($r = 0.56-0.85$) such that fractions with the highest cadmium contents showed the highest concentrations of zinc. The correlation between cadmium and zinc possibly suggests their similar and non-competitive accumulation in protein fractions. Non-significant correlation between copper and both calcium and cadmium indicates that the concentrations of these metals are independent of one another. The weak association of copper and zinc suggests that changes in flaxseed protein fractions or modifications in zinc should have very little effect on copper content.

Table 6
Cadmium:metal ratio of ion-exchange protein fractions of flaxseed cultivars grown at three locations

Fraction	Location			Overall mean
	Morden	Portage	Rosebank	
C:Ca ratio				
Load	6.5a	3.2b	2.4b	4.0
Unbound	0.5	0.2	0.16	0.3
0.10 M	86.2	57.7	45.5	63.1
0.25 M	23.3a	4.0b	14.8ab	14.7
0.50 M	1.9	1.1	2.3	1.8
Cd:Zn ratio				
Load	33.4a	15.9b	15.0b	21.4
Unbound	64.0a	22.3b	50.3ab	45.5
0.10 M	39.9a	18.3b	18.2b	23.8
0.25 M	34.4a	15.1b	17.0b	22.2
0.50 M	72.4	57.0	32.6	55.5
Cd:Cu ratio				
Load	84.1a	31.7b	29.2b	48.3
Unbound	27.5	53.2	64.5	48.4
0.10 M	145a	80.0b	43.5b	89.4
0.25 M	26.0	16.0	9.4	17.1
0.50 M	28.8	56.9	27.9	37.9

'a,b' Means within a row with different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Table 7
Correlation coefficients of metal contents in ion-exchange protein fractions of flaxseed

	Calcium	Cadmium	Copper	Zinc
Protein	0.595	0.646	0.550	0.790
Calcium		0.558	0.202b	0.614
Cadmium			0.275b	0.852
Copper				0.389a

All correlation coefficients significant at $P < 0.0001$, except those with the letters 'a' ($P < 0.001$), and 'b' not significant ($n = 75$).

In flaxseed, the unbound protein fraction was an abundant source of calcium (3969 μg/g). Cadmium accumulated most prominently in the 0.10 M protein fraction (19 μg/g) that was also enriched with zinc (778 μg/g), calcium (1084 μg/g) and copper (249 μg/g). High copper and calcium levels (4142 and 3475 μg/g, respectively) characterized the minor 0.50 M protein fraction. The major 0.25 M protein fraction typically contained equivalent concentrations of calcium, copper and zinc (280, 197 and 115μg/g, respectively) and a minimal amount of cadmium (2.5 μg/g). Cadmium and zinc contents of the 0.25 and 0.50 M protein fractions were similar to those of soybean extracts and gel-filtered fractions (2.5 and 3.5 vs. 2.9 and 4.5 μg/g Cd; 115 and 101 vs. 119 and 132 μg/g Zn) (Yoshida, Tanaka, & Kashimoto, 1985). However, copper contents were 5–100 times higher than those in soy and fababeans (Kostyra, Darewicz, Kostyra, & Markiewicz, 1994; Yoshida et al., 1985). Taken together, the results suggest that the 0.10 M protein fraction may be considered a cadmium-metalothionein while the 0.50 M protein has the characteristics of a copper-metalothionein.

The concurrent presence of zinc and calcium in substantial concentrations in the 0.10 M protein fraction may provide a synergistic mitogenic effect (Huang, Mukherjee, Chung, Crilly, & Kiss, 1999). The potentiating effects of zinc and calcium in inducing cell death (Huang et al., 1999) together with the cytotoxic effect of cadmium (Waalkes & Diwan, 1999) at the cellular level, ensures the use of the 0.10 M protein fraction for positive medical applications. Flax protein fractions, particularly the 0.10 M fraction, enriched in zinc and calcium, considered to be a radiation transfer agent, may be capable of absorbing radiation and re-emitting it as a lower form of energy. This increases the potential of the 0.10 M flaxseed protein fraction for application in cancer radiation therapy, in addition to the anti-tumor effect of cadmium. This study, involving genotypic and environmental variants, provides a general model of cadmium distribution in flaxseed protein and a starting point for its manipulation.

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