

## Isolation of Factors in Linseed Oil Meal Protective against Chronic Selenosis in Rats<sup>1,2</sup>

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**ABSTRACT** Two new cyanogenic glycosides, linustatin and neolinustatin, were isolated from linseed oil meal. Each of the compounds was fed to rats in a corn-based diet at levels of 0.1 and 0.2%. At the 0.2% level, both substances gave significant protection against growth depression caused by 9 ppm selenium as sodium selenite. Both compounds also promoted a significant increase in liver and kidney weight over the selenium control animals. Linustatin and neolinustatin are closely related in structure to linamarin and lotaustralin and were found to be present in linseed oil meal at levels of 0.17 and 0.19%, respectively. Linamarin fed at the level of 0.2% also gave significant protection against growth depression and liver damage. A related cyanogenic glycoside, amygdalin, appeared to give a small but nonsignificant protective response. The isolation of the two new glycosides provides a probable explanation for the protective activity of linseed oil meal against selenium toxicity. *J. Nutr.* 110: 145-150, 1980.

**INDEXING KEY WORDS** selenium toxicity · linseed oil meal · cyanogenic glycosides · linamarin · amygdalin

In 1941, Moxon<sup>3</sup> demonstrated that linseed oil meal (LOM) protected against selenium toxicity when included in the diet at a level of 25%. Olson and Halverson (1) showed that 20% LOM would give protection against 10 ppm selenium in rats as measured by growth and liver damage, but 5 or 10% was not adequate. This was supported in other studies (2). Attempts to fractionate the selenium protective factor showed that it was present in both the embryo and hull (3, 4), and that it was not associated with the anti-B-6 principle of LOM (5). Contrary to earlier assumptions, Halverson et al. (6) demonstrated that the protective factor was not associated with protein, since it could be extracted with hot 50% ethanol and was not precipitated by lead acetate. Fractionation studies by Jensen and Chang (7), using

chicks as the assay organism, showed the factor was partially extracted by methanol and that the active principle in the methanol extract could be partially removed with chloroform-methanol (2:1). Earlier work at our laboratory<sup>4</sup> indicated that absolute methanol was not very effective in removing the factor from LOM directly.

Studies on the mechanism of action of

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<sup>3</sup>Moxon, A. L. (1941) The influence of some proteins on the toxicity of selenium. Ph.D. thesis. University of Wisconsin.

<sup>4</sup>Larson, R. L. (1959) Further attempts to isolate and identify the selenium protective factor of linseed oil meal. M.S. thesis. South Dakota State University.

the protective principle have been limited. Levander et al. (8) suggests that the principle in LOM binds selenium in the tissues in a form unavailable for binding to sensitive cellular sulfhydryl sites. Others have shown that if such selenium binding by LOM occurred, it was not sufficient to accelerate the onset of deficiency symptoms in rats fed diets low in selenium (9).

Our purpose was to further fractionate and purify the selenium protective factor(s) in LOM.

#### MATERIALS AND METHODS

*Animals and diets.* Male, Sprague-Dawley rats (Sasco Inc., Omaha, NE), weighing 60 to 70 g, were housed individually in stainless steel cages and given feed and water ad libitum. The basal diet contained: (in %) corn, 79; casein, 12; brewer's yeast, 2; salts,<sup>5</sup> 3; corn oil, 3; vitamin mix,<sup>6</sup> 1. The treatment diets all contained 9 ppm selenium as sodium selenite, which was added to the basal diet in a 50% ethanol solution prior to mixing. LOM or various fractions were added at the expense of corn. After a 3-week feeding period, the animals were killed and liver, kidney and muscle samples were taken and analyzed for selenium by the method of Olson et al. (10). Effectiveness of treatment was judged by body weight gain and by liver and kidney weights.

*Fractionation of linseed oil meal.* LOM (500 g) was mixed with 3 liters of hot 50% ethanol and stirred intermittently in a 70° water bath. The material was filtered and extracted again for 2 hours with 2.5 liters of 50% ethanol. Extracts were combined and evaporated in a 70° forced air oven. The dried solid was taken up in water (1 part solid to 2.5 parts water). The resulting suspension was centrifuged and the supernatant liquid was decanted and evaporated in the forced air oven. The dried material was extracted three times with methanol (1 part solid to 3.5 parts methanol), and the combined extracts were further fractionated by the addition of 2 volumes of chloroform. The insoluble residue was treated three additional times by dissolving in methanol and precipitating with 2 volumes of chloroform. The com-

bined filtrates were evaporated, dissolved in methanol and subjected to separation on a 7.4 × 7.5 cm, 100 mesh, silicic acid column. The column was loaded with 12 g of sample dissolved in 40 ml of methanol and eluted successively with 400 ml CHCl<sub>3</sub>, 800 ml CHCl<sub>3</sub>-methanol (4:1) and 1,500 ml CHCl<sub>3</sub>-methanol (2:1). The 2:1 CHCl<sub>3</sub>-methanol fraction was retained and evaporated to dryness. It was dissolved in methanol and separated by high performance liquid chromatography (HPLC) employing a 5.7 × 30 cm C<sub>18</sub>-column. Elution was with methanol-water (3:17) at a rate of 250 ml per minute and the load applied to the column was 2.5 g in 10 ml.

Preliminary judgment as to the adequacy of a procedure was aided by examining the fractions by thin-layer chromatography (TLC) on Silica Gel G using CHCl<sub>3</sub>-methanol-17% NH<sub>4</sub>OH (2:2:1) as solvent. The two substances of interest were located in the R<sub>f</sub> region of 0.55 to 0.70.

*Recrystallization of compounds in Peak 2 and Peak 3.* The fractions from HPLC were evaporated to dryness in a rotary evaporator immersed in a 50 to 60° water bath, dissolved in methanol, treated with charcoal, filtered and evaporated to dryness again. The material from Peak 2 was dissolved in 14 volumes of absolute ethanol and allowed to crystallize in the refrigerator. The resulting crystals were redissolved in 35 parts of ethanol and allowed to crystallize again. White crystals were obtained: m.p. 123 to 125.5°.

The material from Peak 3 was dissolved in 90 volumes of ethanol and crystallized in the refrigerator. Recrystallization was

<sup>5</sup> ICN Nutritional Biochemicals, Cleveland, OH. Salt Mixture U.S.P. XIV: (in mg/100 g) cupric sulfate, 7.78; ferric ammonium citrate, 1.528; manganese sulfate, 20.09; ammonium alum, 9.23; potassium iodide, 4.05; sodium fluoride, 50.71; (in %) calcium carbonate, 6.86; calcium citrate, 30.83; calcium biphosphate, 11.28; magnesium carbonate, 3.52; magnesium sulfate, 3.83; potassium chloride, 12.47; dibasic potassium phosphate, 21.88; sodium chloride, 7.71.

<sup>6</sup> Vitamin Diet Fortification Mixture, ICN Nutritional Biochemicals, Cleveland, OH: (in g/kg mix.) vitamin A (200,000 units/g), 4.5; vitamin D (400,000 units/g), 0.25; alpha tocopherol, 5; ascorbic acid, 45; inositol, 5; choline chloride, 75; menadione, 2.25; p-aminobenzoic acid, 5; niacin, 4.5; riboflavin, 1; pyridoxine hydrochloride, 1; thiamin hydrochloride, 1; calcium pantothenate, 3; (in mg/kg mix.) biotin, 20; folic acid, 90; vitamin B-12, 1.35.

carried out in 80 volumes of ethanol. White crystals were obtained: m.p. 190 to 192°.

*Other materials.* Amygdalin and linamarin were commercially obtained (Calbiochem-Behring Corp., San Diego, CA). They were shown to be pure by TLC and were used as obtained. LOM was a solvent-extracted meal available locally and was used without further treatment.

*Statistical analysis.* All values are reported as the mean  $\pm$  standard deviation. Student's *t*-test for unpaired values was used to evaluate differences between treatment groups and the selenium control (11). Correlation coefficients were determined as prescribed by Steel and Torrie (11).

#### RESULTS AND DISCUSSION

The flow diagram in figure 1 illustrates the general procedures used to fractionate LOM. The figures in parentheses are the yields at each step in terms of percent of the original LOM. The first two steps were developed by Halverson et al. (6) and the third and fourth steps were patterned after the work of Jensen and Chang (7). Although the latter workers were able to extract the active principle directly from LOM with methanol, in our experience, a considerable portion of the activity could not be removed in this manner. Therefore, the extractions with aqueous ethanol and water were retained in the procedure. Growth assays were conducted at most steps, but only those for our final step will be reported.

The final purification step (fig. 1) involving HPLC resulted in the separation of the LOM extract into essentially three fractions. Peak 2 and Peak 3 appeared to be pure and could be crystallized from ethanol. The concentration of these two substances in the original LOM was 0.17 and 0.19%. These percentages probably represent the minimum, since no attempt was made to correct for incomplete extraction at the various steps.

Each of the LOM fractions isolated by HPLC was fed to rats in diets containing 9 ppm Se as sodium selenite (table 1). LOM itself, when fed at a level of 20%, significantly increased the 3-week body

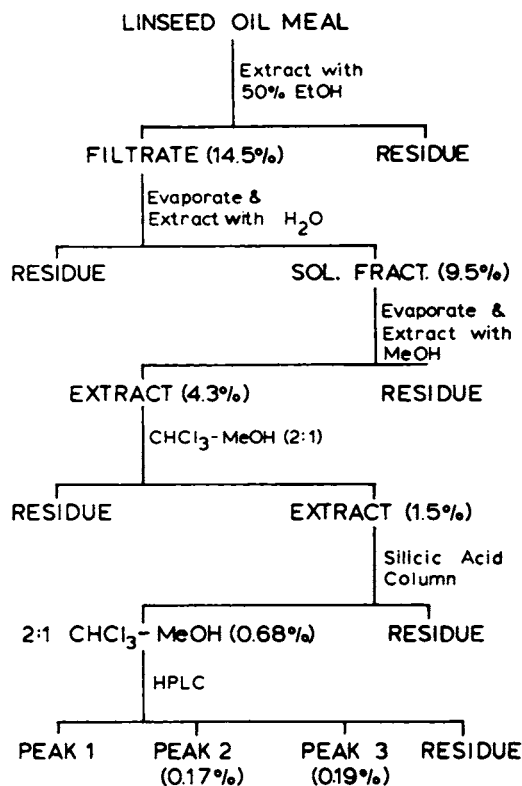


Fig. 1 Flow diagram showing the main features of the system for isolating the selenium protective factors from linseed oil meal. The numbers in parentheses are the percent yields based on original linseed oil meal.

weight gains over the control animals receiving just 9 ppm Se in the diet. The purified compounds obtained from HPLC Peaks 2 and 3 gave some growth response at the 0.1% level but it was slightly less than that required for statistical significance. When fed at 0.2%, both compounds promoted highly significant increases in weight gain. The response of the selenium-fed rats to each of the two compounds was actually greater than that of the rats fed 20% LOM. The efficacy of the two substances against selenium toxicity was about the same. Although only the weight gains at 3 weeks are reported, essentially the same results were observed for weeks 1 and 2.

Peak 1 was shown by TLC to contain a mixture of several substances and it gave no protection against 9 ppm Se at either the 0.1 or 0.2% level. No further attempts

TABLE 1  
Effect of fractions of LOM and of amygdalin on the toxicity of selenium to rats<sup>1</sup>

Additions to diet	Average wt. gains after 3 weeks	Liver wt.	Kidney wt.	Selenium concentration		
				Liver	Kidney	Muscle
				g	g	g
None	165.0 ± 8.7	16.01 ± 1.73	1.013 ± 0.050	0.63 ± 0.06	0.89 ± 0.05	0.20 ± 0.01
20% LOM	170.4 ± 6.6	16.71 ± 0.51	1.049 ± 0.048	1.05 ± 0.62	0.84 ± 0.04	0.21 ± 0.02
Na <sub>2</sub> SeO <sub>3</sub> (9 ppm Se)	57.8 ± 20.4	7.75 ± 2.94	0.692 ± 0.103	5.45 ± 1.34	26.3 ± 12.0	0.36 ± 0.05
20% LOM + 9 ppm Se	108.0 ± 26.3 <sup>2</sup>	13.10 ± 3.70 <sup>2</sup>	0.991 ± 0.085 <sup>2</sup>	4.60 ± 0.45	11.0 ± 2.6 <sup>2</sup>	0.43 ± 0.09
0.1% Peak 2 + Se	75.0 ± 17.7	9.56 ± 0.80	0.806 ± 0.113	5.11 ± 0.80	24.0 ± 5.7	0.39 ± 0.08
0.2% Peak 2 + Se	119.0 ± 14.6 <sup>2</sup>	13.00 ± 3.43 <sup>2</sup>	1.038 ± 0.115 <sup>2</sup>	4.12 ± 0.52	15.9 ± 4.1	0.38 ± 0.06
0.1% Peak 3 + Se	78.6 ± 24.4	10.02 ± 3.51	0.793 ± 0.141	4.66 ± 0.61	22.1 ± 5.3	0.41 ± 0.06
0.2% Peak 3 + Se	123.8 ± 17.7 <sup>2</sup>	15.05 ± 2.23 <sup>2</sup>	1.010 ± 0.143 <sup>2</sup>	4.63 ± 0.88	16.9 ± 8.9	0.42 ± 0.08
0.1% Peak 1 + Se	51.2 ± 24.0					
0.2% Peak 1 + Se	47.8 ± 16.2					
0.1% Amygdalin + Se	49.4 ± 8.5	5.46 ± 1.71	0.723 ± 0.082	7.80 ± 1.32	31.9 ± 10.3	0.32 ± 0.05
0.2% Amygdalin + Se	75.8 ± 20.3	8.19 ± 1.99	0.839 ± 0.138	4.83 ± 1.02	28.8 ± 11.5	0.38 ± 0.06

<sup>1</sup> Values are mean ± SD for five rats. <sup>2</sup> Significantly different at the  $P < 0.01$  level from the 9 ppm Se group by  $t$ -test. <sup>3</sup> Significantly different at the  $P < 0.05$  level from the 9 ppm Se group by  $t$ -test.

were made to characterize this fraction. Preliminary studies indicated that the residual materials, which could be removed from the HPLC column by methanol, were also inactive.

Early work used liver to body weight ratios as a measure of selenium toxicity (6). In our studies, the rats were not as severely affected by the level of selenium used, and therefore the liver to body ratios were not as enlightening. However, the data in table 1 show that selenium greatly reduced liver size, whereas 0.2% levels of Peak 2 or 3 restored liver size to that of the rats fed the basal diet or the selenium diet + LOM. These data, along with the

body weight, suggest that the substances in Peak 2 and Peak 3 can account for the protective activity of linseed oil meal.

Preliminary TLC and spectral data indicated that the two isolated substances resembled cyanogenic glycosides. Amygdalin, a known glycoside with a nitrile constituent, was also fed to rats receiving the toxic selenium diet. Table 1 shows that feeding amygdalin at 0.2% of the diet gave a growth response nearly equal to that obtained from 0.1% of the compounds in Peak 2 and 3, but the response was not significant. There was also a slight but non-significant increase in liver size in the amygdalin-treated rats.

Linamarin and lotaustralin are cyanogenic glycosides which have been reported to occur in flax (12). A commercial source was located for linamarin only, and table 2 shows the effect of feeding the substance to rats receiving the toxic selenium diet. Linamarin at a level of 0.1% was not effective against 9 ppm Se but when fed at 0.2%, the compound gave significant increases in 3-week gains over the selenium controls. The same results were obtained with respect to liver protection; 0.2% linamarin significantly increased liver size.

The structures of the two compounds in Peak 2 and Peak 3 have been determined and the details of structural elucidation will

TABLE 2  
Effect of linamarin on the toxicity of selenium to rats<sup>1</sup>

Additions to diet	Average wt. gain in 3 weeks	
	Liver wt.	
	g	g
None	166.4 ± 17.4	14.97 ± 1.52
Na <sub>2</sub> SeO <sub>3</sub> (9 ppm Se)	44.0 ± 12.4	4.76 ± 0.85
0.1% linamarin + Se	56.4 ± 14.5	5.55 ± 1.82
0.2% linamarin + Se	80.0 ± 24.9 <sup>2</sup>	8.20 ± 2.02 <sup>2</sup>

<sup>1</sup> All values are mean ± SD for five rats. <sup>2</sup> Significantly different from the 9 ppm Se group at the  $P < 0.05$  level. <sup>3</sup> Significantly different from the 9 ppm Se group at the  $P < 0.01$  level.

be reported elsewhere (12). Figure 2 gives the proposed structures along with those of linamarin, lotaustralin and amygdalin. Compound 2, which was isolated as HPLC Peak 2 and has been named linustatin, is closely related to linamarin. Compound 3, isolated from Peak 3, has been named neolinustatin and is closely related to lotaustralin. The unique feature that these protective substances have in common is that all are cyanohydrin glycosides. Upon hydrolysis, they should all yield HCN. It has been established that cyanide is detoxified in mammals by the reaction with thiosulfate under catalysis by rhodanese to form thiocyanate (13). Besides thiosulfate, the sulfur donor substrates include thiosulfonate, persulfides and polysulfides (14). It is likely that selenium analogs of the sulfur compounds may also be formed and it has been suggested that selenopersulfides play an active role in selenium metabolism (15, 16). Conceivably, cyanide released from the glycosides might react with such forms of selenium to form selenocyanates. Studies by Prohaska et al. (17) support this hypothesis. By such a mechanism, excessive levels of the element could be detoxified. Injection and feeding studies with cyanide (18) support this mechanism and explain adequately the protective nature of the cyanogenic glycosides but not the observed structural specificity.

From the feeding trial (table 1) it seems that the various cyanogenic glycosides have different efficacy against selenium toxicity. Amygdalin appeared to be relatively inactive. Although linamarin did show some protective effect, the levels required were equivalent to much higher cyanide levels than required for linustatin and neolinustatin. Some of the apparent differences in activity may be due to differences in ease of hydrolysis of the compounds. For instance, it is known that amygdalin is subject to hydrolysis by emulsion but linamarin is only slowly hydrolyzed by the same  $\beta$ -glucosidase (14). Some workers have reported that animals lack the  $\beta$ -glucosidases necessary to hydrolyze the glycosides when fed in pure form (12). In these studies, the cyanide was apparently re-

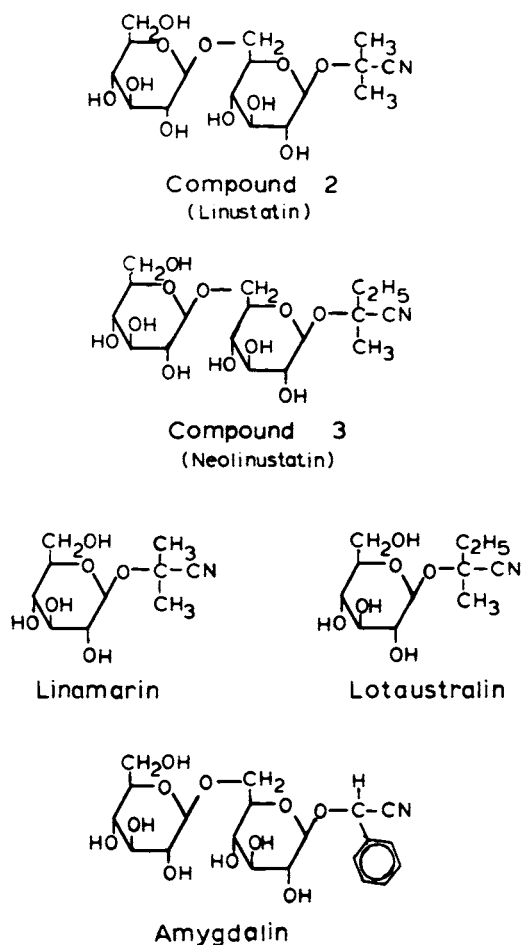


Fig. 2 Structures of the two cyanogenic glycosides isolated from linseed oil meal along with some related glycosides. Compound 2 was obtained from HPLC Peak 2 and Compound 3 from HPLC Peak 3. Trivial names which have been assigned are shown in parentheses.

leased either by animal enzymes or by intestinal bacterial action.

Table 1 also gives data on kidney size and selenium concentration in the liver, kidney and muscle. Supplementing the diet with 20% LOM or 0.2% of linustatin (Peak 2) or neolinustatin (Peak 3) significantly increased kidney weights over those from rats fed diets with just 9 ppm Se. With respect to selenium concentration in the tissues, there was no significant effect of LOM or the purified glycosides on the selenium concentration in liver and muscle. Previous reports in the literature have shown increased tissue selenium concen-

trations in the presence of LOM (2, 8). In the data reported here, there appears to be a trend in which LOM or the compounds in Peak 2 and 3 lower the concentration of selenium in the kidney as compared to that in the kidneys of the control animals. However, the differences were significant for only LOM itself.

The data tend to show that the smaller the average organ weight, the higher the selenium concentration. When correlation coefficients were determined for average size versus average selenium concentration, fairly high negative correlations were observed ( $r = -0.75$ , liver;  $r = -0.84$ , kidney). The same trend was observed when the values for individual animals were considered except that slightly lower coefficients were obtained ( $r = -0.54$ , liver;  $r = -0.66$ , kidney).

The data presented in this study suggest that the two cyanogenic glycosides, linustatin and neolinustatin, are responsible for the protective action of linseed oil meal against selenium toxicity.

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