

Insecticidal Components from Field Pea Extracts: Soyasaponins and Lysolecithins

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Extracts from field peas (*Pisum sativum* L.) have previously been shown to have a utility to control insect pests. To identify potentially new bioinsecticides in field crops, we describe the fractionation of impure extracts (C8 extracts) derived from protein-rich fractions of commercial pea flour. The activity of separated fractions was determined by a flour disk antifeedant bioassay with the rice weevil [*Sitophilus oryzae* (L.)], an insect pest of stored products. Bioassay-guided fractionation showed that the triterpenoid saponin fractions were partly responsible for the antifeedant activity of C8 extracts. Soyasaponin I (soyasaponin Bb), isolated from peas and soybeans, and mixtures of soyasaponins, comprised of soyasaponins I–III and isolated from soybeans, were inactive antifeedants, but dehydrosoyasaponin I (the C-22 ketone derivative of soyasaponin I), a minor component found in C8 extracts, was shown to be an active component. Dehydrosoyasaponin I (soyasaponin Be) and soyasaponin VI (soyasaponin β g) coeluted under conditions of silica gel thin-layer chromatography and C18 high-performance liquid chromatography. However, dehydrosoyasaponin I could be isolated from saponin-enriched fractions with a reversed phase column of styrene/divinylbenzene operated at alkaline pH. Phospholipids of the lysolecithin type were also identified in saponin fractions of C8 extracts from peas. Three of the lysolecithins were inactive alone against rice weevils, but mixtures of these phospholipids enhanced the insecticidal activity of dehydrosoyasaponin I.

KEYWORDS: *Pisum sativum*; soyasaponins; lysolecithins; yellow field peas; bioinsecticides

INTRODUCTION

Certain legume plants, including seeds of the pea (*Pisum sativum* L.), are toxic to insects (1–4). Bodnaryk et al. (5) showed that the flour from air-classified field peas was insecticidal to stored-product insects. Protein-rich pea flour was more effective against stored-product insects than starch or fiber-enriched fractions (5, 6). An extraction procedure was developed with hot 80% methanol to obtain crude insecticidal pea extracts. The aqueous methanol extracts from defatted, protein-rich flour were partially purified with reversed phase C8 silica (5). Activity was found in fractions obtained by elution of the C8 silica column with methanol. These C8 extracts displayed insecticidal and antifeedant activities against rice weevil [*Sitophilus oryzae* (L.)] and other stored-product insects, but the active ingredients of C8 extracts were not identified.

The purpose of this work was to isolate and identify the antifeedant components of C8 extracts derived from air-classified protein-rich field pea flour. We report here the bioassay-guided fractionation of C8 extracts by silica gel chromatography, describing active components contained in

fractions of intermediate polarity. An accompanying publication describes the characterization of highly polar end fractions from silica gel chromatography.

MATERIALS AND METHODS

Materials. Protein-rich pea flour (~54% protein, 24% fiber, 8% starch, 7% water, 5% ash, and 2% fat) was supplied by Parrheim Foods (Saskatoon, SK). Soybean flour (type I), Dowex 50WX8-400 resin, 3-hydroxy-2-methyl-4-pyrone (maltol), L- γ -linoleoyl- α -lysolecithin (L-18:2), L- γ -oleoyl- α -lysolecithin (L-18:1), and L- γ -palmitoyl- α -lysolecithin (L-16:0) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). MCI gel CHP20P (Mitsubishi Chemical Industries) was purchased from Supelco Inc. (Bellefonte, PA). Organic solvents were either OmniSolv glass-distilled grade (Merck, Darmstadt, Germany) or high-performance liquid chromatography (HPLC) grade (Fisher Scientific, Nepean, ON). Water was purified in the laboratory with a Millipore Super-Q system (Bedford, MA).

Thin-Layer Chromatography (TLC). Precoated silica gel 60 F₂₅₄ plastic sheets (Merck) of 0.2 mm layer thickness were used and developed with a solvent mixture of the lower layer of chloroform–methanol–water, 65:35:10.

HPLC. The samples were prepared in 80% methanol at 1–4 mg/mL and syringe filtered (0.45 μ m pore size, nylon membrane type, Chromatographic Specialties, Brockville, ON) into 0.25 mL glass autosampler vial inserts (Fisher no. 03-375-3A). The solutions were maintained at 15 °C before injection (10 μ L volume). The instrument

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consisted of an Alliance 2690 separations module (Waters Canada, Mississauga, ON) equipped with vacuum solvent degassing, a Waters 996 photodiode array detector, and a PL-EMD-960 evaporative light scattering detector (ELSD) (Polymer Laboratories, Amherst, MA) controlled by Waters Millennium software. A Waters reversed phase C18 Symmetry column (3.0 mm \times 150 mm, 5 μ m particle size) maintained at 30 °C was used. The mobile phase consisted of 0.05% trifluoroacetic acid (TFA) in water (solvent A) and 0.05% TFA in acetonitrile (solvent B), delivered at a flow rate of 0.4 mL/min. The gradient elution program consisted of 95% A and 5% B at time 0. After 30 min, the composition was 5% A and 95% B (linear, curve 6 gradient) and maintained at that proportion for 5 min. The gradient changed back to 95% A and 5% B from 35 to 43 min. The run time was 45 min.

Mass Spectrometry. Positive ion electrospray ionization (ESI) mass spectra were obtained with a benchtop tandem quadrupole mass spectrometer (Quattro LC, Micromass UK Limited) equipped with an atmospheric pressure ESI source interfaced directly to a Waters Alliance 2690 separations module. Nitrogen gas was used for nebulization and desolvation. The instrument was controlled by Micromass MassLynx software (version 3.3) running under Microsoft Windows NT. A reversed phase C18 Symmetry column (2.1 mm \times 150 mm, 5 μ m particle size) held at 30 °C was used for HPLC/MS. The mobile phase (flow of 0.2 mL/min) consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The starting mixture was 95% solvent A–5% solvent B. A 20 min linear gradient was applied, ending with 5% solvent A–95% solvent B and held for 5 min before recycling back to the original conditions. Collision-induced dissociation (CID) experiments were conducted on the Quattro LC in the positive ion mode with argon as the collision gas with collision energies of 30–35 eV. The Waters Alliance 2690 module was used for sample introduction (as above) but with a union replacing the C18 column. Solvent A and solvent B were delivered isocratically (0.2 mL/min) at a constant 1:1 ratio. Quasimolecular ions (MH⁺) were used to generate daughter ion spectra.

IR and NMR Spectroscopy. IR spectra were recorded on an ATI Mattson Genesis series Fourier transform (FT) spectrometer, using potassium bromide disks. FT-NMR spectra were obtained (in pyridine-*d*₅) with a Bruker AVANCE 400 spectrometer operating at 100 MHz (for carbon-13).

Isolation of C8 Extracts. Protein-rich pea flour, obtained from the pea mill following air classification (7), was extracted in the laboratory (5). The C8 extracts were isolated in the last step using C8 SepPak Vac cartridges (Waters Corp., Milford, MA). Utilizing two C8 SepPak Vac cartridges per 100 g of flour, the C8 extracts (in methanol) were combined, rotary evaporated, and concentrated to dryness at 43 °C with a centrifugal evaporator (model SC 110A Savant SpeedVac Plus). Starting with 100 g of defatted, protein-rich flour, C8 powder (beige in color) was obtained in 0.7–0.9% yield (*N* = 10).

Bioassays. The antifeedant activity was assessed with a flour disk bioassay (8) with 70% ethanol as the solvent. Twenty-five *S. oryzae* adults (1–2 weeks old) were held on five wheat flour disks for 3 days at 30 °C and 70% relative humidity. Flour disks (ca. 0.1 g/disk) were weighed before and after exposure to the insects. The antifeedant activity was determined by expressing the consumption of treated disks as a percentage of control disks (70% ethanol). Positive controls using the same C8 extract were run with each bioassay. Over the interval that bioassays were conducted, the C8 extract at a dose of 1.6 mg/200 mg flour gave a mean food consumption value of 25.2% (*N* = 17), a standard deviation of 11.0%, a minimum of 9.8%, a maximum of 42.7%, and a 95% confidence interval of 5.6%. In some bioassay experiments, the insecticidal activity was assessed as median survival time by Kaplan–Meier survival analysis (log-rank) using SigmaStat (SPSS Inc., Chicago, IL). After the disks were weighed, *S. oryzae* and flour disks were returned to the Petri dishes and survivors were noted each day until the insects had been on the disks for a total of 14 days. A close correlation was previously found between the antifeedant activity and the toxicity of pea extracts (5, 9).

Fractionation of C8 Extracts by Silica Gel Column Chromatography. C8 powder (130 mg) was applied to a glass column (1.2 cm \times 20.5 cm) filled with silica gel (Mallinckrodt SilicAR cc-7). The column

was eluted with chloroform (75 mL) and then with 75 mL volumes of various mixtures of chloroform and methanol followed by pure methanol. The column was washed with more methanol (75 mL) and finally with two additional volumes of methanol (250 mL each). Each of the 12 fractions was evaporated on a Buchi Rotavapor R-114 apparatus with the aid of a Buchi B-169 vacuum system and a water bath (maintained at \leq 45 °C). The residue that remained in each flask was transferred to test tubes by washing successively with 95% ethanol (1 mL), water (0.5 mL), and 95% ethanol (2 mL). A final evaporation was performed with a Savant apparatus (43 °C). The tubes were weighed, capped, and transferred to the bioassay laboratory. Each sample was dissolved in 70% ethanol (0.5 mL), and 0.2 mL of that solution was added to the wheat flour (200 mg) for preparation of the disks. Dose–response experiments were conducted on active extracts (C8-1a and C8-1b), by taking 200, 100, 50, 25, 12.5, and 6.25 μ L aliquots of the original bioassay solution and adjusting the final volume for bioassays to 0.2 mL with 70% ethanol. The control disks were prepared with 0.2 mL of 70% ethanol.

Fractionation of C8 Extracts by Silica Flash Chromatography. A FLASH 40 M apparatus (Biotage Inc., Charlottesville, VA) equipped with a prepacked 90 g (4 cm \times 15 cm) KP-Sil (Biotage) cartridge (32–63 μ m, 60 Å silica) was used. C8 extracts (250 mg) were prepared for chromatography by mixing with Biotage silica (5 g). The mixture was transferred to a sample injection module, and the solvent flow rate of the lower layer of chloroform–methanol–water (65:35:10) was maintained at approximately 20 mL/min. Initially, fractions were collected in 8 mL test tubes and examined by TLC, visualizing the chromatograms by UV light and then by spraying with ninhydrin followed by the Liebermann–Burchard reagent. The fractions were combined on the basis of high, intermediate, and low *R*_F values, corresponding to compounds eluting with 0–365 (combined fraction 1; low polarity), 366–730 (combined fraction 2; intermediate polarity), and 731–1000 (combined fraction 3; high polarity) mL. Continued elution with 250 mL of methanol gave fraction 4 of very high polarity. All fractions were rotary evaporated, and the residue was transferred to test tubes using 70–95% ethanol before Savant evaporation.

Soyasaponin I (S-I). The isolation of soyasaponins from soybean meal was carried out by the procedures of Kitagawa et al. (10, 11) in a pilot plant (POS Pilot Plant Corp., Saskatoon, SK). Small portions of the crude soyasaponin mixture, isolated as sodium salts, were purified by the following methods:

(a) *With Diaion HP20.* Using a FLASH 40 M chromatography apparatus, the crude mixture (0.5 g) dissolved in water (5 mL) was syringe injected onto a 180 mL cartridge (Biotage) of Diaion HP20 (250–600 μ m, 300–600 Å) and eluted with water (200 mL), with water containing 50% methanol (200 mL) and finally with methanol (400 mL), using a flow rate of 30 mL/min. The methanol fraction was concentrated and dried in a vacuum desiccator to give 246 mg of a soyasaponin mixture (sodium salts), as an off white solid.

(b) *With Dowex 50WX8-400.* This resin (10 g, H form) was mixed with water (25 mL) and slurry packed in a 1.5 cm \times 12 cm polypropylene column followed by rinsing successively with 1 M hydrochloric acid (30 mL), water (120 mL), and methanol (90 mL). The soyasaponin mixture (sodium salts) (136 mg) in methanol (100 mL) was passed through the column at 5 mL/min. The methanol was collected and evaporated (rotary then Savant). A soyasaponin mixture (free acids) was obtained, as an off white, hygroscopic solid (155.6 mg) that was highly soluble in methanol and aqueous methanol or ethanol mixtures. The crude soyasaponin mixture (sodium salts) (106 mg) without Diaion HP20 cleanup gave 101 mg of a free acid mixture.

(c) *With Silica Gel.* A sample of the soyasaponin mixture (1.85 g) cleaned up with Diaion HP20 was subjected to silica gel flash chromatography as described. Fractions eluting with the first 600 mL gave 774 mg of a brown solid that contained low S-I content by TLC and HPLC. The 200 mL fraction that followed on solvent evaporation gave 444 mg of a white solid, composed principally of the sodium salt of S-I. The last fraction (800–1200 mL) on evaporation gave an additional quantity (327 mg) of S-I but of lower purity.

(d) *With Reversed Phase Silica Gel.* A cartridge of C18 silica gel (55 g of Biotage KP-C18-HS, 35–70 μ m, 60 Å) in a FLASH 40 M apparatus was conditioned with a mixture of 95% water and 5%

methanol (300 mL total) followed by 50% methanol (250 mL) and 90% methanol (250 mL). The cartridge was equilibrated with 250 mL of 70% methanol. A portion (180 mg) of the main sample from method (c) was neutralized with Dowex 50WX8-400 (method b), and the resulting free acid mixture (165 mg) in 70% methanol (1.65 mL) was injected. After it was eluted at 15 mL/min with 70% methanol (250 mL), the solvent was switched to 90% methanol (400 mL) and the center fractions contained mostly S-I. Savant evaporation at 43 °C gave 95 mg of a white solid, identified as the free acid of S-I by ESI mass spectrometry (molecular weight of 942) and by recording the FT-IR and carbon-13 FT-NMR spectra and comparing the spectra to those described in the literature (11, 12).

Soyasaponin VI (S-VI). Defatted, protein-rich pea flour (50 g) was homogenized for 1 min in a Waring blender with ice-cold 80% methanol (500 mL) and centrifuged at 8000g for 10 min, according to Tsurumi et al. (12). The supernatant was concentrated on a rotary evaporator (bath temp < 30 °C), and the concentrated extract (80 mL) was subjected in 20 mL portions to Diaion HP20 flash chromatography. The resulting methanol extracts were concentrated by rotary evaporation, diluted with water, and freeze-dried to give 284 mg of a light brown solid, which was shown by TLC to consist of a complex mixture. However, some of the components appeared to be common with those of C8 extracts, including at least two soyasaponins (Liebermann–Burchard positive spots) and other spots that were ninhydrin-positive. This mixture (130 mg) was subjected to flash chromatography with the lower layer of chloroform–methanol–water (65:35:10) at a flow rate of 7 mL/min using a Biotage FLASH 12i apparatus with a prepacked 8 g (1.2 cm × 15 cm) KP-Sil (Biotage 12M) cartridge (32–63 μm, 60 Å silica).

Additional Fractionation of C8-2a. MCI gel CHP20P was washed with methanol and water according to instructions from the supplier. The gel was slurry packed into a 1.6 cm × 45 cm column (XK model, Amersham Biosciences Inc., Baie d'Urfé, Québec, Canada). A C8-2a extract (46 mg) in 80% methanol (5 mL) was loaded onto the column with a pump (model RP-D, Fluid Metering Inc., Syosset, NY). With a flow rate of 3 mL/min (2 bed volumes/h), the column was eluted with a step gradient of 100% water to 90% methanol in 10% increments (50 mL fractions). Elution was continued with 100% methanol. Evaporation of the solvent showed that the first eluting material was contained in the 80% methanol fraction. Appropriate fractions were combined on the basis of similar TLC profiles with Liebermann–Burchard spray.

Isolation of Dehydrosoyasaponin I (D-I). An AKTAEplorer 100 medium-pressure LC (Amersham Biosciences) equipped with dual P-901 pumps, a UV-900 absorption monitor with a 2 mm (3 μL) flow cell, and a fraction collector (Frac-901) were used. The system was controlled by Unicorn software (version 3.00). Separations were achieved at ambient temperature with a 3 mL (6.4 mm × 100 mm) reversed phase column packed with 15 μm polystyrene/divinylbenzene beads [Resource 15 RPC (reversed phase chromatography), Amersham Biosciences]. The mobile phase delivered at a flow rate of 3 mL/min consisted of (A) 0.035% (10 mM) ammonium hydroxide (prepared by dilution with water of a 100 mM analytical concentrate from J. T. Baker Chemical Co., Phillipsburg, NJ) and (B) 0.018% ammonium hydroxide in 50% acetonitrile (prepared by dilution of eluent A with an equal volume of acetonitrile). The initial conditions were 90% A and 10% B (5% acetonitrile) for 5 min. A linear gradient was applied by increasing the acetonitrile to 25% over 13.3 min (13.3 column volumes). Another more shallow gradient to 35% acetonitrile was applied during the next 35 min. Thereafter, a 50% acetonitrile concentration was achieved during 7 min and maintained for 5 min before recycling to the initial conditions. Samples for RPC were dissolved in 80% methanol (typically at a concentration of 15 mg/mL) and filtered, and about 0.225 mL (3.5 mg) was directed with a peristaltic pump (model P-910) to a sample loop (0.5 mL) and the column. Fractions (1 mL) were collected, combined as appropriate, and bubbled with nitrogen gas before transferring to test tubes for Savant evaporation at 43 °C.

RESULTS AND DISCUSSION

Samples of the C8 extract isolated in powder form were moderately soluble in water. The pH was 6. Ethyl acetate

Table 1. Fractionation of a Blank Sample and a C8 Extract (130 mg) by Column Chromatography with Silica Gel and Antifeedant Activity of Resulting Fractions

eluent composition			isolated fractions			
			blank ^a		C8	
chloroform (%)	methanol (%)	volume (mL)	weight (mg)	f.c. ^b (%)	weight (mg)	f.c. ^b (%)
100	0	75	<1	123	18	99
98	2	75	<2	109	2	100
95	5	75	<2	104	2	76
90	10	75	<1	94	4	56
80	20	75	1	106	3	119
60	40	75	<1	106	8	113
40	60	75	<2	116	12	15 ^c
20	80	75	<1	117	12	45
0	100	75	<1	117	6	42
0	100	75	1	110	5	36
0	100	250	2	101	6	24 ^c
0	100	250	1	106	3	42

^a Chromatography was performed without the C8 extract. ^b Food consumption in the rice weevil antifeedant bioassay, expressed as % of control. The total amount of each isolated fraction was tested in 200 mg of flour. ^c Dose–response experiments were performed on these active fractions, designated C8-1a and C8-1b, respectively (see Figure 1).

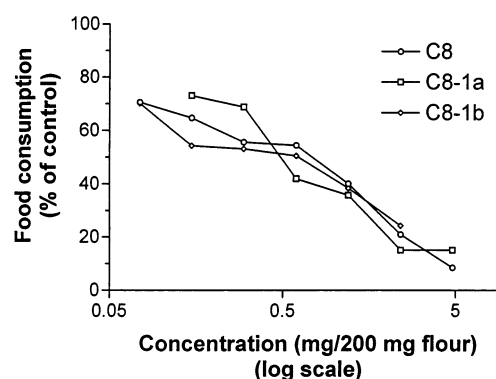


Figure 1. Comparison of dose–response curves in antifeedant bioassays with rice weevils (*S. oryzae*) using a crude C8 extract and partially purified extracts from silica gel column chromatography with 60% methanol–40% chloroform (C8-1a) and 100% methanol (C8-1b).

extraction at pH values of 6, 7.5, and 9.5 gave trace quantities of organosoluble material that did not show activity in the rice weevil bioassay. Fractions containing active components were obtained by extraction of the aqueous layer with *n*-butanol.

Following column chromatography (Table 1), C8 powders gave two chromatographically distinct, high-polarity bands that showed good antifeedant activity; the first (12 mg, 15% food consumption) appeared in the 40% chloroform (60% methanol) fraction, designated C8-1a. The second active, very highly polar band eluted gradually with 80–100% methanol and was collected as five separate fractions at the end of the experiment. These end fractions, collectively representing about 25% of the mass of applied C8 extract, gave food consumption values of 24–45%. The most active of these five fractions, designated as C8-1b, was compared in a dose–response experiment to C8-1a and to a C8 extract (Figure 1). Dose–response curves were similar for the three extracts.

Some of the TLC spots in the sample of C8-1a gave a gray-colored response to Liebermann–Burchard, indicative of the presence of triterpene saponins (13), whereas most of the spots from C8-1b were positive to ninhydrin, indicating that this fraction probably contained nitrogen-containing compounds such

Table 2. Fractionation of a C8 Extract (250 mg) by Flash Chromatography with a Biotage Silica Cartridge

fraction	R_f^a	weight (mg)	f.c. ^b	relative polarity
1	>0.5	65	109	low
2 (C8-2a)	0.25–0.5	48	39	intermediate
3	<0.25	7	71	high
4 (C8-2b) ^c	<0.25	28	52	high

^a With silica gel TLC plates. Detection techniques are discussed in the text.

^b Food consumption, expressed as % of control. Experimental samples were tested in the rice weevil antifeedant bioassay at a concentration of 1.6 mg/200 mg flour (1.2 mg for fraction 3). ^c This end fraction was obtained by elution with methanol (250 mL).

as amino acids or peptides (14). This implied that at least two chemically distinct insecticidal components were present in C8 extracts.

Flash chromatography with silica gel was used to fractionate the C8 mixture into the equivalent of C8-1a and C8-1b, ensuring that there was a clear separation between these active fractions. Antifeedant activity could not be demonstrated in the major, nonpolar fraction (Table 2). Fraction 2, designated C8-2a and representing about 20% of the mass of applied C8 extract, was similar in activity and TLC profile (Liebermann–Burchard-positive spots) to C8-1a from the silica gel column. Combined fractions 3 and 4, designated as C8-2b, represented a low yield of high-polarity, ninhydrin-positive components that were similar to C8-1b from the column.

Evidence from TLC and HPLC suggested that extracts of C8-2a, readily obtained by flash chromatography, contained S-I as a major component. Thus, authentic samples of S-I showed the same TLC properties as the major component of C8-2a, using various solvent systems and spray reagents. HPLC clearly illustrated the similarity of these two samples, as illustrated by the ELSD traces shown in Figure 2. Although several late-eluting minor components were found in C8-2a, it was reasonable to suspect that S-I might be responsible for the antifeedant properties of the C8-2a extract. However, Bodnaryk et al. (5) showed previously that their sample of S-I was nearly inactive in the rice weevil bioassays. In the present work, samples of S-I of various purities were isolated from soybean meal. None of the preparations of S-I approached the antifeedant activity of C8-2a.

Because S-I could not explain the activity of extracts of C8-2a, the findings of Tsurumi et al. (12) were considered because they reported that pea seedlings contained S-I in the form of a 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyrone (DDMP) conjugate at the C-22 hydroxyl group (S-VI, also termed soyasaponin β g, soyasaponin BeA, or chromosaponin I). They suggested that S-I does not occur in the free form in peas but is formed from S-VI during extraction. This type of conjugation has also been demonstrated in dehulled, mature pea seeds (15, 16) and in other legume seeds, including alfalfa (17), soybeans (18–20), scarlet runner beans (21), and adzuki beans (22). The hydrolytic reaction at C-22, generating maltol and S-I, is reputed to occur gradually in solution, is promoted by heat, and is catalyzed by mild alkaline conditions. It was therefore of interest to determine if S-VI was present in C8 extracts and whether this compound (or maltol) possessed antifeedant activity.

A mixture containing S-VI was isolated by the method of Tsurumi et al. (12) and purified by silica gel flash chromatography (Table 3). Antifeedant activity of early fractions 1–3 was absent, but the activity tended to increase in fractions 4–6. Fraction 5 was of particular interest because the sample contained S-I and S-VI predominantly, and unlike fraction 6,

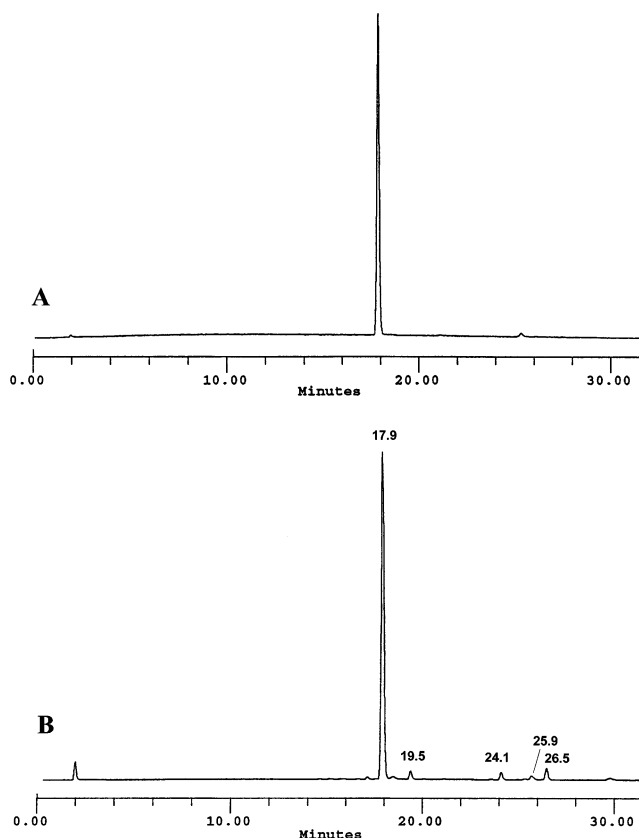


Figure 2. Comparison by HPLC with a reversed phase C18 Symmetry column and an ELSD of (A) a reference sample of inactive S-I with a retention time of 17.9 min and (B) an active S-I-enriched extract (C8-2a) isolated from C8 material by silica flash chromatography.

Table 3. Fractionation of Cold Methanol Extracted (12) and Freeze-Dried Material (130 mg) by Flash Chromatography with a Biotage Silica Cartridge

fraction	elution volume (mL)	weight (mg)	soyasaponins ^a		f.c. ^b
			I	VI	
1	0–42	32	–	–	99
2	43–57	8	–	–	112
3	58–72	9	–	–	111
4	73–87	9	+	+	78
5	88–102	7	+ ^c	+ ^c	55
6	103–500	9 ^d	+	+	28
maltol					110

^a The presence (+) or absence (–) of saponins as shown by TLC and HPLC.

^b Food consumption, expressed as % of control, in the rice weevil antifeedant bioassay. Experimental samples were tested at a concentration of 1.6 mg/200 mg of flour. ^c Also detected by HPLC/MS. ^d This sample contained primarily ninhydrin-positive components by TLC.

ninhydrin-positive TLC spots that could contribute to activity were undetectable. Because S-I was already shown to be inactive, it seemed that S-VI might be contributing to the activity. However, the potential influence of minor or cochromatographing components in this fraction needed to be evaluated.

We found that S-VI, a complex molecule of molecular mass 1068, was unstable in solution. During TLC and HPLC analyses, the content of S-I gradually increased in enriched samples of S-VI dissolved in aqueous alcohol solutions. Samples containing S-VI, freshly prepared in 80% ethanol, were shown to contain intact S-VI by HPLC/MS with ESI, by the appearance of the strong quasimolecular ion at mass 1069. However, the samples

Table 4. HPLC and ESI Mass Spectral Data on Identified Components of Active Fraction C8-2a (Fraction 2 of **Table 2**)

retention time (min)	relative peak areas ^a (%)	quasimolecular ion ^b (<i>m/z</i> of MH ⁺)	designated structure in Figure 5
17.9	93.2	943	S-I
19.5	1.7	941	D-I
19.5	c	1069	S-VI
24.1	1.6	520	L-18:2
25.9	1.0	496	L-16:0
26.5	2.5	522	L-18:1

^a Obtained by integration of HPLC peaks from a representative ELSD chromatogram (see **Figure 2B**) by peak area of indicated component/sum of peak areas \times 100. ^b These are *m/z* values for protonated molecular ions found in the corresponding peaks of the total ion chromatogram during HPLC/MS analysis.

^c Compounds D-I and S-VI coeluted during conditions used for HPLC so the area of these two components represented 1.7% of the mixture. During HPLC/MS, the leading edge of this peak showed predominantly the ion at *m/z* 941 whereas the trailing edge showed predominantly the *m/z* 1069 ion.

were always contaminated with S-I (quasimolecular ion at 943) and other components. Maltol, the other hydrolysis product that was inactive as an antifeedant (see **Table 3**), could easily be detected by TLC as a mobile, UV-active spot.

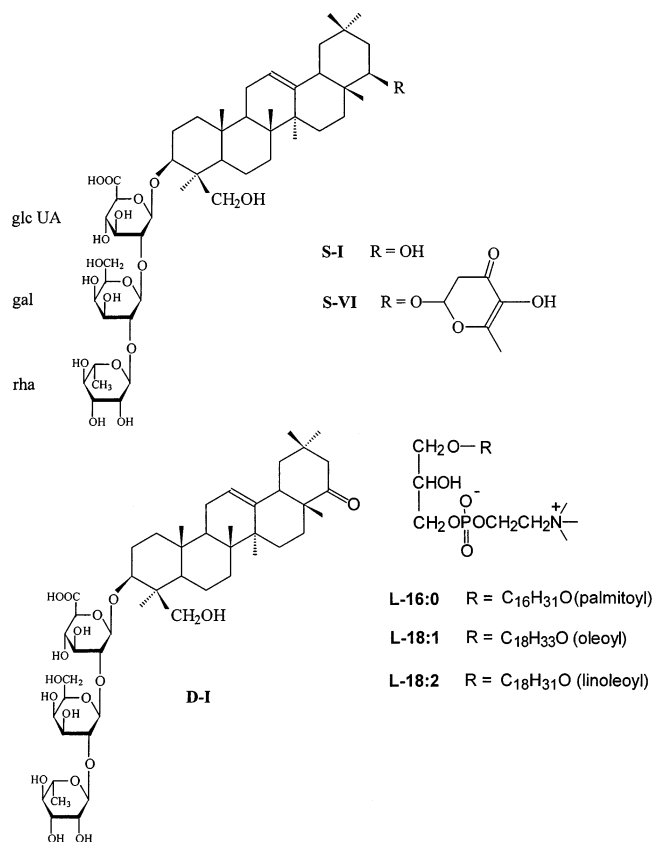
The HPLC properties of fraction 5 of **Table 3** were similar to fraction 2 (C8-2a) of **Table 2** except that fresh solutions of fraction 5 showed S-VI as the major peak at 19.5 min. The availability of this reference sample confirmed that S-VI was present as a minor component in C8-2a, at 19.5 min (see **Figure 2**). It therefore seemed unlikely that S-VI, of weak antifeedant activity, could account for the moderate to high activity of C8-2a, which contained S-VI as a minor component. A logical explanation might be that another active natural product with similar properties to S-I and S-VI coexisted in these extracts. S-VI has recently been reported (23) to stimulate the sugar taste receptor cells of the blowfly, *Phormia regina*, perhaps implicating this compound as a feeding stimulant for certain insects.

Identification of the minor components of C8-2a was attempted by HPLC and HPLC/MS (**Table 4**). The derived chemical structures are shown in **Figure 3**. The major HPLC peak at 17.9 min in C8-2a was confirmed as S-I by HPLC/MS. The peak at 19.5 min was of particular interest because not only the quasimolecular ion for S-VI was observed but also another prominent ion at *m/z* 941 was observed that corresponded to the molecular weight of D-I, a natural product that has recently been isolated from immature green peas (24). Previous workers have shown that D-I, which occurs as a minor component in soybeans, also coeluted with S-VI under reversed phase HPLC conditions (18).

Three late-eluting peaks representing about 5% of the mixture were found by HPLC/MS to represent phospholipids of the lysolecithin (lyso-phosphatidylcholine) type (25). Their identities were readily confirmed by comparison with authentic standards. Reference samples of L-16:0 and L-18:1 were inactive antifeedants, both giving values for food consumption of 102% in the rice weevil bioassay (1.6 mg/200 mg of flour). The reference sample of L-18:2, purchased as a mixture of lysolecithins, showed food consumption of 152% (114% at 1.44 mg/200 mg of flour).

There were very small peaks that eluted close to S-I, two with shorter retention times (17.3 and 17.7 min) and two with longer retention times (18.7 and 19.2 min). These four unidentified peaks gave observable ions during HPLC/MS at *m/z* 1029, 1045, 536 (534, 518), and 536 (534, 518), respectively.

The C8-2a extract was purified further by MCI gel chromatography (**Table 5**). The major component of fractions 1 and 2

**Figure 3.** Structures of the soyasaponins and lysolecithins identified in C8-2a extracts.

was S-I. Both fractions were nearly inactive. Fraction 3, representing the main fraction from the column, contained not only S-I as the major component but also D-I as a minor component. This fraction and fraction 4 were active, the latter appearing to be free of both S-I and S-VI but enriched in D-I. The major components in this fraction were the lysolecithins. This evidence suggested that D-I was the insecticidal factor in the C8-2a extracts.

D-I is known to occur as a minor component in alfalfa (26), soybeans (18), and other legumes (27–31). In mammalian *in vitro* experiments (31, 32), D-I was shown to be a high-affinity activator of calcium-dependent potassium channels and was 60-fold more potent than S-I as a potassium channel opener. To our knowledge, the influence of D-I on insects has not been reported.

Although MCI gel provided reasonable separation of S-I and D-I, the latter compound could not be isolated in pure form because of the coeluting lysolecithins. HPLC with C18 reversed phase columns did not offer a logical solution because D-I and S-VI coeluted under acidic conditions. Using RPC, we achieved good separation of these components with a column of polystyrene/divinylbenzene beads operated at pH 10.5 and a gradient composed of dilute ammonium hydroxide and acetonitrile.

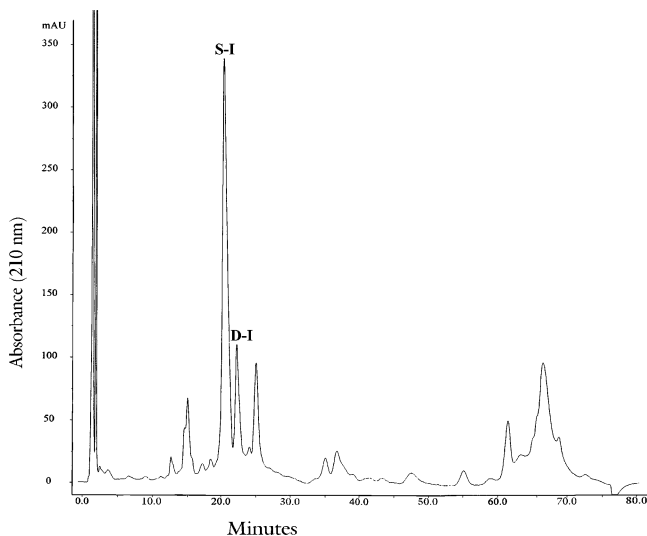
A chromatogram (210 nm trace) from RPC separation of D-I and S-I in a MCI gel fraction (fraction 3 of **Table 5**) is shown (**Figure 4**). Lysolecithins L-16:0, L-18:1, and L-18:2 were strongly retained under these conditions, eluting from 60 to 70 min. With RPC at pH 9 with ammonium bicarbonate buffer, the lysolecithins were less retained, eluting near 40 min.

The identity of D-I, isolated as a white solid, was established by CID experiments. Daughter ion fragments of S-I have been assigned previously (33). It was found that D-I fragmented under

Table 5. Fractionation of a C8-2a Extract by Liquid Chromatography with MCI Gel

fraction	elution solvent (volume)	weight (mg)	soyasaponins ^a		lysolecithins ^a			f.c. ^b
			S-I	D-I	L-18:2	L-16:0	L-18:1	
1	80–90% methanol (100 mL)	4.2	+	–	–	–	–	82
2	methanol (0–35 mL)	8.2	+	–	+	+	±	85
3	methanol (36–70 mL)	13.7	+	+ ^c	+	+	–	35
4	methanol (71–125 mL)	7.6	–	+ ^d	+	+	+	45

^a The indicated compounds were detected (+), were probably detected at trace concentrations (±), or were undetectable (–) as determined by TLC and HPLC. ^b Food consumption, expressed as % of control, in the rice weevil antifeedant bioassay. Experimental samples were tested at a concentration of 1.6 mg/200 mg of flour. ^c D-I (but not S-VI) was detected by HPLC/MS. The major component was S-I (see **Figure 4**). ^d Although D-I was the only saponin detected, the major components were lysolecithins.

**Figure 4.** Chromatogram illustrating the separation by medium pressure RPC (Resource 15 RPC column) of S-I and D-I contained in S-I-enriched fraction 3 from MCI gel liquid chromatography (3 mg injected).

appropriate CID conditions in an entirely analogous manner, yielding the same daughter ions as S-I but 2 mass units lower, corresponding to the difference in molecular masses of the aglycones. The daughter ion spectra suggested that the trisaccharide sequence (see **Figure 3**) was the same in both molecules.

Additional D-I was isolated by RPC on MCI gel fraction 4 and by RPC on saponin-enriched C8-2a extracts. It was also possible to isolate additional small samples of S-I and S-VI by RPC.

The RPC isolates of S-I, S-VI, and D-I gave food consumption values in the rice weevil bioassay of 92 (1.6 mg dose), 80 (1.6 mg), and 17% (1.7 mg), respectively. Saponins have previously been implicated as possible factors in the prevention of insect attacks on legume seeds (34, 35). Our findings implicate D-I as the insect-active soyasaponin of pea seeds.

Although the activity of D-I exceeded the activity of S-VI (and S-I), it did not seem reasonable that the presence of the low concentrations of D-I could account for the complete antifeedant effect of C8-2a extracts or of fractions of C8-2a obtained by MCI gel chromatography. Additionally, the S-VI isolated by RPC showed weaker antifeedant activity than the isolate of S-VI obtained by silica flash chromatography (fraction 5 of **Table 3**). This observation could be rationalized from HPLC/MS analyses because the latter isolate was contaminated not only with D-I but also with the lysolecithins.

It should be noted that samples of C8-2a, including the MCI gel-purified fractions of C8-2a, appeared to be free of high-polarity ninhydrin-positive components; that is, the TLC components of $R_F < 0.25$ (see C8-2b of **Table 2**). In addition, we were unable to detect these highly polar substances during

Table 6. Antifeedant and Insecticidal Activity of Pea Soyasaponins and Some Potentially Synergistic Mixtures

trial	soyasaponins (mg)			lysolecithins (mg)			f.c. ^a	median survival time (days, ± SE) ^b
	D-I	S-I	S-VI	L-18:2	L-16:0	L-18:1		
1	1.7						17	5.8 ± 0.2 a
2	1.3						65	>14 d
3	0.5						80	>14 d
4	0.4			0.4	0.4	0.4	33	6.7 ± 0.3 b
5	0.4	0.7 ^c		0.5	0.2	0.5	37	9.0 ± 0.5 c
6		0.4 ^c		0.4	0.5	0.4	85	>14 d
7				0.6	0.7	0.6	109	>14 d
8		1.6 ^c					77	>14 d
9			1.6 ^d				80	>14 d

^a Food consumption, expressed as % of control, in the rice weevil bioassay. Samples were tested at the indicated amounts in 200 mg of flour. ^b Kaplan–Meier survival analysis was used to estimate median survival times, and multiple comparisons were made with the Holm–Sidak method, $P = 0.05$. Medians followed by a different letter are significantly different. All insects in controls survived to 14 days. ^c Purified by methods a, c, b, and d. ^d Isolated by RPC.

HPLC of C8-2a, which were subsequently shown to elute near 15 min (see **Figure 2**).

We therefore examined the possibility that the lysolecithins were contributing to the antifeedant activity of C8-2a, by enhancing the activity of D-I. Low-dose experiments and mixing experiments were therefore performed, using available quantities of D-I. It was indeed found (**Table 6**) that the lysolecithins synergized the activity of D-I when tested with a mixture of the phospholipids. Without the addition of lysolecithins, D-I at a dosage of 0.4 mg/200 mg flour would be expected to produce little activity, similar to the 80% food consumption value and >14 day survival time with the 0.5 mg dose of D-I (trial 3). With the four component mixture, the food consumption was 33% and the median survival time was 6.7 days (trial 4). The activity was not enhanced by the addition of S-I either to the mixture of lysolecithins and D-I (trial 5) or to the lysolecithins only (trial 6). The lysolecithin mixture by itself (trial 7) was inactive, like S-I (trial 8) and S-VI (trial 9).

Lysolecithins, like saponins, are excellent detergents and can modify the function of membrane proteins including ion channels (36). However, the mechanism of synergism reported here and the optimal proportions of active ingredients will require further study. As further quantities of D-I become available, the contribution of these mixtures to the insecticidal activity of pea flour will also be clarified.

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