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Fractionation of Lentil Seeds (*Lens culinaris* Medik.) for Insecticidal and Flavonol Tetraglycoside Components

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Crude methanol extracts from four cultivated varieties of mature lentil seeds (*Lens culinaris* Medik.) were found to possess antifeedant and insecticidal properties in laboratory tests with the rice weevil (*Sitophilus oryzae* L.), an insect pest of stored products. Flash chromatography with silica gel on active Diaion HP-20 methanol extracts gave flavonol, lysolecithin, soyasaponin, and peptide fractions, as determined by HPLC and electrospray ionization LC/MS. The flavonol fraction was shown by high-resolution NMR experiments to contain a mixture of kaempferol 3-*O*-β-glucopyranosyl(1→2)-*O*-[α-rhamnopyranosyl(1→6)]-β-galactopyranoside-7-*O*-α-rhamnopyranoside and, tentatively, kaempferol 3-*O*-β-glucopyranosyl(1→2)-*O*-[α-rhamnopyranosyl(1→6)]-β-glucopyranoside-7-*O*-α-rhamnopyranoside. These inactive tetraglycosides, although inseparable under the reported HPLC conditions, were detected by NMR spectroscopy in nearly equal proportions. Three lysolecithins were identical to those previously identified in pea extracts. Soyasaponin I (soyasaponin Bb) and soyasaponin VI (soyasaponin βg) were found in Diaion HP-20 methanol extracts. An insecticidal lentil peptide with a mass of 3881 Da, isolated from an Eston variety in small quantities by anion exchange chromatography, was related to the cysteine-rich pea albumin 1b class of botanical insecticides. Binary mixtures of the insecticidal lentil peptide and soybean soyasaponin I were synergistic in tests with *S. oryzae*.

KEYWORDS: ***Lens culinaris*; insecticidal lentil peptide; soyasaponin; synergy; kaempferol**

INTRODUCTION

A few legume crops, including seeds of the field pea (*Pisum sativum* L.) are toxic to insect pests (*1*−*3*). Insecticidal and insect antifeedant properties of flours and crude extracts from field peas have been reported for stored-product insects (*3*−*5*). A number of compounds active against stored-product weevils, notably *Sitophilus oryzae* L. (rice weevil), have been isolated including dehydrosoyasaponin I or soyasaponin Be (*6*) and peptides of the pea albumin (PA1b) type (*7*−*9*). Insect-toxic seed albumins have also been detected in soybean (*Glycine max* L.), French bean (*Phaseolis vulgaris* L.), and *Medicago truncatula* Gaertner (*10*). Seeds of additional legume species have recently been evaluated for insecticidal activity (*11*).

Bodnaryk et al. (*3*) demonstrated that flours of lentil seeds (*Lens culinaris* Medik.) were feeding deterrents in a wheat flour disk bioassay with *S. oryzae* (*12*). Brown and orange seeded varieties tended to be more effective than green seeded lentil, but the active ingredients were not identified. Crude aqueous extracts of *L. culinaris* were recently found to be insecticidal in other tests with *S. oryzae* (*11*). Earlier, Applebaum et al. (*13*) found that saponins of lentil had little effect on the Azuki bean beetle (*Callosobruchus chinensis* L.). From a nutritional viewpoint, Ruiz et al. (*14*) reported levels of soyasaponin VI (S-VI, a major but unstable saponin of lentils) of 0.07−0.11% in two varieties grown in Spain. Beige and green seeded lentil varieties had higher S-VI levels than brown seeded types (*15*). Concentrations of saponins, phytic acid, condensed tannins, low molecular weight phenolic acids, and other compounds from lentils have also been reported (*16*−*18*).

El-Negoumy et al. (*19*) reported that four triglycosides of kaempferol (3,4′,5,7-tetrahydroxyflavone) were isolated from the seeds of *Lens esculenta* Baumg., a synonym of *L. culinaris*. These included kaempferol 3-*O*-β-D-glucopyranosyl-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranoside, kaempferol 3-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside 7-*O*-α-L-rhamnopyranoside, kaempferol 3-*O*-α-L-rhamnopyranoside 7-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside, and kaempferol 3-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside 7-*O*-β-D-galactoside. Flavonol diglycosides of *L. esculenta* have been described (*20*), namely, kaempferol 3-*O*-α-L-rhamnopyranoside 7-*O*-β-D-glucopyranoside and kaempferol 7-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside. Earlier, D'Arcy and Jay (*21*) reported the occurrence of a major unidentified 3,7-substituted kaempferol tetraglycoside in the cotyledons of *L. culinaris*, in addition to 5-deoxykaempferol.

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The purpose of this study was to identify the compounds of lentils responsible for insecticidal activity, using *S. oryzae* as a bioassay insect. A major lentil tetraglycoside (LTG), isolated as an isomeric flavonol mixture (LTGa and LTGb) from four cultivated varieties, was also identified.

MATERIALS AND METHODS

Materials. Lentil seeds were obtained from the Crop Development Centre (CDC), University of Saskatchewan (Saskatoon, SK). The varieties studied (accession numbers of Plant Gene Resources of Canada genebank) were Eston (CN 40010), Laird (CN 35415), CDC Redwing (CN 105582) and Indianhead (CN 105579). Organic solvents were either OmniSolv glass distilled grade (Merck, Darmstadt, Germany) or HPLC grade (Fisher Scientific, Nepean, ON). Methanol-d_4 (100 atom % D) and 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). Water was purified in the laboratory with a Millipore Super-Q system (Bedford, MA). Samples of soyasaponin I (S-I) for testing were isolated from soybean meal and purified by methods a and b as described previously (*6*). A reference sample of dehydrosoyasaponin I was also available (*6*).

Thin-Layer Chromatography (TLC). Precoated silica gel 60 F_{254} plastic sheets (Merck) of 0.2 mm layer thickness were developed with a solvent mixture of *n*-propanol−ethyl acetate−water (7/2/1), with *n*-butanol−ethanol−ammonia (7/2/5) or with the lower layer of chloroform−methanol−water (65/35/10). Samples for spotting were prepared at 4 mg/mL in 80% methanol. After development, the plates were examined under UV light and then sprayed with Liebermann−Burchard reagent (useful for detection of saponins), molybdenum blue (phospholipids), and phosphomolybdic acid (saponins and flavonoids). A few plates were sprayed with ninhydrin in attempts to detect proteins and peptides, but the sensitivity was poor.

HPLC. The instrument consisted of an Alliance 2690 separations module (Waters Canada, Mississauga, ON) equipped with a Waters 996 photodiode array detector (PDA) and a PL-EMD-960 evaporative light scattering detector (ELSD) (Polymer Laboratories, Amherst, MA) controlled by Waters Millennium or Empower software. Samples were prepared in 80% methanol at 4 or 8 mg/mL (with and without 0.25 mg/mL of α-hederin as internal standard) and syringe filtered (0.45 μm) before injection (5 or 10 μL) onto a Waters reversed phase C18 Symmetry column (3.0 mm × 150 mm, 5 μm particle size) maintained at 30 °C. The mobile phase (0.4 mL/min) consisted of 0.05% trifluoroacetic acid (TFA) in water (solvent A) and acetonitrile (solvent B). The gradient elution program consisted of 95% A and 5% B at time 0. The composition was 65% A and 35% B after 10 min. The linear gradient progressed to 50% A and 50% B over 15 min and to 5% A and 95% B over 5 min. The column was held at 5% A/95% B for 5 min before reverting to 95% A and 5% B from 35 to 43 min.

Mass Spectrometry. Positive ion electrospray ionization (ESI) mass spectra were obtained with a bench top 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 and higher). A C18 Symmetry column (2.1 mm × 150 mm, 5 μm) held at 30 °C was used for LC/MS. The mobile phase (0.2 mL/min) consisted of 0.1% formic acid in water (solvent A) or acetonitrile (solvent B). The starting mixture was 95% solvent A and 5% solvent B (65% A and 35% B after 10 min, 50% A and 50% B over 15 min and 5% A and 95% B over 5 min). Collision induced dissociation (CID) experiments were conducted in the positive ion mode with argon and collision energies of 30−35 eV. Quasimolecular ions (MH^+) were used to generate daughter ion spectra. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained as previously described (*8*, *9*).

Initial Lentil Fractionation. Whole seeds of lentils (*L. culinaris*, var Eston, a common small-seeded variety) were ground with a Wiley mill equipped with a 40 mesh screen. With a procedure from Bodnaryk et al. (*3*), the flour (121 g) was defatted with chloroform (1000 mL) at room temperature. A chloroform extract was obtained by rotary evaporation (5.94 g). The defatted flour (103 g), collected by Buchner filtration, was extracted with 80% methanol under reflux for 5 min and filtered, and the filtrate was concentrated by rotary evaporation (bath temperature <40 °C) until approximately 25% methanol remained. The concentrated solution was diluted with water (200 mL) and stirred for 24 h with water-washed Diaion HP 20 beads (particle size 250−600 μm; porosity 300−600D) from a 180 mL cartridge (Biotage Inc., Charlottesville, VA). After the beads were collected by filtration (the filtrate was designated as the <30% MeOH extract), the beads were washed with 30% methanol (500 mL). Both of the aqueous extracts were concentrated by rotary evaporation to give 7.2 g (<30% MeOH extract) and 0.58 g (30% MeOH extract). The beads were rewashed with 100% methanol (500 mL), and the methanol filtrate was concentrated by rotary evaporation. Evaporation was completed at 43 °C with a centrifugal evaporator (model SC 110A Savant SpeedVac Plus), and the brown powder that remained, designated as a Diaion HP-20 methanol fraction, was dried under vacuum in a desiccator (0.84 g). The above procedures were also applied to seedstocks of Laird (a common large-seeded variety), CDC Redwing (a small-seeded variety similar to Eston), and Indianhead (a small-seeded black variety), also starting with 120 g of flour.

Purification of Flavonols from Lentils. A portion of the Diaion HP-20 methanol fraction (var Eston, 250 mg) was mixed with silica (5 g) and purified with a FLASH 40 M apparatus (Biotage Inc., Charlottesville, VA) equipped with a 90 g (4 cm × 15 cm) KP-Sil (Biotage) cartridge (32−63 μm, 60 Å silica). The solvent was *n*-propanol−ethyl acetate−water (7/2/1), delivered at 15−20 mL/min. This solvent system was also utilized during TLC (usually with the Liebermann−Burchard spray reagent) to monitor the appropriate fractions for pooling. A yellow flavonol fraction eluting with 15−85 mL was obtained after evaporation of the solvent (42−61 mg of this fraction were isolated in six experiments). This fraction, designated as the LTG, was further purified on an AKTAExplorer 100 medium-pressure LC (Amersham Biosciences) equipped with dual P-901 pumps, a UV-900 absorption monitor, a fraction collector (Frac-901) and a 4.6 mm × 250 mm stainless steel column packed with Sephasil C18 peptide (12 μm, Amersham Biosciences). LTG samples were dissolved in 80% methanol (at a concentration of 10 mg/mL) and filtered, and about 1 mL (10 mg) was directed with a peristaltic pump (model P-910) to a sample loop (2 mL) and the column. The mobile phase (1.5 mL/min) consisted of (A) 0.1% formic acid in water and (B) 0.01% formic acid in 90% acetonitrile. The initial conditions were 95% A and 5% B for 12.8 min. A linear gradient was applied by increasing eluent B to 14% over 4.14 min. Another shallower gradient to 16% eluent B was applied during the next 27.6 min. Thereafter, a 100% eluent B concentration was achieved during 2.8 min and maintained for 19.4 min before recycling to the initial conditions. Similar fractions (0.5 mL) were combined, bubbled with nitrogen gas, and Savant evaporated at 43 °C (20 mg of Diaion HP-20 MeOH material from Eston gave 1.58 mg of LTGa/LTGb; 20 mg of HP-20 MeOH from Laird gave 2.93 mg of LTGa/LTGb; 20 mg of HP-20 MeOH from CDC Redwing gave 2.21 mg of LTGa/LTGb; 18 mg of HP-20 MeOH from Indianhead gave 1.97 mg of LTGa/LTGb).

NMR Spectroscopy. Spectra were obtained at 25 °C on LTGa/LTGb mixtures in methanol-d_4 (1−4 mg/mL) with a Bruker AVANCE 500 spectrometer (TopSpin 1.3 software) equipped with a Bruker BioSpin 5 mm CryoProbe with z-gradient. Chemical shifts (δ in ppm) were referenced to solvent resonances at 3.30 and 49.00 ppm for ^{1}H and ^{13}C respectively.

Anion Exchange Chromatography. An AKTAExplorer 100 medium-pressure LC and a 20 mL (16 mm × 100 mm) column of HiLoad Q Sepharose Fast Flow (Amersham Biosciences) were used. The Diaion HP-20 methanol extract (406 mg) from Eston was dissolved in 41 mL of 50 mM ammonium acetate (pH 9) and transferred during syringe filtration (0.45 um) to a Superloop (Amersham Biosciences), and 20 mL (200 mg) was injected onto the column. Chromatography was performed at room temperature with 140 mL of 50 mM ammonium acetate (adjusted to pH 9) at 5 mL/min followed by a linear gradient of water containing sodium chloride (0−2 M NaCl over 40 min). The flowthrough (7 mL) and salt-retained (6 mL) fractions were collected from 0 to 28 min (140 mL volume) and 32−52 min (100 mL)

Table 1. Isolated Yield and *S. oryzae* Bioassay Data from Crude Lentil Extracts of Eston (E), Laird (L), CDC Redwing (R), and Indianhead (I) Varieties

	yield of extract (%)				food consumption (% of control)[a]				mortality (%) after day14[a]				
extract	E	L	R	I	E	L	R	I	E	L	R	I	status
chloroform	4.9[b]	1.9	1	1.9	114	105	105	118	0	4	0	2	inactive
HP-20 <30% MeOH	7	9.2	5.9	10.9	109	99	99	110	0	4	4	0	inactive
HP-20 30% MeOH	0.6	0.6	0.5	0.4	93	112	118	106	0	0	0	0	inactive
HP-20 100% MeOH	0.8[c]	0.4	0.6	0.7	30	26	43	42	96	100	100	100	active[d]

[a] Experimental extracts were tested at a single concentration of 0.8 mg/100 mg of wheat flour (with 70% ethanol as solvent). Food consumption was expressed as % of control and mortality as % of dead insects ($n = 25$). [b] This sample probably contained residual chloroform. [c] In seven additional extractions of Eston flour, the yield of this fraction was 0.5–0.6% (470–558 mg). [d] See **Table 2** for bioassay data obtained from dose–response experiments.

respectively. The experiment was repeated with the remaining sample (195 mg), and the appropriate fractions were combined. The flowthrough (unretained) fraction (~170 mL) was subjected to ultrafiltration with an Amicon YM3 membrane (3000 molecular weight cutoff) using a pressurized stirred cell (model 8200, Millipore Corp., Bedford, MA). The retentate was obtained by washing the membrane with 80% methanol (3 × 25 mL) and concentrating the solvent on a rotary evaporator. A final concentration was done by Savant evaporation at 43 °C. A gray-brown powder was obtained (8.51 mg), designated as the insecticidal lentil peptide (ILP). The flowthrough filtrate (FF) that remained with the stirred cell was freeze-dried to give a brown solid (124 mg), designated as the ILP FF fraction.

A combined fraction (~170 mL) collected during application of the salt gradient was freeze-dried to give a beige solid (4.82 g), designated as the ILP salt-retained fraction. A portion of this powder (4.05 g) was stirred in methanol (100 mL) at room temperature. After 1 h, the mixture was filtered (scintered glass), and the filtrate was rotary evaporated to give 1.96 g of an off-white powder containing a reduced concentration of sodium chloride.

Insect Bioassays. A wheat flour disk bioassay with *S. oryzae* was used to assess the antifeedant activity and toxicity (*12*). The samples for bioassay were dissolved in 70% ethanol and mixed with wheat flour. Twenty-five adult *S. oryzae* (1−2 weeks old) were held on five wheat flour disks for 3 days at 30 °C, 70% relative humidity. Flour disks (ca 0.02 g/disk) were weighed before and after exposure to the insects. Mortality was noted each day until insects had been on the disks for a total of 14 days. Antifeedant activity was determined on day 3 by expressing consumption of treated disks as a percentage of control disks (70% ethanol), whereas mortality was assessed on day 7 or day 14. A sample concentration of 0.8 mg/100 mg of wheat flour was used for the single dose experiments. Effective concentrations (EC) to reduce feeding by 50% were calculated by reverse confidence intervals (*8*). Lethal concentrations (LC) for 50% of the population were calculated by probit analysis (*22*) with ILP concentrations (by weight in flour) of 0.016, 0.048, 0.096, 0.192, 0.4, 0.8, 1.6, and 3.2 mg/100 mg of flour. For S-I, the concentrations were 0.192, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/100 mg of flour. Mixtures of ILP and S-I were evaluated with nine concentrations in a ratio of 1:1 (0.008, 0.024, 0.048, 0.096, 0.2, 0.4, 0.8, 1.6, and 3.2 mg/100 mg of flour) and with eight concentrations in a ratio of 1:9 (0.0016, 0.0048, 0.0096, 0.0192, 0.04, 0.08, 0.16, and 0.32 mg of ILP/100 mg; 0.0144, 0.0432, 0.0864, 0.1728, 0.36, 0.72, 1.44, and 2.88 mg of S-I/100 mg of flour). Synergy was determined from EC and LC data by calculating the synergistic ratio (SR) and percent synergism (% S) (*23*).

RESULTS AND DISCUSSION

Crude samples obtained by defatting of lentil flours and by Diaion HP-20 treatment of methanol extracts were isolated for initial testing at single doses in the rice weevil bioassay (**Table 1**). Activity of all four varieties was associated with the Diaion HP-20 methanol extracts, which were isolated as brown-red powders in yields of 0.4−0.8%. Based on our experience with polymeric adsorbents of the HP-20 type, the active extracts were expected to contain a number of polar natural products, including phospholipids, soyasaponins and PA1b peptides (*6*, *22*, *24*).

Table 2. Comparison of Different Sources of Diaion HP-20 Methanol Extracts in *S. oryzae* Bioassays

source	time (min) of reflux during extn in 80% MeOH	EC_{50} (mg/100 mg flour)[a,c]	LC_{50} (mg/100 mg flour)[b,c]
Eston	5	0.57 (0.43–0.76) b	0.69 (0.57–0.84) c
Eston[d]	5	0.46 (0.33–0.69) ab	0.84 (0.57–1.20) c
Eston[e]	5	0.30 (0.24–0.39) a	0.56 (0.44–0.71) bc
Eston[f]	30	0.37 (0.29–0.49) ab	0.78 (0.63–0.95) c
Eston[g]	180	0.51 (0.37–0.76) ab	0.80 (0.51–1.31) c
Laird	5	0.36 (0.26–0.49) ab	0.36 (0.29–0.44) b
CDC Redwing	5	0.56 (0.44–0.73) b	0.77 (0.62–0.95) c
Indianhead	5	0.37 (0.25–0.58) ab	0.62 (0.36–1.00) bc
field pea extract[h]	5	0.40 (0.25–0.75) ab	0.12 (0.09–0.16) a

[a] Effective concentration to reduce feeding by 50%; R^2 values of the fitted lines used to estimate EC_{50} were equal to or greater than 0.93. [b] Lethal concentration for 50% of the population after 7 days. For estimates of LC_{50}, the *g* values (index of heterogeneity or significance for potency) were equal to or less than 0.28. [c] Values in brackets were 95% confidence intervals. Within columns, values followed with different letters were significantly different (non-overlapping confidence intervals). [d] This extract was the same material as entry 1 but subsampled and tested 2 years later. [e] This extract was obtained in 0.5% yield from the same batch of Eston lentil flour as entry 1. [f] The yield was 0.5%. [g] The yield was 0.6%. [h] This extract was obtained from protein-rich pea flour supplied by Parrheim Foods (Saskatoon, Canada).

Diaion HP-20 methanol extracts from four sources of lentils were isolated and tested in dose−response experiments against the rice weevil (**Table 2**). Reduction in feeding, expressed by EC_{50} values, was the same for all lentil samples, assessed by overlapping confidence intervals. With Eston lentils, storage of the flour for about a year or prolonged extraction with hot aqueous methanol had little effect on activity of the Diaion HP-20 methanol extract. The active ingredients therefore appeared to be heat stable. In terms of insecticidal potency, Laird was slightly more active than the other lentil varieties. A Diaion HP-20 methanol extract from field pea flour (*22*) was more toxic than any of the lentil extracts.

The main components of Diaion HP-20 methanol extracts from the four lentil varieties were separated by reversed phase HPLC (**Figure 1**). A major component of the bioactive lentil extracts eluted at 8.2 min. PDA recordings on this component showed a UV absorption band at 266 nm and a weaker band near 345 nm, consistent with a flavonol structure (*25*). LC/MS gave a protonated molecular ion at *m*/*z* 903 [MH^+] for extracts from the four varieties. CID experiments on the *m*/*z* 903 ion gave daughter ions at *m*/*z* 741, 595, 433, and 287, consistent with the sequential loss of hexose (−162), deoxyhexose (−146), hexose, and deoxyhexose units. An ion at *m*/*z* 757, attributed

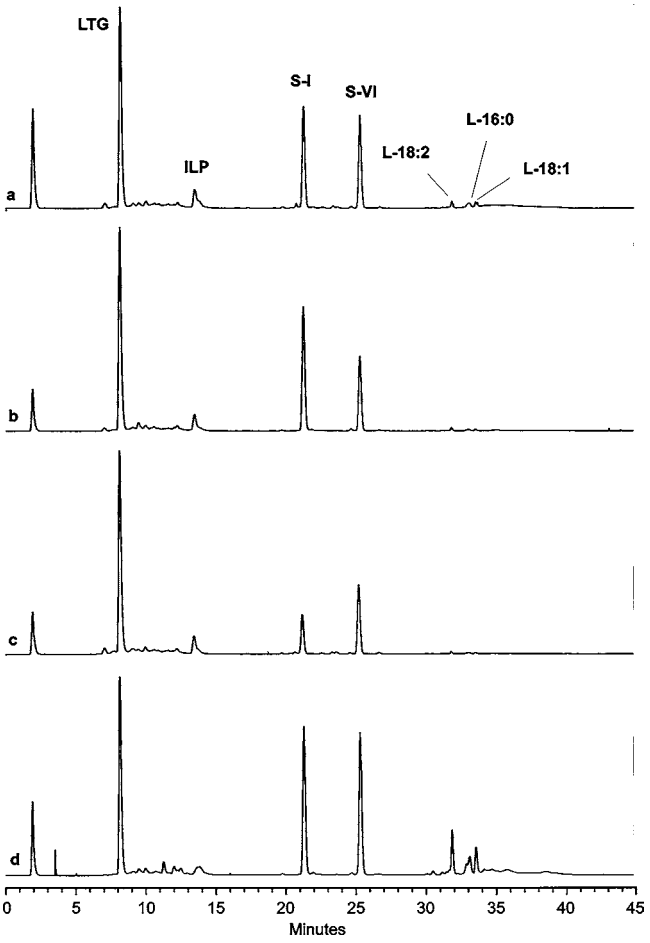

Figure 1. Representative HPLC/ELSD traces of Diaion HP-20 methanol extracts from defatted lentil seeds of (a) Eston, (b) Laird, (c) CDC Redwing, and (d) Indianhead varieties. Labeled peaks are as follows: LTG, lentil tetraglycoside; ILP, insecticidal lentil peptide; S-I, soyasaponin I; S-VI, soyasaponin VI; L-18:2, L-γ-linoleoyl-α-lysolecithin; L-16:0, L-γ-palmitoyl-α-lysolecithin; L-18:1, L-γ-oleoyl-α-lysolecithin.

to loss of rhamnose from the ion at *m*/*z* 903, was also observed. The ion at *m*/*z* 287 corresponded to the MH^+ of kaempferol. Therefore, the MS data suggested the major component was a kaempferol tetraglycoside, perhaps the same as the unidentified flavonol tetraglycoside reported by D'Arcy and Jay (*21*). One of the units appeared to be a deoxyhexose, probably rhamnose, as a terminal sugar. Existing structural data on lentil flavonoids suggested that both the C-3 and C-7 positions of kaempferol were probable sites of O-glycosylation (*19*−*21*).

Flavonols with a molecular weight of 902 led us to propose the lentil glycoside as kaempferol 3-*O*-β-glucopyranosyl(1→2)-*O*-[α-rhamnopyranosyl(1→6)]-β-galactopyranoside-7-*O*-α-rhamnopyranoside (LTGa, **Figure 2**), a flavonol glycoside previously isolated from *Cephalocereus senilis* (*26*) and *Ateleia chicoasensis* (*27*) and detected in other species (*27*). Another possibility was kaempferol 3-*O*-β-glucopyranosyl(1→2)-*O*-[α-rhamnopyranosyl(1→6)]-β-glucopyranoside-7-*O*-α-rhamnopyranoside (LTGb, **Figure 3**). 3,7-*O*-Diglycosides, 3-*O*-tetraglycosides, and 3-*O*-triglycosyl-*7*-*O*-rhamnosides of kaempferol with various combinations of glucose, galactose, and rhamnose have also been identified in various plant species (*28*−*31*).

Under conditions of flash chromatography with silica gel and a solvent mixture of *n*-propanol−ethyl acetate−water, the Diaion HP-20 methanol extracts gave an early eluting fraction of LTG. This fraction was further purified by C18 medium-pressure LC for structural analysis by NMR spectroscopy (**Table 3**). In methanol-d_4, ^{13}C NMR signals from purified LTG samples from Eston and Laird varieties suggested the presence of a kaempferol skeleton because chemical shift values for these 15 carbons were very similar to those reported in this solvent for 3,7-O,O-substituted kaempferol triglycosides isolated from *Alangium premnifolium* (*32*) and from *Arabidopsis thaliana* (*33*). Two-dimensional correlation spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments on LTG samples fully supported this skeletal assignment.

In purified LTG samples from the four varieties, ^1H NMR spectra and their integrals suggested that four anomeric protons of the attached sugars were present, two of which resonated at 5.56 ppm (broad singlet) and 4.75 ppm (doublet, $J = 7.25$ Hz). The remaining anomeric protons were divided into two doublets (integrating for a total of one proton) of nearly equal intensity at 5.31 ppm ($J = 7.65$ Hz) and 5.39 ppm ($J = 7.7$ Hz) and two singlets (also integrating for one proton) of nearly equal intensity at 4.47 and 4.48 ppm. HMBC experiments showed that the broad singlet at 5.56 ppm was correlated to C-7 whereas each of the doublets at 5.31 and 5.39 ppm were correlated to C-3. HMBC cross-peaks were observed with the anomeric proton of glucose at 4.75 ppm and C-2″ signals from the primary sugar attached to C-3. The two singlets at 4.47 and 4.48 ppm, representing an anomeric proton of rhamnose, were correlated to the C-6″ signals from the primary sugar. Therefore, the isolated LTG samples were isomeric mixtures of 3,7-O,O-substituted kaempferol glycosides with two hexoses and a rhamnose at C-3 and one rhamnose at C-7. The primary monosaccharide at C-3 appeared to be a nearly equal mixture of glucose and galactose, which would explain the pair of doublets at 5.31 and 5.39 ppm. In addition, the rhamnose attached to C-6″ via a glycosidic bond gave two doublets for the C-6‴ methyl group, at 1.06 and 1.16 ppm. This observation also suggested that the primary sugar at C-3 was a mixture of glucose and galactose, with the axial and equatorial orientations of the 4″-OH affecting the chemical shift of the C-6‴ methyl group.

By comparison of our spectral data to ^1H NMR data reported (in methanol-d_4) for the anomeric and C-6‴ methyl protons of kaempferol 3-*O*-β-glucopyranosyl(1→2)-*O*-[α-rhamnopyranosyl(1→6)]-β-galactopyranoside-7-*O*-α-rhamnopyranoside (*26*), it could be concluded that one of the isomers in the lentil mixture had this identical structure (i.e., LTGa of **Figure 2**). The assignment was supported by ^{13}C NMR data as well (**Table 3**).

The other isomer was tentatively identified as kaempferol 3-*O*-β-glucopyranosyl(1→2)-*O*-[α-rhamnopyranosyl(1→6)]-β-glucopyranoside-7-*O*-α-rhamnopyranoside (LTGb) by comparison to the ^1H NMR data reported (in methanol-d_4) for kaempferide 3-*O*-β-glucopyranosyl(1→2)-*O*-[α-rhamnopyranosyl(1→6)]-β-glucopyranoside (*34*). Notably, the anomeric glucose C-1″ proton of LTGb was downfield and C-2″ proton of LTGb was upfield compared with the same protons of galactose in LTGa. There was also a slight upfield shift reported for the C-6‴ methyl group of rhamnose compared with the same signal from LTGa.

Concentrations of some additional lentil components present in late-eluting flash chromatography fractions were estimated by HPLC/ELSD relative to an internal standard (**Table 4**). LC/MS analysis confirmed the presence of S-I (MH^+ 943) and S-VI (MH^+ 1069) in Diaion HP-20 methanol extracts. These saponins were found by HPLC in flash chromatography fractions 3−5, with the highest levels in fraction 4. The small peak preceding S-VI (at 24.8 min in Figure 1) was initially believed to represent dehydrosoyasaponin I (MH^+ 941), a compound previously

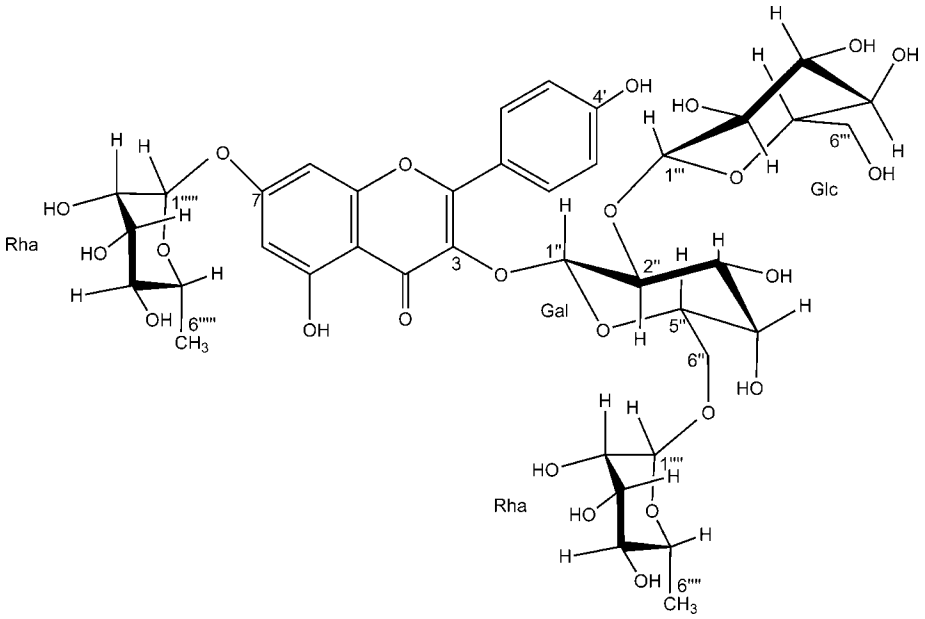

Figure 2. Structure and numbering system of the lentil tetraglycoside designated as LTGa.

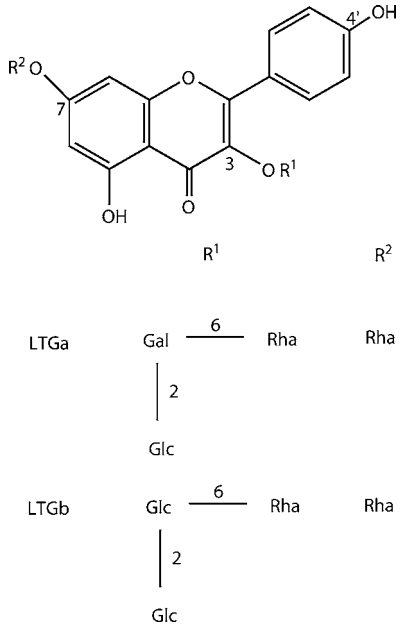

Figure 3. Comparison of LTGa and LTGb.

identified in peas as an insecticidal saponin (*6*). Although trace concentrations of dehydrosoyasaponin I were detected by LC/MS in extracts of the four lentil varieties, the small component at 24.8 min was principally composed of a substance with a molecular weight of 720 (MH^+ 721). This unidentified material, with a slightly shorter retention time than dehydrosoyasaponin I, was detected during LC/MS at higher concentrations in Eston and CDC Redwing extracts compared with those from Laird and Indianhead. It was also interesting to find that lysolecithins L-18:2, L-18:1, and L-16:0 were elevated in extracts from Indianhead, not only in flash chromatography fractions 4 and 5 but also in the Diaion HP-20 methanol extract before fractionation. Single dose bioassay experiments, utilized for targeting fractions with anti-insect activity, indicated that the LTG-containing fraction 2 and impure fraction 3, usually composed of a mixture of LTG, soyasaponins, and lysolecithins, were always inactive. Fraction 4 containing a large proportion of soyasaponins ranged from active with Eston to inactive with the other varieties. LC/MS and MALDI/MS indicated that the main peak corresponding to the ILP had a molecular mass of 3881 Da (MH^+ 3882). The ILP was present in fraction 5 as a

Table 3. ^1H and ^{13}C NMR Data for the Lentil Tetraglycoside (LTG) Mixture Obtained in Methanol-d_4

position	^{13}C δ	^1H δ, multiplicity ($J_{H,H}$ in Hz)	HMBC correlations (H → C)
2	159.6		
3	135.0		
4	179.8		
5	162.8		
6	100.7	6.48, d (2.05)	5, 7, 8, 10
7	163.6		
8	95.8	6.78, d (2.10)	6, 7, 9, 10
9	158.1		
10	107.4		
1′	122.6		
2′, 6′	132.5	8.08, d (8.90)	2, 4′
3′, 5′	116.3	6.90, d (8.90)	1′, 4′
4′	161.7		
3-gal or 3-glc			
1″ gal	101.6	5.31, d (7.65)	3
1″ glc	100.9	5.39, d (7.70)	3
2″ gal	80.0	4.04, m	1″
2″ glc	82.4	3.73, m	1″
6″ gal	67.1	3.40 and 3.68, m	
6″ glc	68.1	3.32 and 3.78, m	
(2→1)-glc			
1‴ glc	104.7 and 104.8	4.75, d (7.25)	2″
6‴ glc	62.6	3.70 and 3.79	
(6→1)-rha			
1⁗ rha	101.9 and 102.2	4.47 and 4.48, s	3⁗, 5⁗, 6″
2⁗ rha	72.0	3.53, m	
3⁗ rha	72.3	3.44, m	
4⁗ rha	73.6	3.25, m	
5⁗ rha	69.7	3.40 and 3.50, m	
6⁗ rha	17.9 and 18.0	1.06 and 1.16, d (6.15)	4⁗, 5⁗
7-rha			
1⁗′ rha	99.9	5.56, broad s	3⁗′, 5⁗′, 7
2⁗′ rha	71.7	4.01, s	
3⁗′ rha	72	3.82, m	
4⁗′ rha	73.5	3.45, m	
5⁗′ rha	71.3	3.60, m	
6⁗′ rha	18.2	1.27, d (6.10)	4⁗′, 5⁗′

Table 4. HPLC and *S. oryzae* Bioassay Data from Fractionation of Diaion HP-20 MeOH Extracts (250 mg) by Silica Flash Chromatography

			HPLC peak area ratios of main components to α-hederin[a] (retention time)						
variety	flash chromatography fraction	weight (mg) of fraction	flavonoid (LTG, 8.2 min)	peptide (ILP, 13.5 min)	soyasaponins (S-I + S-VI, 21.2/25.2 min)	lysolecithins (31.9/33.2 /33.6 min)	food consumption (% of control)[b]	mortality (%) after day14[b]	status
Eston	1 (0–14 mL)	12.1					94	0	inactive
	2 (15–105 mL)	56.7	30.8				102	4	inactive
	3 (106–200 mL)	26.6	5.4		2.5	0.4	93	0	inactive
	4 (201–350 mL)	24.8	0.2		33.3	0.1	68	68	active
	5 (351–700 mL)	24.8	0.2	0.05	1.8	0.2	53	84	active
Laird	1 (0–28 mL)	21.1	5.7				99	0	inactive
	2 (29–85 mL)	49.5	42.8				99	0	inactive
	3 (86–170 mL)	20.9	14.8		2.4	0.3	114	0	inactive
	4 (171–350 mL)	51.4	0.7		44	0.1	81	8	inactive
	5 (351–700 mL)	24.1	0.6	0.02	16.1	0.1	78	8	inactive
CDC Redwing	1 (0–14 mL)	12.7[c]					108	4	inactive
	2 (15–70 mL)	42.4	33.8				117	8	inactive
	3 (71–170 mL)	42.3	4.9		0.1		102	16	inactive
	4 (171–315 mL)	39.7	0.3		31.1		90	0	inactive
	5 (316–670 mL)	21.0[d]	0.4	0.02	7.2	0.2	54	84	active
Indianhead	1 (0–21 mL)	19.1[c]					108	0	inactive
	2 (22–85 mL)	44.2	28.9				105	0	inactive
	3 (86–135 mL)	16.1	7.7		0.2	0.3	99	0	inactive
	4 (136–350 mL)	25.5	0.6		36.2	0.7	92	4	inactive
	5 (351–700 mL)	16.4	0.7	0.03	5.3	1.3[f]	97	0	inactive

[a] The concentrations during HPLC were 8 mg/mL (for experimental fractions) and 0.25 mg/mL (for α-hederin, the internal standard eluting at 26 min). All fractions were also analyzed by TLC. Traces of dehydrosoyasaponin I (molecular weight of 940) eluted at 25 min during HPLC. The lysolecithins were oleoylphosphocholine, palmitoylphosphocholine, and linoleoylphosphocholine, respectively (see ref *6*). [b] Experimental extracts were tested at a single concentration of 0.8 mg/100 mg of wheat flour (with 70% ethanol as solvent). Food consumption was expressed as % of control and mortality as % of dead insects ($n = 25$). [c] These fractions were not analyzed by HPLC. [d] This fraction gave an additional peak at 20.9 min (1.2 peak area ratio), which gave an MH^+ ion of *m*/*z* 1045 by LC/MS. [e] This peak had a retention time of 13.4 min. [f] An additional, partially resolved component was evident at 32.9 min (see Figure 1d).

Table 5. HPLC and *S. oryzae* Bioassay Data from Fractionation of a Diaion HP-20 Methanol Extract of an Eston Lentil Variety by Q Sepharose Anion Exchange Chromatography

	HPLC peak area ratios relative to α-hederin (retention time)[a]						
fraction	flavonoid (LTG, 8.2 min)	peptide (ILP, 13.5 min)	soyasaponins (S-I + S-VI, 21.2/25.2 min)	lysolecithins (31.9 /33.2 / 33.6 min)	food consumption (% of control)[b]	mortality (%) at day14[b]	status
unfractionated	7.4	1.1	7.5	0.2	33	100	active
ILP	0.2	27.8			42	100	active
ILP FF	10.7	0.4			58	80	active
ILP salt-retained		[c]	0.1	[c]	96	16	inactive
1.6[d]					97	48	slightly active
3.2[d]					91	76	slightly active
6.4[d]					80	100	active
12.8[d]					66	100	active

[a] The concentrations during HPLC were 8 mg/mL (for experimental fractions) and 0.25 mg/mL (for α-hederin). [b] Experimental extracts were tested at a single concentration of 0.8 mg/100 mg of wheat flour (with 70% ethanol as solvent). Food consumption was expressed as % of control and mortality as % of dead insects ($n = 25$). [c] ILP and lysolecithins were detected in low concentrations during HPLC with a PDA detector set at 210 nm. [d] These numbers represent increasing doses (in mg/100 mg of flour) of the salt-retained fraction. EC_{50} and LC_{50} values obtained on this fraction were relatively high, 80 mg/100 mg of flour and 9 mg/100 mg of flour respectively (see Tables 2 and 6 for comparisons).

minor component, and this fraction from Eston and CDC Redwing displayed activity against *S. oryzae*. Diaion HP-20 methanol extracts showed higher levels of the ILP than fractions from flash chromatography. Diaion HP-20 methanol extracts from Eston were selected for additional fractionation by anion exchange chromatography.

Following Q Sepharose chromatography and ultrafiltration of the flowthrough fraction with a YM3 membrane, the ILP of mass 3881 Da was isolated. Peptides of this mass have not previously been reported from lentils. A small, purified sample of the ILP was shown in a single dose bioassay experiment to be active against the rice weevil (**Table 5**), giving a food consumption value of 42% and mortality of 100%. Although the concentration of ILP had increased about 25-fold compared with the unfractionated Diaion HP-20 methanol extract, activity was similar in these fractions. This suggested that a synergistic interaction was occurring between the ILP and one or more of the other components in the Diaion HP-20 methanol extract. Flowthrough filtrate (ILP FF) and ILP salt-retained fractions were also isolated during Q Sepharose chromatography. Their activity (**Table 5**) was considerably reduced compared with the ILP, which was probably related to contamination of these isolates with low concentrations of the active peptide. Ultrafiltration would not be expected to completely remove the ILP,

Table 6. Antifeedant and Insecticidal Bioassay Data with *S. oryzae* Utilizing a Purified Sample of Insecticidal Lentil Peptide (ILP) and a Sample Mixed with an Experimental Sample of Soyasaponin I (S-I) in Ratios of 1:9 and 1:1 by Weight

	antifeedant data					insecticidal data				
ILP/S-I	EC_{50}[a]	CI[b]	sEC_{50}[c]	SR[d]	% S[e]	LC_{50}[f]	CI[b]	sLC_{50}[c]	SR[d]	% S[e]
1:0	0.23	0.16–0.33				0.19	0.14–0.26			
1:9	0.27[g]	0.20–0.38	0.027	8.5	90	0.36[g]	0.22–0.58	0.036	5.3	81.1
1:1	0.20[g]	0.13–0.31	0.1	2.3	56.5	0.16[g]	0.09–0.25	0.08	2.4	57.9

[a] Concentrations were expressed in mg/100 mg flour. R^2 values of the fitted lines used to estimate EC_{50} were equal to or greater than 0.94. [b] 95% confidence intervals. [c] Synergized EC (or LC) according to Brindley and Selim (*23*), obtained here by multiplying the EC (or LC) of ILP only by the proportion of ILP (0.1 or 0.5) in the mixture. [d] Synergist ratio, SR = EC/sEC (or LC/sLC). [e] Percent synergism, %S = 100(EC − sEC)/EC [or 100(LC − sLC)/LC]. [f] Concentrations were expressed in mg/100 mg of flour. For estimates of LC_{50}, the *g* values (index of heterogeneity) were equal to or less than 0.23. [g] The EC and LC values obtained from bioassays with mixtures were utilized for calculating sEC and sLC. S-I was evaluated in tests with mixtures at sublethal concentrations (≤3.2 mg/100 mg of flour).

whereas, by analogy to the isolation of plant peptides from crude pea extracts with strong anion exchange resins (*8*), some of the ILP would be found in salt-retained fractions.

After reduction of the ILP with dithiothreitol and alkylation with iodoacetamide (*9*), MALDI/MS showed a prominent molecular ion at *m*/*z* 4229, indicative of six cysteine residues (three disulfide bonds) from a gain of 348 mass units. This indicated that the ILP was closely related to insecticidal PA1b peptides of peas (*7*−*9*), beans (*10*), and other legumes (*11*).

It was hypothesized that insecticidal activity of the 3881 Da peptide from lentils might be enhanced by the presence of S-I, recently identified as a synergist for insecticidal pea peptides of the PA1b type (*24*). Dose−response experiments were therefore designed to test experimental samples of the ILP isolate alone and mixed with S-I (**Table 6**). Values obtained for synergistic ratios and percent synergism (*23*) indicated that both the antifeedant and insecticidal properties were enhanced in tests with the two mixtures. This observation helped to explain the enhanced activity of Diaion HP-20 methanol extracts because the ILP contained in those extracts was already synergized by the presence of S-I. In Eston extracts, the ratio of ILP to S-I plus S-VI determined by HPLC/ELSD was about the same as the 1:9 ratio employed in one of the experimental mixtures. The 1:1 mixture also showed synergistic effects.

Only small differences in insecticidal activity were apparent among the four varieties (**Table 2**). HPLC/ELSD analysis of the Diaion HP-20 methanol extracts showed varying ratios of ILP to total soyasaponins, increasing from about 1:10 for Eston to greater than 1:20 for Indianhead. This implied that the ratio of ILP to soyasaponins for synergism to occur was probably greater than 1:9 (as studied), perhaps 1:20 or higher. In fact, one of the active Eston fractions (fraction 4, **Table 4**) did not show levels of ILP above the detection limit. This work has demonstrated that the ILP can represent a minor component of the synergized insecticidal mixture. That may be fortunate because the ILP occurred at low concentrations in the examined varieties, much lower than the PA1b insecticidal peptides from field peas (*8*, *24*). The ILP might represent a suitable candidate for genetic expression in crops susceptible to stored-product insect pests once a partial or complete amino acid sequence has been established.

ABBREVIATIONS

LTG, lentil tetraglycoside; ILP, insecticidal lentil peptide; FF, flowthrough filtrate; S-I, soyasaponin I; S-VI, soyasaponin VI; L-18:2, L-γ-linoleoyl-α-lysolecithin; L-16:0, L-γ-palmitoyl-α-lysolecithin; L-18:1, L-γ-oleoyl-α-lysolecithin; % S, percent synergism; SR, synergistic ratio.

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