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Insecticidal Components from Field Pea Extracts: Isolation and Separation of Peptide Mixtures Related to Pea Albumin 1b

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Chromatographic fractionation of crude extracts (C8 extracts) from the protein-enriched flour of commercial field peas (*Pisum sativum* L.) has been shown here to yield peptide mixtures related to the pea albumin 1b (PA1b) family of cysteine-rich plant peptides. The mixtures were obtained initially by flash chromatography with silica gel. Following elution of soyasaponins and lysolecithins, the end fractions obtained with the use of two flash chromatographic solvent systems displayed activity in a flour disk antifeedant bioassay with the rice weevil [*Sitophilus oryzae* (L.)]. Chemical properties of these mixtures were compared by thin-layer chromatography, high-performance liquid chromatography (HPLC), IR, MS, and amino acid analyses. The major peptides of C8 extracts, with average masses of 3752, 3757, and 3805 Da, were isolated by anion exchange chromatography. Samples enriched in the peptide of mass 3752 were isolated by cation exchange chromatography. Reduction plus alkylation experiments in combination with electrospray ionization mass spectrometry showed that C8 extracts contained about 10 peptides and, like PA1b, each peptide possessed six cysteine residues (three disulfide bonds). Disulfide bond reduction with 2-mercaptoethanol destroyed the antifeedant activity. The native peptides of C8 extracts were found to be resolved into nine peaks with XTerra HPLC columns operating at alkaline pH. These columns were employed to assess the distribution of pea peptides in the isolated fractions, with photodiode array and electrospray detection.

KEYWORDS: ***Pisum sativum*; pea albumins; PA1b; bioinsecticides; flash chromatography; ion exchange chromatography; XTerra HPLC**

INTRODUCTION

Extracts of field peas (*Pisum sativum* L.) from defatted protein-rich pea flour have utility to control insect pests (*1*). During a previous study on the fractionation of crude pea extracts (C8 extracts) by silica gel chromatography with chloroform−methanol mixtures, we found that the saponin and lysolecithin components of medium polarity were responsible in part for the insecticidal activity (*2*). Mixtures of highly polar end fractions that eluted from silica gel with methanol also displayed activity in a weevil bioassay.

The objectives of the present study were to isolate, separate, and identify some of the highly polar insecticides found in C8 extracts. We found that these methanol soluble components were complex mixtures of cysteine-rich peptides related to pea albumin 1b (PA1b), a family of 37 amino acid peptides discovered by Higgins et al. (*3*). Delobel et al. (*4*) claimed that variants of PA1b have insecticidal properties. Gressent et al. (*5*) have studied the binding of these variants to microsomal fractions of susceptible and resistant weevils.

Initially, we isolated pea peptide mixtures from end fractions during silica gel flash chromatography on C8 extracts. These isolates helped to confirm that C8 extracts contained insecticidal peptides and resulted in mixtures enriched in peptides of interesting molecular masses, determined primarily by electrospray ionization (ESI) mass spectrometry. It was subsequently found that the peptides of C8 extracts could also be isolated by anion exchange chromatography but the major peptides appeared not only in the flowthrough (unretained) fraction but also in the fractions eluting on the application of a salt gradient.

MATERIALS AND METHODS

Chemicals. Pentafluoropropionic anhydride was purchased from Supelco (Bellefonte, PA). Molybdenum blue thin-layer chromatography (TLC) spray reagent, constant boiling hydrochloric acid, 2-mercaptoethanol, 5,5′-dithio-bis(2-nitrobenzoic acid), and the L-amino acids (supplied as a kit) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). Organic solvents were of OmniSolv glass-distilled grade (Merck, Darmstadt, Germany), and water was purified in the laboratory with a Millipore Super-Q system (Bedford, MA).

General. Pea flour (approximately 54% protein) supplied by Parrheim Foods (Saskatoon, SK) was extracted as before to produce C8 extracts (*1*, *2*). Diaflo ultrafiltration membranes were obtained from Amicon, Inc. (Beverly, MA). Instruments and conditions for high-

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performance liquid chromatography (HPLC) and HPLC/MS with C18 Symmetry columns have been described (*2*). Micromass deconvolution software (MaxEnt) was used to process the ESI mass spectra. The mass spectrometer (Quattro LC, Micromass UK Ltd.) was calibrated with myoglobin. The cone voltage was typically set at 20 V, the capillary voltage was at 3.5 kV, and the mass range scanned was 300−2000 Da. The source block and desolvation temperatures were 120 and 350 °C, respectively. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed with Voyager Elite time-of-flight mass spectrometer (Perseptive Biosystems, Framingham, MA) in the positive ion linear mode with a matrix solution of α-cyano-4-hydroxycinnamic acid (20 μg/μL) in 90% acetonitrile, 10% water, and 0.1% acetic acid. Processing of the spectra was performed with Perseptive GRAMS 386 software. IR spectra were recorded on an ATI Mattson Genesis series Fourier transform (FT)-IR spectrometer, using potassium bromide disks. Precoated silica gel 60 F_{254} 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 (solvent system 1), or with *n*-butanol−ethanol−ammonium hydroxide, 7:2:5 (solvent system 2). The sheets were examined under UV light and after treatment with ninhydrin and Liebermann−Burchard sprays. Lysolecithins were detected with molybdenum blue (*6*).

Antifeedant and insecticidal activities were assessed in a flour disk bioassay with rice weevils [*Sitophilus oryzae* (L.)], as previously described (*2*, *7*). Sufficient quantities of most of the extracts were available to conduct dose−response experiments. Each extract was tested in a flour disk bioassay at concentrations of 0.016, 0.048, 0.096, 0.192, 0.384, 0.80, 1.6, and 3.2% (0.016−3.2 mg/100 mg flour). AIEX NaCl was tested at additional concentrations of 6.4 and 12.8%. The effective dose required to reduce feeding to 50% of feeding in the control disks (ED_{50}) was estimated using reverse confidence intervals. Reverse confidence intervals from regression were computed by a method similar to Sokal and Rohlf (*8*) but considering Y_0 as a population mean rather than a single observation. After exposure of the insects to the disks for 7 days, the dose required to kill 50% of the population (LD_{50}) was estimated using probit analysis (*9*).

Silica Gel Flash Chromatography. Fractionation of C8 extracts (250 mg) with solvent system 1 was performed as previously described (*2*), obtaining the end fraction (designated as system 1 isolate) by elution with methanol. With solvent system 2, C8 extracts (150 mg) were subjected to flash chromatography on a FLASH 12i apparatus (Biotage Inc., Charlottesville, VA) with a prepacked cartridge (8 g) and a flow rate of 2 mL/min. Five fractions (with 6−7 mL per fraction) were collected, and the solvent was evaporated, first with a N-EVAP apparatus (Organomation Associates, Berlin, MA) with nitrogen gas and then with a Savant SpeedVac Plus (Holbrook, NY). Fractions 3 and 4 were designated as the system 2 isolate.

Amino Acid Analyses. Amino acid analyses were performed by separately transferring the system 1 and 2 isolates (1 mg) to test tubes containing 1 mL of 6 M hydrochloric acid (constant boiling). The mixture was heated for 16 h at 110 °C with the Pierce Reacti-Therm system, equipping the test tubes with a Teflon-lined screw cap. Excess hydrochloric acid was evaporated (N-EVAP). The residue that remained was dissolved in acidified 2-propanol (0.5 mL; prepared at 2.8 M hydrochloric acid by the addition of 0.25 mL of acetyl chloride per mL of 2-propanol), heated at 110 °C for a further 45 min, and cooled (−15 °C). A dry residue was obtained (N-EVAP), which was dissolved in methylene chloride (0.25 mL), and pentafluoropropionic anhydride (0.1 mL) was added. After it was heated at 110 °C for 15 min, excess reagent was removed (N-EVAP), and the samples were transferred with hexane (0.2 mL) into autosampler vials for analysis by electron impact (70 eV) gas chromatography (GC)/MS (Hewlett-Packard model 5989A) with a HP-1701 column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and helium as the carrier gas (under electronic pressure control). Samples (1 μL), including derivatized L-amino acids as references, were injected splitless (45 s) at an initial oven temperature of 50 °C. After 1 min, the temperature rose at 25 °C/min to 100 °C and then at 10 °C/min to 250 °C. The column was held at 250 °C for 10 min. Background-subtracted mass spectra were obtained with a scan range of 35−800 mass units. The temperature of the ion source was 250 °C. Confirmation of molecular weights of the pentafluoropropionamide isopropyl ester derivatives was obtained by chemical ionization mass spectrometry with isobutane as the reagent gas. In attempts to detect tryptophan, acid hydrolyses were also conducted in the presence of 1% phenol (*10*).

Reductions with 2-Mercaptoethanol and Ellman Assays. Samples of C8 powder (25 mg) were dissolved in 8 M urea [containing 0.5 M Tris hydrochloride at pH 8.5 plus 5 mM ethylenediaminetetraacetic acid (EDTA)] and treated with 2-mercaptoethanol (50 μL) according to a literature procedure (*11*). After the mixture was heated at 40 °C for 2 h, the incubate was transferred to a Sep-Pak Vac (5 g) C8 silica cartridge (Waters Corp., Milford, MA) that had been previously conditioned with methanol and water. After they were washed with water (100 mL) and with a 1:1 mixture of water−methanol (100 mL), the (reduced) peptides were eluted with methanol (200 mL). Evaporation of the solvent gave 18 mg of a solid that was inactive in the rice weevil antifeedant bioassay (food consumption of 102%). At the same dose (1.6 mg/200 mg flour), a sample of C8 extract treated identically but without addition of 2-mercaptoethanol gave a food consumption value of 30%.

In the Ellman assay for thiols, performed in 6 M guanidinium chloride with 0.1 M phosphate buffer at pH 7.3 and 1 mM EDTA according to Creighton (*12*), the reduced solid of C8 gave a strong absorbance at 412 nm for released nitrothiobenzoate, indicating the presence of free thiol groups. Unreduced samples of C8 extracts as well as system 1 and system 2 isolates were negative in this assay.

A subsample of a C8 extract (25 mg) was reduced with 2-mercaptoethanol and with introduction of an alkylation step after the 2 h incubation (*11*). The alkylating agent was iodoacetamide (67 mg), added as a solution in 0.5 M Tris buffer (pH 8.5). After it was stirred in the dark at room temperature for 15 min, the mixture was desalted with a Sep-Pak Vac (5 g) C8 silica cartridge as before. Evaporation of the methanol gave 21 mg of a light brown solid that was inactive in the rice weevil antifeedant bioassay (food consumption of 96%). The product was also a useful reference for mass spectral determination of the number of cysteine residues.

Anion Exchange Chromatography on Crude C8 Extracts. These experiments were carried out with an AKTAExplorer 100 instrument (Amersham Biosciences Inc., Baie d'Urfé, PQ, Canada), initially with a 1 mL (7 mm × 25 mm) column prepacked with HiTrap Q Sepharose Fast Flow (Amersham Biosciences). The starting buffer was 50 mM ammonium acetate, adjusted to pH 9 with ammonium hydroxide, and delivered to the column at a flow rate of 1 mL/min. C8 extracts were prepared in this buffer at a concentration of 4 mg/mL and syringe filtered (0.45 μm). After injection of 0.25 mL, the column was eluted with 22 mL of buffer before a linear gradient of sodium chloride was applied from 0 to 2 M over 20 min.

Scale-up was achieved with a 53 mL (26 mm × 100 mm) column prepacked with HiLoad Q Sepharose Fast Flow (Amersham Biosciences). In a typical experiment, the C8 extract (700 mg) in 35 mL of 50 mM ammonium acetate (pH 9) was transferred to a Superloop (Amersham Biosciences) and 32 mL (640 mg) injected onto the column. The column was eluted with 400 mL of 50 mM ammonium acetate at 5 mL/min followed by a linear gradient of 50 mM ammonium acetate containing sodium chloride (0−2 M NaCl over 106 min). The flowthrough and salt-retained fractions were collected from 0 to 80 (400 mL volume) and 81−166 min (425 mL), respectively.

The flowthrough (unretained) fraction was subjected to ultrafiltration with an Amicon YM3 membrane (3000 molecular weight cutoff) using a pressurized stirred cell (model 8200, Millipore Corp.). 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 transferring the residue (with 80% methanol) to a test tube followed by Savant evaporation. A white powder (163.5 mg), designated as AIEX YM3, was obtained. Repetition of the experiment with a different batch of C8 material and using an Amicon YM1 membrane (1000 molecular weight cutoff) gave an off-white powder (115.6 mg), designated as AIEX YM1.

A 400 mL fraction collected during application of the salt gradient was freeze-dried, and the resulting powder 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 2.1 g of a white solid, designated AIEX NaCl.

In a separate experiment, a 400 mL fraction collected during application of the salt gradient on freeze-drying gave a white powder (15.5 g). A portion (7 g) of this powder was dissolved in water (250 mL) and ultrafiltered (YM3 membrane). The retentate was isolated as described for the flowthrough fraction. A tan solid (80.3 mg), designated as AIEX NaCl YM3, was obtained.

Base Hydrolysis of the Salt-Retained Material. AIEX NaCl (1 g) was dissolved in 80% methanol (25 mL) containing 25 mM NaOH and stirred at 4 °C for 18 h. The mixture was neutralized with 10% hydrochloric acid and concentrated on a rotary evaporator. The opaque aqueous solution that remained (12 mL) was transferred to a conical centrifuge tube with the aid of water (5 mL). *n*-Butanol (4 mL) was added with vortex mixing. After it was centrifuged at 2600*g* for 10 min, the *n*-butanol layer was transferred to a test tube. Extraction with *n*-butanol was repeated. Evaporation of the combined *n*-butanol layers with an N-EVAP at 50 °C gave 33.1 mg of a brown solid, designated as AIEX NaCl NaOH. Additional material (11.9 mg), similar in chromatographic and antifeedant properties to the first isolate, was isolated by re-extracting the remaining aqueous layer with *n*-butanol (2 × 4 mL). This experiment was repeated as described but with 250 mM NaOH at 22 °C for 2 h. A brown powder (22.2 mg) was obtained on extraction with *n*-butanol (8 mL). Additional extractions with *n*-butanol (2 × 5 mL) gave 30.5 mg of an off white powder.

Cation Exchange Chromatography. These experiments were carried out with an AKTAExplorer 100 instrument, initially with a 1 mL strong (sulfopropyl) cation exchange column of SP Sepharose Fast Flow (Amersham Biosciences). Using 1 mg test samples of AIEX YM3, appropriate separation conditions were demonstrated with 50 mM sodium acetate (pH 3) and a linear salt gradient to 0.4 M. These conditions gave three resolved peaks at 280 nm, the first being relatively unretained whereas the main components eluted during the salt gradient. Resolution of these components was much less apparent at pH 4 and, with ammonium acetate buffer, at pH 6. Experiments with 1 mL columns of HiTrap SP Sepharose Fast Flow and Resource S (Amersham Biosciences) gave similar results. Scale-up was achieved with a 20 mL (16 mm × 100 mm) column prepacked with HiLoad SP Sepharose Fast Flow. In a typical experiment, an AIEX YM3 fraction (140 mg) in 35 mL of 50 mM sodium acetate (prepared from sodium acetate trihydrate and adjusted to pH 3 with acetic acid) was syringe filtered (0.45 μm) and transferred to a Superloop, and 22 mL (88 mg) was injected onto the column. The column was eluted with 200 mL of 50 mM sodium acetate (pH 3) at 5 mL/min followed by a linear gradient of 50 mM sodium acetate (pH 3) containing sodium chloride (0−0.4 M NaCl over 240 min). The peak eluting before the gradient (24−36 min postinjection, CIEX fraction 1), the first peak eluting during the gradient (66−139 min, CIEX fraction 2), and the second peak eluting during the gradient (148−207 min, CIEX fraction 3) were collected and processed as follows.

A portion (15 mL of 60 mL) of the eluent containing CIEX fraction 1 was transferred to a centrifugal filter device (Centriprep YM3, Millipore Corp.) that had been prerinsed with 50 mM sodium acetate solution (pH 3). After centrifugation at 3000*g*, the filtrate was decanted and the retentate and chamber were rinsed with 5 mL of 0.05% acetic acid (pH 3.4). After recentrifugation, the retentate and chamber were rinsed with water (5 mL) and centrifuged. The retentate was dissolved in 80% methanol (3 × 2 mL) with vortex mixing, decanting the solvent after each wash. The combined solution was Savant evaporated (43 °C) to give 1.65 mg of a white solid. Using the above-described Centriprep procedure, portions (15 mL) of the eluent containing CIEX fraction 2 and CIEX fraction 3 gave 1 and 0.7 mg, respectively.

The eluent containing CIEX fraction 2 (350 mL of 365 mL) was ultrafiltered with a stirred cell containing a YM3 membrane. After the membrane was washed with 80% methanol and after Savant evaporation, 42.3 mg of a white powder of CIEX fraction 2 was obtained. Using the same techniques, CIEX fraction 3 (280 mL of 295 mL) gave 32.5 mg of a white solid.

Reduction and Alkylation Experiments for ESI Mass Spectrometry. Small scale reduction−alkylation experiments were conducted with the same reagents as described (*11*), using 0.5 mg of peptide samples in a buffered solution of urea (130 μL) plus 2-mercaptoethanol (10 μL) and iodoacetamide (6.7 mg). Sample cleanup and concentration were performed using pipet tips containing C18 silica (ZipTip $_{C18}$, Millipore Corp.), using procedures described by the manufacturer and a Gilson P-20 manual pipettor. The elution buffer (100 μL total volume), which consisted of 0.1% formic acid in a 1:1 mixture of methanol and water, was transferred to an autosampler vial equipped with a glass insert, for analysis by HPLC/MS under acidic conditions.

XTerra HPLC. HPLC experiments under alkaline conditions were performed with a Waters Alliance 2690 separations module equipped with a Waters 996 photodiode array detector (PDA), a PL-EMD-960 evaporative light scattering detector (ELSD) (Polymer Laboratories, Amherst, MA), and Waters XTerra C18 columns (3.0 mm × 150 mm, 5 or 3.5 μm particle size) maintained at 50 °C. The mobile phases were 10 mM (0.035%) ammonium hydroxide in water (solvent A) and 10 mM ammonium hydroxide in 80% acetonitrile (solvent B), delivered at a flow rate of 0.4 mL/min. The gradient elution program consisted of 60% A and 40% B at time 0. After 35 min, the composition was 40% A and 60% B (5 μm column) or 55% A and 45% B (3 μm column) (linear, curve 6 gradient), maintained at those proportions for 5 min. The gradient changed back to 60% A and 40% B from 40 to 43 min. The total run time was 45 min.

Using a triple quadrupole mass spectrometer (Quattro LC, Micromass UK Limited) interfaced directly to a Waters Alliance 2690 separations module, HPLC/MS experiments under basic conditions were performed with a Waters XTerra C18 column (2.1 mm × 150 mm, 3.5 μm particle size) at 50 °C using the HPLC gradient already described for the 3.5 μm XTerra column. Samples dissolved in 80% methanol were diluted with an equivolume mixture of solvents A and B (1:1) to give a concentration of 0.5−1 mg/mL. The flow rate was 0.2 mL/min.

RESULTS AND DISCUSSION

Flash Chromatography. Our previous work (*2*) showed that end fractions from flash chromatography with silica gel and solvent system 1 contained at least one other insecticide that was chemically unrelated to the saponins. In addition to the pink spots detected on TLC plates with ninhydrin, other common spray reagents (Dragendorff, Ehrlich, chlorine-tolidine, iodoplatinate, potassium permanganate, and fluorescamine) gave positive reactions. These sprays, as well as the Schlittler reagent (*13*), collectively suggested that the other insecticide was probably an amino acid, peptide, protein, alkaloid, or other nitrogen-containing compound. The end fractions were impure by TLC, but one main spot was detected, tailing from the origin with solvent system 1 and with an R_F of 0.5 with solvent system 2. HPLC comparison to a crude C8 extract (**Figure 1**) illustrated the complexity of the mixture and suggested that other components, including the major component of C8 extracts, were probably retained on the silica gel, even after eluting with methanol.

To address this difficulty, we investigated the purification of C8 extracts by silica gel flash chromatography with solvent system 2. The main HPLC component of C8 extracts (at 14.9 min in **Figure 1**) appeared to have an R_F of 0.28, which was much lower than the main component of the system 1 isolate (R_F of 0.5) and the least mobile ninhydrin-positive spot detected in C8 mixtures (**Table 1**).

C8 extracts could be quickly fractionated by flash chromatography using relatively small quantities of solvent system 2. The first fraction was practically inactive in antifeedant and survival tests (**Table 1**), reminiscent of nonpolar fractions from previous experiments. Fraction 2, isolated as a highly complex mixture, was active. The main active fraction (fraction 3) was compared to the main fraction from flash chromatography with solvent system 1 by FT-IR spectroscopy, MALDI mass spectrometry, and amino acid analyses.

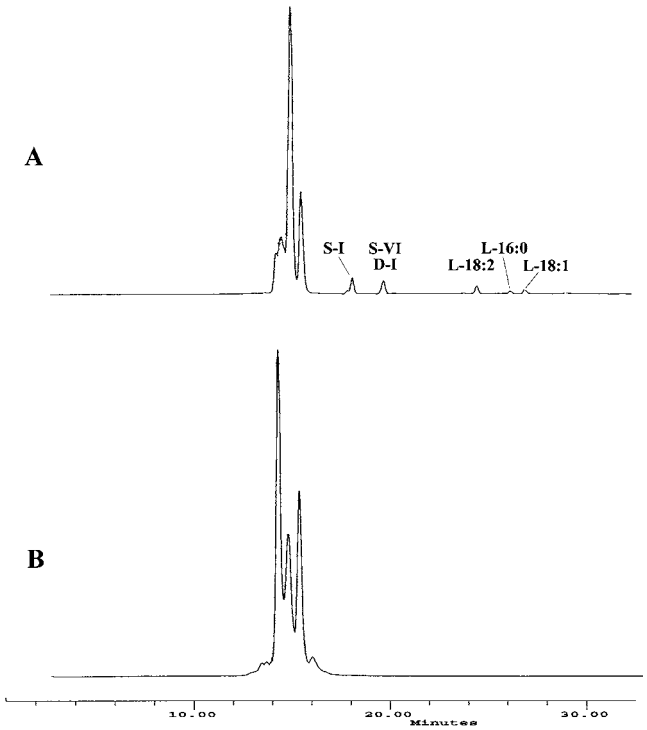

Figure 1. Comparison by HPLC with a reversed phase C18 Symmetry column and an ELSD illustrating the major components eluting from 14 to 16 min in (**A**) a crude C8 extract and (**B**) a system 1 isolate from silica flash chromatography on a C8 extract with chloroform–methanol–water and then methanol. Labeled peaks (*2*) are as follows: S-I, soyasaponin I; S-VI, soyasaponin VI; D-I, dehydrosoyasaponin I; L-18:2, L-γ-linoleoyl-α-lysolecithin; L-16:0, L-γ-palmitoyl-α-lysolecithin; and L-18:1, L-γ-oleoyl-α-lysolecithin.

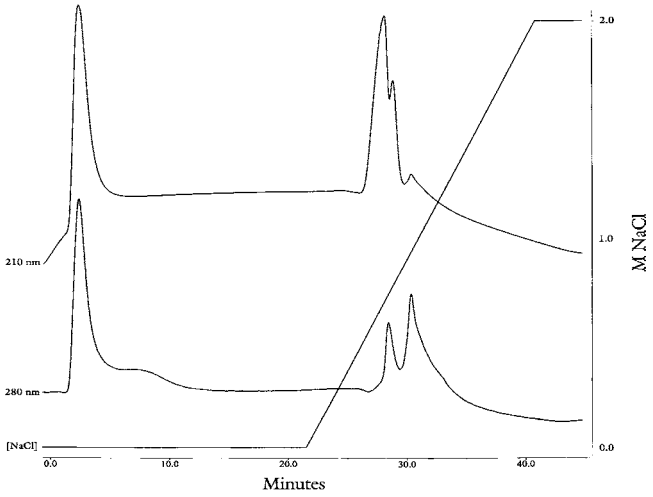

Figure 2. Anion exchange chromatogram (HiTrap Q Sepharose Fast Flow 1 mL column) of crude C8 material illustrating the flowthrough and salt-retained fractions. The starting buffer was 50 mM ammonium acetate, pH 9.

The strong IR absorption band near 1655 cm $^{-1}$ indicated that these two isolates contained amide functional groups. MALDI mass spectrometry showed prominent molecular ions near 3800, at approximately 3790 for the system 1 isolate and 3755/3805 for system 2. Following acid hydrolysis, amino acid determinations by GC/MS analysis of pentafluoropropionamide isopropyl ester derivatives (*14*, *15*) revealed that both isolates contained all of the common amino acids, except tryptophan. Standard samples of histidine were not reliably detected under these conditions, and asparagine-aspartic acid or glutamine-glutamic acid cannot be differentiated on acid hydrolysis. Detection of cystine suggested that the cysteine residues were probably disulfide-linked. In fact, samples of the system 1 and system 2 peptide isolates were negative in the Ellman assay for thiols (*16*), suggesting that the cysteine thiol groups in the native peptides were indeed disulfide-linked.

The cumulative evidence at this stage indicated that the insecticidal peptide mixtures were related to PA1b, a family of cysteine-rich 37 amino acid peptides derived from a precursor pea albumin PA1 (*3*). Nucleotide and amino acid sequence data indicated that there were at least four PA1 genes expressed in pea cotyledons from a single pea genotype (*3*). Isoforms or variants of PA1b could result from different genetic loci and allelic variation at these loci. This genetic evidence was consistent with the findings of Delobel et al. (*4*) who extracted pea flour with acetate buffer and found that three insecticidal peptides were variants of PA1b.

Ion Exchange Chromatography. Our strategy was to attempt to isolate the insecticidal peptides of crude C8 extracts with Q Sepharose, a strong anion exchange gel of the quaternary ammonium type (**Figure 2**). On the basis of literature reports on the isolation with Q Sepharose of plant peptides of similar mass (*17*−*19*), it was predicted that the peptides of interest would elute from the small Q Sepharose column in the flowthrough fraction (i.e., with the first eluting peak of **Figure 2**). It was more difficult to predict if this technique would separate the desired peptides from other components in the C8 extracts. By collecting the two main fractions, preliminary

Table 1. Fractionation of a C8 Extract (150 mg) by Flash Chromatography with a Biotage Silica Cartridge and Solvent System 2

fraction	yield (mg)	ninhydrin-positive TLC spot of R_F 0.50 [a]	0.35 [b]	0.28 [c]	soyasaponins	lysolecithins	f.c.[d]	median survival time (days ± SE) [e]
1	13	–[f]	–	–	–	–	81	12.6 ± 0.4 c
2	36	+[f]	+	+	+	+	35	5.7 ± 0.3 a
3[g]	37	±[f]	+	+	–	–	32	5.6 ± 0.3 a
4[g]	7	±	±	+	–	–	45	7.3 ± 0.5 b
5	6	±	±	+	–	–	60	8.7 ± 0.5 b
C8[h]		+	+	+	+	+	25	5.3 ± 0.3 a

[a] The HPLC retention time was 14.4 min (see **Figure 1**). [b] The HPLC retention time was 15.4 min. [c] The HPLC retention time was 14.9 min. [d] Food consumption, expressed as % of control, in the rice weevil bioassay. Each fraction was tested at a concentration of 1.6 mg/200 mg of flour. [e] 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. [f] The indicated compounds were detected (+), were probably detected in trace concentrations (±), or were undetectable (–) as determined by TLC (solvent system 2) and HPLC. [g] These fractions are considered as the system 2 peptide isolate. [h] The same batch of C8 powder used for fractionation.

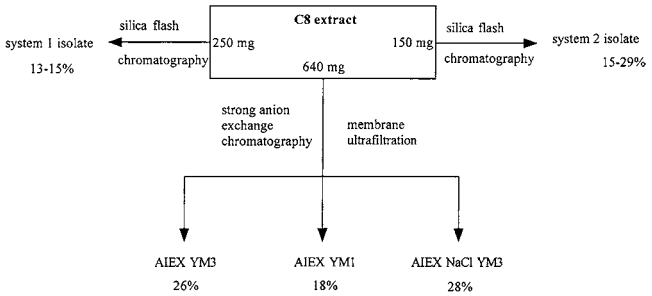

Figure 3. Yield of pea peptide mixtures isolated by silica and anion exchange chromatography. Starting amounts of C8 extract for each of the separation methods are shown in the box.

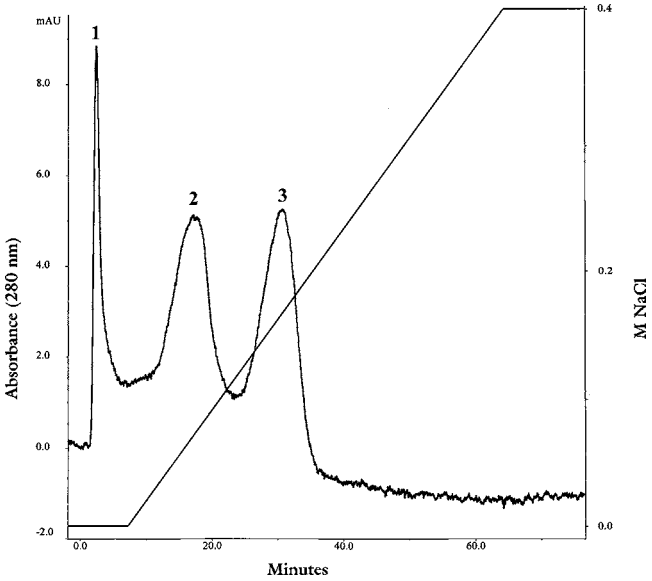

Figure 4. Cation exchange chromatogram (SP Sepharose Fast Flow 1 mL column) of a flowthrough fraction from anion exchange chromatography showing the separation of peptide fractions 1, 2, and 3. The starting buffer was 50 mM sodium acetate, pH 3.

evidence from TLC and HPLC suggested that the peptides of interest were indeed concentrated in the flowthrough fraction whereas the saponins and lysolecithins eluted during the salt gradient (i.e., within the cluster of peaks eluting around 30 min). However, the collected samples were very dilute and contaminated with buffer and salt.

With a large Q Sepharose column, ammonium acetate buffer, and a delayed salt gradient, the peptides of C8 extracts were isolated from the flowthrough fraction by ultrafiltration with a membrane of 3000 molecular weight cutoff. This isolate was designated AIEX YM3. A membrane of 1000 molecular weight cutoff could also be used, yielding AIEX YM1.

It was found that additional peptides could be isolated in quantity from the salt-retained fractions, after freeze-drying and dissolving the mixture in methanol (AIEX NaCl) or water (AIEX NaCl YM3). The yields of peptide mixtures isolated by the various techniques are shown (**Figure 3**).

We selected a flowthrough fraction (AIEX YM3) for further purification by strong cation exchange chromatography with a column of SP Sepharose. Under appropriate conditions of pH and salt concentration, the mixture could be resolved into three peaks **(Figure 4**). The first peak, eluting before application of the salt gradient, represented a minor component that was not characterized. Peptides contained in the second (CIEX fraction 2) and third peaks (CIEX fraction 3) were isolated in quantity by ultrafiltration. **Table 2** shows the properties of these fractions.

The TLC solvent of butanol−ethanol−ammonia (*20*) was useful to observe the distribution of peptides, saponins, and lysolecithins in the isolated samples. Both the flowthrough and the salt-retained fractions from anion exchange chromatography were enriched in (at least two) peptides. These fractions appeared to be enriched in the same peptides. After workup, the salt-retained fractions contained detectable concentrations of saponins and lysolecithins, suggesting that the ultrafiltration process was not completely effective in removing these lower molecular weight compounds. The treatment of AIEX NaCl with 25 mM sodium hydroxide resulted in the detection of all three peptide bands that were found in C8 extracts. A higher concentration of sodium hydroxide (250 mM) gave similar TLC and HPLC profiles, except that the lysolecithins were undetectable.

As indicated by food consumption and the ED_{50} values, fractions isolated by ion exchange chromatography showed moderate to good antifeedant activity (**Table 2**). The most effective samples were AIEX YM1 and AIEX YM3 (both equal in activity to a C8 reference extract), whereas the least effective were CIEX fraction 3, CIEX fraction 1, and the salt-contaminated AIEX NaCl. Systems 1 and 2 isolates from flash chromatography displayed ED_{50} values equivalent to the C8 extract. These conclusions were in parallel with survival time and LD_{50} measurements with the rice weevils.

Reduction and Alkylation Experiments. Although HPLC with a C18 column and an acidic mobile phase indicated three major peptides of C8 extracts (see **Figure 1**), we believed that the mixture was much more complex. With the available fractions and by use of ESI HPLC/MS, it was possible to find the molecular ions of 11 peptides in the mixture. The quasi-molecular ions ranged in mass from *m*/*z* 3732 to 3958 (**Table 3**, top). Four of these ions corresponded in mass to peptides previously reported from peas, the PA1b variant (3788) of Higgins et al. (*3*), and the insecticidal variants of PA1b (3736, 3741, and 3941) described by Delobel et al. (*4*).

The samples examined by ESI mass spectrometry were found to exist as mixtures, although CIEX fraction 1 could not be conclusively shown to contain any of these peptides. Because the peaks were inadequately resolved during HPLC with the Symmetry column, we could not determine the relative concentrations of peptides in these mixtures. However, CIEX fraction 2 seemed to be enriched in peptides of mass 3757 and 3805 whereas CIEX fraction 3 appeared to contain the 3752 peptide as a major component.

The 3788 and 3741 peptides from peas have been sequenced (*3*, *4*) and were found to be linear, 37 amino acid peptides containing six cysteine residues (three intramolecular disulfide bonds). It was therefore of interest to determine the number of cysteine residues in our peptide isolates, particularly in samples that appeared to be enriched in 3752, 3757, and 3805 peptides, the major components. This was accomplished by reducing selected mixtures with 2-mercaptoethanol, followed by alkylation with iodoacetamide and analysis of the derivatives by ESI HPLC/MS. Collectively, the derivatized samples showed appropriate quasimolecular ions corresponding to the addition of six S-carbamidomethyl groups (348 mass units) to the native peptides (**Table 3**, bottom). This suggested that the peptides of C8 extracts, with the possible exception of the 3841 peptide, were closely related to PA1b.

XTerra HPLC. C18 HPLC columns that are stable over a wide pH range have recently become commercially available (*21*), and their utility in peptide separations at alkaline pH has been recognized (*22*−*24*). We found that Waters XTerra C18 columns, operating at 50 °C with a gradient of aqueous

Table 2. Properties of Peptide Fractions Isolated by Ion Exchange Chromatography

sample	ninhydrin-positive TLC spot of R_F 0.50[a]	0.35[a]	0.28[a]	soyasaponins	lysolecithins	f.c.[b]	median survival time (days ± SE)[c]	dose to reduce feeding by 50% ED_{50} (%)[d]	CI (%)	dose to reduce survival by 50% LD_{50} (%)[e]	CI (%)
AIEX YM3	+[f]	±[f]	+	–[f]	–	34	5.8 ± 0.2 bc	0.21	0.14–0.31	0.14	0.10–0.18
AIEX YM1	+	±	+	–	–	30	6.3 ± 0.4 bcd	0.10	0.06–0.15	0.17	0.10–0.28
AIEX NaCl[g]	±	±	±	+	+	52	7.7 ± 0.4 d	0.76	0.59–1.01	0.85	0.33–2.01
AIEX NaCl YM3	±	±	+	±	+	33	6.1 ± 0.3 bc	0.31	0.24–0.41	0.28	0.19–0.42
AIEX NaCl NaOH[h]	+	+	+	+	+	35	5.4 ± 0.3 b	0.28	0.24–0.33	0.32	0.25–0.41
CIEX fraction 1[i]	–	–	–	–	–	47	8.1 ± 0.5 d				
CIEX fraction 2	+	–	+	–	–	39	6.1 ± 0.2 bc	0.25	0.15–0.44	0.19	0.11–0.31
CIEX fraction 3	±	±	+	–	–	42	8.0 ± 0.5 d	0.34	0.17–0.73	0.47	0.28–0.90
system 1 isolate[j]	+	±	±	–	–	32	5.0 ± 0.3 ab	0.15	0.09–0.22	0.10	0.06–0.30
system 2 isolate[j]	±	+	+	–	–	17	5.7 ± 0.4 bc	0.08	0.02–0.16	0.06	0.03–0.10
C8[k]	+	+	+	+	+	13	4.2 ± 0.2 a	0.10	0.05–0.18	0.08	0.06–0.10

[a] With solvent system 2. [b] Food consumption, expressed as % of control, in the rice weevil bioassay. For comparison to bioassay data in **Table 1** and ref *2*, the samples were tested at a concentration of 1.6 mg/200 mg of flour. [c] Kaplan–Meier survival analysis was used to estimate median survival times at a concentration of 1.6 mg/200 mg of flour. Multiple comparisons were made with the Holm–Sidak method, $P = 0.05$. Medians followed by a different letter are significantly different. All insects in untreated controls survived to 14 days. [d] The effective dose (mg/100 mg of flour) to reduce the feeding to 50% of the control after 3 days, followed by the 95% confidence intervals, dose–response data. [e] The lethal dose (mg/100 mg of flour) to reduce populations by 50% after 7 days, followed by the 95% confidence intervals, dose–response data. [f] The indicated compounds were detected (+), were probably detected in trace concentrations (±), or were undetectable (–) as determined by TLC (solvent systems 1 and 2) and HPLC. [g] A freeze-dried sample (methanol soluble portion) contaminated with salt. [h] A freeze-dried sample (methanol soluble portion) was treated with 25 mM sodium hydroxide in methanol. [i] Insufficient quantities were isolated for dose–response experiments. [j] Isolates from flash chromatography with silica gel. [k] From the same batch of C8 powder used for anion exchange chromatography.

Table 3. Quasimolecular Ions of Isolated Peptides and of Their S-Carbamidomethyl Derivatives Found during ESI Mass Spectrometry[a]

	m/z of MH^+ (Native Peptides)											
sample	3732	3737[b]	3742[b]	3753	3758	3789[c]	3790	3806	3842	3858	3942[b]	3958
C8	+[d]	+	+	+	+	+	+	+	+	+	–[d]	+
system 1 isolate	+	±[d]	–	–	±	+	–	±	±	–	–	–
system 2 isolate	–	+	–	+	+	±	+	+	–	+	–	±
AIEX YM3	±	+	+	+	+	+	+	+	+	+	±	±
AIEX NaCl[e]	–	–	–	±	+	±	±	+	±	+	±	±
CIEX fraction 1	–	–	–	±	±	±	±	±	–	–	–	–
CIEX fraction 2	±	–	±	–	+	–	+	+	+	+	–	–
CIEX fraction 3	–	±	–	+	±	+	+	±	±	–	–	±
	m/z of MH^+ (S-Carbamidomethyl Derivatives)[f]											
derivatized sample	4080	4085	4090	4101	4105	4137	4138	4154	4190	4205	4290	4305
C8	+	+	–	+	+	+	+	+	–	+	–	+
AIEX YM3	+	+	+	+	+	+	+	+	±	+	–	+
CIEX fr. 2	–	–	+	–	+	–	+	+	–	+	–	–
CIEX fr. 3	+	+	–	+	±	+	+	–	–	–	–	±

[a] Samples were analyzed by HPLC/MS without complete separation of the various peptides or their derivatives. [b] Peptides of these masses were reported to occur in the albumin fraction of peas grown in France (*4*). [c] A peptide of this mass was first described by Higgins et al. (*3*). From their reported 37 amino acid sequence, the calculated average mass is 3788.4 (MH^+ of 3789 expected under our reported HPLC/MS conditions). [d] The indicated peptides and their derivatives were detected (+), were probably present in trace concentrations (±), or were undetectable (–) by searching for the appropriate quasimolecular ions following HPLC/MS. [e] This sample had been treated with 25 mM sodium hydroxide. [f] These derivatives corresponded in molecular mass to the alkylation of six cysteine residues with iodoacetamide (addition of 348 mass units).

ammonium hydroxide and acetonitrile, resolved most of the peptides of C8 extracts. Furthermore, these elution solvents were compatible with ESI mass spectrometry so it was therefore possible to confirm the molecular mass of the separated peptides. This is illustrated with the peptide sample AIEX YM3 (**Figure 5**), which shows chromatograms from the PDA detector (**A**) and from positive ESI current (**B**) from a single injection. As indicated, nine distinct peaks were observed under optimum conditions. Only the peptides of mass 3736 and 3857 coeluted. This technology was useful to obtain the distribution of peptides in C8 extracts and in the various chromatographic fractions derived from this material without the need to rely on cochromatographing peptides observed during TLC or Symmetry HPLC. **Table 4** presents the results from analysis of these samples with XTerra columns.

XTerra HPLC and XTerra HPLC/MS confirmed that the peptides of mass 3752, 3757, and 3805 were major peptides in C8 extracts. Seven minor peptides were also detected in samples of C8 extracts. The peptide of mass 3788 was of particular interest because its molecular weight was the same as that of a PA1b variant described by Higgins et al. (*3*). This 3788 peptide was found in highest concentrations in the end fractions from silica gel flash chromatography of C8 extracts with chloroform−water−methanol (system 1 isolate). The distribution of peptides in the system 2 isolate and in the anion exchange flowthrough fractions was similar. The peptide fraction that eluted during application of the salt gradient was enriched in the 3805 peptide, followed by the 3757 and 3752 peptides. Mild base treatment did not alter the peptide distribution. The occurrence of peptides in fractions eluting during the salt gradient might be related to

Table 4. Peak Area Distribution (%) Determined by XTerra HPLC of Ten Pea Peptides Found in Experimental Samples[a]

sample[b]	3752[c]	3757	3736	3857	3805	3741	3841	3789	3731	3788
system 1 isolate	9.8	7.3	6.9[d]		11.0	1.1[e]		8.5	55.3[f]	
system 2 isolate	18.7	18.8	12.5		25.5	10.7[e]		13.7	0.2[f]	
AIEX YM3	24.9	18.9	15.3		16.3	7.6	1.2	5.3	2.1	8.3
AIEX YM1	25.5	22.1	14.6		21.7	6.3	1.1	5.9	0.1	2.6
AIEX NaCl YM3	10.9	22.7	3.2		52.5	2.2[e]		3.2	0.3[f]	
AIEX NaCl NaOH[g]	6.6	18.9	7.6		57.1	2.3[e]		5.8	1.6[f]	
CIEX fraction 2	6.9	47.6	–		37.9	5.8[e]		1.5	0.2[f]	
CIEX fraction 3	68.5	2.6	9.5		5.0	0.8[e]		2.2	11.4[f]	
C8[h]	22.4	29.5	8.6		24.0	5.7	0.4	5.4	1.0	3.0
C8[h]	21.8	28.6	9.0		23.3	6.3	0.4	6.4	1.1	3.1
C8[h]	24.7	25.8	7.0		28.1	4.3	0.5	5.8	0.7	2.9

[a] At 210 nm. Peak area distribution was calculated by peak area of indicated peptide/sum of the peptide peak areas × 100. [b] See **Table 2** and **Figure 3**. [c] The peptides are listed in order of their elution from the column. Assignments were confirmed in most cases by ESI HPLC/MS with an XTerra column (see **Figure 5**). [d] Peak areas obtained for these data column represent an unresolved mixture of the 3736 and 3857 peptides. These peptides remained unseparated on a 3.5 or 5 μm particle size HPLC column. [e] These peak areas represent a mixture of the 3741 and 3841 peptides (5 μm particles). [f] These peak areas represent a mixture of the 3731 and 3788 peptides (5 μm particles). [g] This sample had been treated with 25 mM sodium hydroxide (see footnote *h* of **Table 2**). [h] These extracts were obtained from different commercial batches of air-classified pea flour.

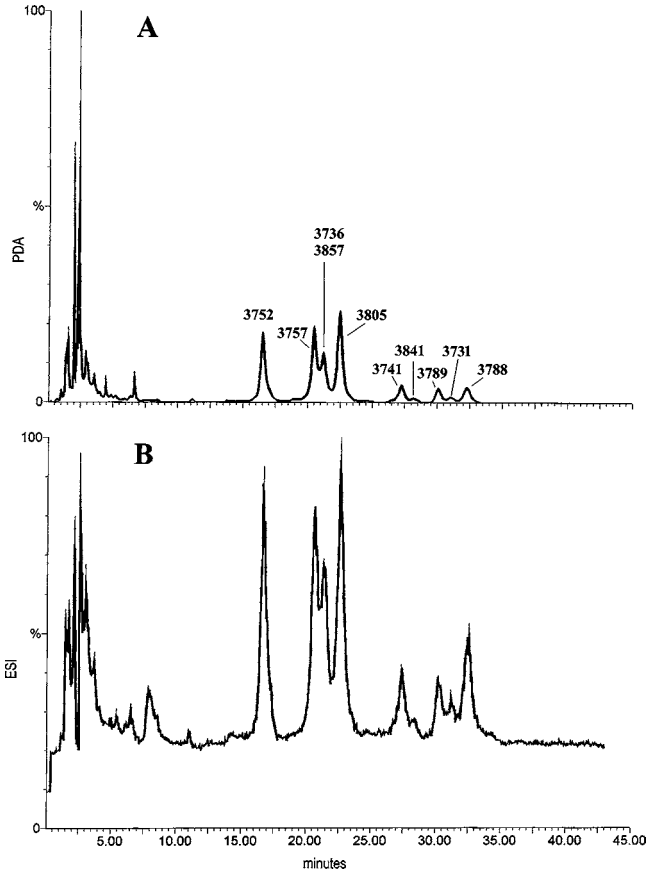

Figure 5. Separation by HPLC/MS of pea peptides in a sample of AIEX YM3 (0.5 mg/mL dissolved in 80% methanol and diluted with an equivolume mixture of 10 mM ammonium hydroxide in water and 10 mM ammonium hydroxide in 80% acetonitrile) with a Waters XTerra C18 column (2.1 mm × 150 mm, 3.5 μm particle size). Trace **A** represents a total PDA chromatogram (sum of the absorbance from 195 to 400 nm), whereas trace **B** is a total ion chromatogram obtained during ESI. The traces were recorded concurrently with the same sample. The top trace is labeled with the peptide masses found by ESI mass spectrometry.

the potential formation of a modified hydrophobic phase component by plant extracts applied to ion exchange gels (*25*). According to that assertion (*25*), a substantial amount of the available ionic sites on the Q Sepharose would become occupied by ligands such as saponins in the C8 mixture, resulting in a modified gel capable of retaining a portion of the peptides by hydrophobic interaction chromatography.

On the basis of molecular masses, only three of the minor peptides that were detected in C8 extracts have been isolated previously from peas. Sequences of the major peptides isolated here are of interest, and they are described in an accompanying publication. Other proteinaceous insecticidal toxins, often of high cysteine content, have been described in the literature (*26*−*29*).

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