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Insecticidal Components from Field Pea Extracts: Sequences of Some Variants of Pea Albumin 1b

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Methanol soluble insecticidal peptides with masses of 3752, 3757, and 3805 Da, isolated from crude extracts (C8 extracts) derived from the protein-enriched flour of commercial field peas [*Pisum sativum* (L.)], were purified by reversed phase chromatography and, after reduction and alkylation, were sequenced by matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry with the aid of various peptidases. These major peptides were variants of pea albumin 1b (PA1b) with methionine sulfoxide rather than methionine at position 12. Peptide 3752 showed additional variations at positions 29 (valine for isoleucine) and 34 (histidine for asparagine). A minor, 37 amino acid peptide with a molecular mass of 3788 Da was also sequenced and differed from a known PA1b variant at positions 1, 25, and 31. Sequence variants of PA1b with their molecular masses were compiled, and variants that matched the accurate masses of the experimental peptides were used to narrow the search. MALDI postsource decay experiments on pronase fragments helped to confirm the sequences. Whole and dehulled field peas gave insecticidal C8 extracts in the laboratory that were enriched in peptides with masses of 3736, 3741, and 3789 Da, as determined by high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry. It was therefore concluded that oxidation of the methionine residues to methionine sulfoxide occurred primarily during the processing of dehulled peas in a mill.

KEYWORDS: *Pisum sativum*; pea albumins; sequence variants; mass spectrometry; oxidation; methionine; methionine sulfoxide

INTRODUCTION

In a preceding publication (*1*), we showed that insecticidal extracts (C8 extracts) from protein-enriched flour of field peas [*Pisum sativum* (L.)] yielded complex mixtures of linear, cysteine-rich peptides of the pea albumin 1b (PA1b) type (*2*). Three major, methanol soluble peptides with masses of 3752, 3757, and 3805 Da were detected in C8 extracts by electrospray ionization (ESI) mass spectrometry, and these insecticidal peptide mixtures could be isolated from C8 extracts by ion exchange chromatography.

The objective of the present study was to sequence these three peptides using matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry and peptidase enzymes (ladder sequencing). An additional objective was to purify and sequence a minor peptide with a mass of 3788 Da, which was isobaric with the reported sequence of a variant of PA1b.

Exopeptidases gradually shorten linear peptides at either one of their termini, creating C-terminal (carboxypeptidases A, P, and Y) or N-terminal (aminopeptidase M) sequence ladders that can be analyzed directly by MALDI mass spectrometry (*3*). Sequence information can also be obtained by employing endoproteinases such as endoproteinase Asp-N, a site specific enzyme that cleaves peptides at the amino side of aspartic acid. Pronase, a proteolytic enzyme mixture containing carboxypeptidases, aminopeptidases, and endopeptidases, has found utility as well (*4*). The technique of MALDI postsource decay (PSD) (*5*) generates amino acid immonium ions (to aid in determining amino acid compositions) as well as series of N-terminal product ions (a, b, c, and d type fragments) and C-terminal product ions (x, y, and z type fragments). Because PSD fragmentation mainly occurs at peptide bonds, b and y type fragment (decay) ions are predominantly observed in MALDI PSD spectra and are the most useful ions for sequencing peptides directly. With some peptides, y ions representing uninterrupted sequences of several amino acid residues can be found (*6*). Internal fragments, representing two or more amino acids derived from neither termini, are also observed in MALDI PSD spectra.

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In theory, ladder sequencing with exopeptidases results in the sequential hydrolysis of C- or N-terminal amino acids and the quasimolecular ions of resulting peptide fragments are detected in the incubation mixtures by MALDI mass spectrometry. In practice, the detection of every possible peptide fragment is rarely possible, mainly because the peptidases have different specificities toward peptide bonds. According to the manufacturer, aminopeptidase M does not cleave at the amino side of proline, alanine, glutamine, or aspartic acid containing peptides whereas the C-terminal release of glycine and aspartic acid is considerably retarded with carboxypeptidase Y. Carboxypeptidases P and Y do not readily cleave long peptides or those with disulfide bridges (*7*). These authors, using mixtures of carboxypeptidases P and Y, found that degradation of peptides containing carboxymethylated cysteine residues was terminated one residue before the cysteine.

MATERIALS AND METHODS

Enzymes. Carboxypeptidase A, endoproteinase Asp-N, and endoproteinase Glu-C were purchased from Sigma-Aldrich Canada Limited (Oakville, ON). Carboxypeptidase P, carboxypeptidase Y, pronase containing 20% calcium acetate, and aminopeptidase M as a suspension in ammonium sulfate were purchased from Roche Diagnostics (Laval, PQ).

Peptide Samples and Purification. Samples enriched in the 3788 peptide were obtained from C8 extracts by silica gel flash chromatography, eluting with chloroform−methanol−water and then with methanol, whereas samples containing the 3752, 3757, and 3805 peptides were isolated from C8 extracts by anion exchange chromatography with Q Sepharose followed by cation exchange chromatography with SP Sepharose on the flowthrough fraction (*1, 8*). The 3757 and 3805 peptides were also obtained from a salt-retained fraction from a Q Sepharose column, followed by reversed phase chromatography (RPC).

RPC was performed with an AKTAExplorer 100 instrument (Amersham Biosciences Inc., Baie d'Urfé, PQ) by the conditions described (*8*). Separations were achieved at ambient temperature with a 3 mL (6.4 mm × 100 mm) reversed phase column of polystyrene/divinyl benzene beads (Resource 15 RPC, 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 plus 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 shallower 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.

The six peptide 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 centrifugal evaporation (Savant SpeedVac Plus) at 43 °C. Each peptide was obtained in quantities of 0.2−0.7 mg.

ESI Mass Spectrometry. Using a quadrupole mass spectrometer (Quattro LC, Micromass UK Limited) interfaced to a Waters Alliance 2690 separations module equipped with a Waters 996 photodiode array detector, high-performance liquid chromatography (HPLC)/MS experiments to confirm purities and molecular masses of the isolated peptides were performed with a Waters XTerra C18 column (2.1 mm × 150 mm, 3.5 μm particle size) under basic conditions at 50° C as previously described (*1*). Samples dissolved in 80% methanol were diluted with an equivolume mixture of the elution solvents to give a concentration of 0.5 (for purified peptides) or 1 mg/mL (for crude C8 extracts).

MALDI Mass Spectrometry. Accurate mass measurements were made with a Voyageur DE-STR time-of-flight mass spectrometer (Perspective Biosystems, Framingham, MA) in reflectron mode using

adrenocorticotropic hormone (ACTH fragment 7−38; monoisotopic mass of 3656.92) and bovine insulin (5729.61) as calibrants. Sequencing experiments were performed in linear mode (typically from 800 to 5000 Da) on the same instrument equipped with updated acquisition (Voyager Instrument Control Panel, ver. 5.1) and processing (Data Explorer ver. 4.0) software. The instrument was externally calibrated with angiotensin I, ACTH (1−17), ACTH (7−38), and bovine insulin. This instrument was also used for PSD experiments.

Sequencing Experiments. Samples (50 μg) of the purified peptides were dissolved in 40 μL of 100 mM Tris HCl buffer (pH 8.5) containing 60 mM dithiothreitol (Sigma) as the reducing agent. The mixture was heated at 45 °C for 1 h. Iodoacetamide (in 5 μL of Tris) was added to a final concentration of 190 mM, and the mixture was heated in the dark at 37 °C for 1 h. The reaction was quenched by the addition of dithiothreitol (150 mM) followed by heating at 37 °C for 1 h. The mixture was acidified with 2.5% trifluoroacetic acid (TFA) (50 μL). Sample cleanup and concentration were performed on the acidified solution, using 10 μL portions for each pipet tip containing C18 silica (ZipTip $_{C18}$, Millipore Corp., Bedford, MA). The ZipTip procedures followed those of the manufacturer, using a final elution solvent of 0.1% TFA in 50% acetonitrile (10 μL per ZipTip). The solvent from six ZipTips was combined (60 μL) and evaporated with nitrogen (N-EVAP), and the residue was redissolved in 20 μL of buffer. At 100% recovery from the reduction, alkylation, and ZipTip procedures, the peptide concentration was approximately 1.5 μg/μL (415 pmol/μL). For incubation, 3 μL (4.5 μg) was transferred to a 0.6 mL microcentrifuge tube (Rose Scientific, Edmonton, AB).

Solutions of the various peptidases were prepared at appropriate concentrations in the same buffer as the peptide sample, and 2 μL was delivered to the microcentrifuge tube to initiate the reactions (5 μL total volume). Incubations were conducted at room temperature unless otherwise specified with different enzyme-to-substrate ratios (1:1 to 1:500 by weight, depending on the peptidase) and incubation times ranging from 0.5 to 120 min (up to 9 h for endoproteinase Glu-C). Control tubes with substrate plus buffer (enzyme-depleted) and enzyme plus buffer (substrate-depleted) were included for comparisons.

At a specified incubation time, a portion (0.5 μL) of the incubation mixture was transferred to the MALDI plate and the reaction was quenched with a solution (0.5 μL) of the matrix (α-cyano-4-hydroxycinnamic acid at 5 mg/mL in 75% acetonitrile containing 0.1% TFA). In experiments with endoproteinase Asp N in phosphate buffer and in a few experiments with carboxypeptidase A and Y in ammonium citrate buffer, the incubation mixture (0.5 μL) was added to 0.1% TFA (5−10 μL) before desalting by the described ZipTip procedure, using a final elution mixture of α-cyano-4-hydroxycinnamic acid at 5 mg/mL in 75% acetonitrile containing 0.1% TFA. The desalted mixture (1−2 μL) was transferred to the MALDI plate for analysis.

Mass Calculations. A list of all possible variants of PA1b with their molecular masses, assembled within the constraints of Higgins et al. (*2*), was compiled using a batch file and spreadsheet (Microsoft Excel).

Isolation of Unoxidized C8 Extracts. Whole field peas obtained from Parrheim Foods (Saskatoon, SK) were ground with a Wiley mill (0.4 mm mesh sieve), and a subsample (120 g) was defatted with chloroform (*9*). The air-dried, defatted meal that remained (117 g) was extracted with hot 80% methanol (2.2 L). The methanol soluble fraction was diluted with water, and a C8 extract was isolated (*8*) using two C8 SepPak Vac cartridges (Waters Corp., Milford, MA). Evaporation of the methanol gave 211 mg of a light gray C8 powder, which was analyzed by HPLC/MS with a Waters XTerra C18 column. Commercially dehulled field peas (from Parrheim Foods) processed in this manner gave 476 mg of a C8 powder.

RESULTS AND DISCUSSION

Purifications. Peptide-enriched isolates obtained by either flash or ion exchange chromatography were purified by RPC at pH 10.5 to obtain the peptides with masses of 3752, 3757, 3788, and 3805 Da. Their elution order during RPC was 3752, 3757, 3805, and 3788. The success of these purifications was dependent on the use of enriched peptide fractions; otherwise,
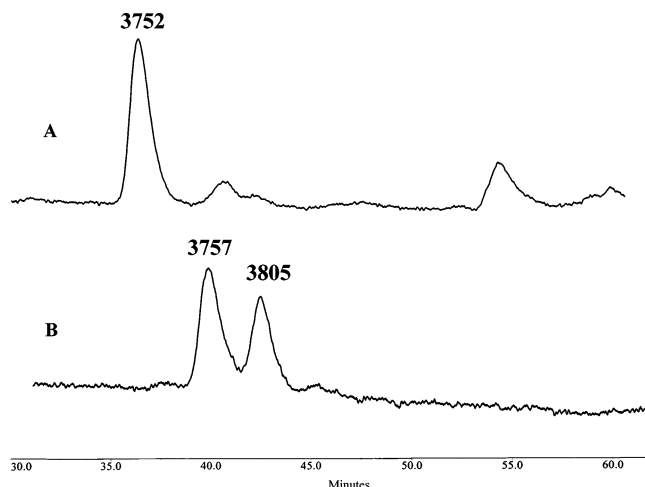
Figure 1. Chromatogram (280 nm traces) illustrating the purification of peptides found in (**A**) cation exchange fraction 3 and (**B**) cation exchange fraction 2. About 1.6 mg of each sample (designated as CIEX fraction 3 and CIEX fraction 2 in ref *1*) dissolved in 80% methanol was injected.

the isolates could be contaminated with minor peptides. For example, peptides with masses of 3736 and 3857 appeared to coelute with the peptide with a mass of 3757. Peptides 3731 and 3788 also coeluted. The minor peptide with a mass of 3741 eluted between the 3805 and the 3789 peptides. The peptide with a mass of 3752 could possibly be isolated directly from crude peptide mixtures because it eluted before the other peptides. However, we were unable to determine the relative retention time of a minor peptide with a mass of 3957 during RPC.

Figure 1 illustrates the purification by RPC of the 3752 (trace A) and 3757/3805 (trace B) peptides. After anion and cation exchange chromatography, RPC showed that CIEX fraction 3 was enriched in the 3752 peptide whereas CIEX fraction 2 yielded a mixture of the 3757 and 3805 peptides, in agreement with the results from XTerra HPLC on these fractions (*1*). Although fraction 3 contained later eluting peptides, collection of the first peak gave a sample of the desired 3752 peptide. In a similar manner, fraction 2 yielded samples of the 3757 and 3805 peptides. These three peptides were major components in crude C8 extracts.

The peptide fraction that eluted during application of the salt gradient during anion exchange chromatography (AIEX NaCl YM3 of ref *1*) was also subjected to RPC. Two main peptides were isolated and were shown by ESI and MALDI mass spectrometry to have masses of 3757 and 3805. The 3788 peptide was found at the highest concentrations in the end fractions from silica gel flash chromatography of C8 extracts with chloroform−water−methanol and then methanol as the eluent (system 1 isolate) (*1, 8*). A purified sample of this peptide was therefore obtained by RPC on the system 1 isolate.

Accurate Masses. Because the purified peptide with a (average) mass of 3788 corresponded to that of a known (*2*) PA1b variant (see **Figure 3**), it was reasonable to conclude that we had isolated this peptide or an isobaric variant (isoform) of the same (or nearly the same) molecular mass. It also seemed reasonable that the other isolated peptides of lower or higher mass were sequence variants of PA1b because Higgins et al. (*2*) had shown that two or three amino acid substitutions were possible at 10 sites (boldfaced in **Figure 3**), always with conservation of the six cysteine residues (three disulfide bonds). Within these constraints of Higgins et al. (*2*), it was shown that 3456 variants (including isoforms) of PA1b were possible and

Table 1. Some Chemical Features of Pea Albumin (PA1) Described by Higgins et al. (*2*)[a]

	leader[b]	PA1b	spacer	PA1a	carboxyl end
chain length	26	37	5	53	8
number of cysteines	0	6	0	4	0
variant possibilities	0	3456[c]	0	2	0
molecular mass	2696	3618−4023	634	5927−6018	817

[a] PA1 is first synthesized as a preproprotein (molecular weight ∼13900 Da) consisting of a leader sequence (∼2700 Da) at the amino terminal end and a proprotein (∼11200 Da), which contains the sequences of both PA1a (∼6000 Da) and PA1b (∼3800 Da). [b] The leader sequence, consisting of a nine and 17 amino acid peptide linked by an intron (in the gene sequence), is presumably removed cotranslationally, and the proprotein is thought to be cleaved posttranslationally (endoproteolytically) to yield two polypeptides, which, after removal of some carboxyl-terminal amino acids, represent the mature forms of PA1a and PA1b. [c] Determined in the present work.

Table 2. Quasimolecular Ions of Four Purified Peptide Samples Found during MALDI Mass Spectrometry and Sequence Variant Possibilities Derived from the PA1b Model of Higgins et al. (*2*)

sample designate[a] (average mass)	m/z[b] of MH+ (monoisotopic)	molecular mass (monoisotopic)	PA1b variants[c] M	m
3752	3750.5	3749.5	10	35
3757[d]	3755.6	3754.6	10	7
3805[e]	3803.6	3802.6	4	14
3788[f]	3786.7	3785.7	30	−[g]

[a] The peptides are listed in order of their elution during RPC and correspond to molecular masses derived from ESI data (*1*). These average masses were confirmed by linear MALDI experiments. The 3788 peptide corresponded in molecular weight to a PA1b variant of Higgins et al. (*2*). [b] The accuracy of the peptide masses was estimated at ±0.2 Da. Monoisotopic masses of these peptides were about 2.8 Da less than average masses. [c] Values in these columns represented the number of variants (isoforms) that were found in the calculations to have identical masses (within ±0.2 Da) to those determined by reflectron MALDI mass spectrometry, considering either methionine (M) or methionine sulfoxide (m) as the amino acid at position 12. [d] Another source (AIEX NaCl YM3, ref *1*) of the 3757 peptide gave an MH+ of 3755.5. [e] Another source (AIEX NaCl YM3, ref *1*) of the 3805 peptide gave an MH+ of 3803.4. [f] The 3788 peptide isolated from a different sample of the system 1 isolate gave an MH+ of 3786.8. [g] Variant possibilities are irrelevant because the 3788 peptide had isoleucine at position 12.

that these variants in theory would span an average molecular mass range of 3618−4023 Da (**Table 1**). All of the detected peptides in C8 extracts (*1*), ranging in molecular mass from 3731 to 3957 Da, were well within that mass range. The accurate masses, calculated for each of the 3456 possible variants, ranged from 3615.56 to 4020.7411.

Following accurate mass determinations (**Table 2**) and considering that the mass accuracy with the spectrometer was ±0.2 Da, the calculated sequences that corresponded to the accurate mass of an isolated peptide were selected as possible variants. This strategy resulted in selection of a reasonable number of likely sequence variants. For example, 30 sequences could be predicted for the 3788 peptide that fell within the selected mass range. Each of the other peptides showed 4−35 possible variants.

Sequencing of the 3788 Peptide. Reflectron MALDI analysis of the purified 3788 peptide sample confirmed the close match between the monoisotopic mass (3785.6) and that of a reported (*2*) PA1b variant (3785.7). However, the mass calculations showed 30 sequence variants with monoisotopic masses in the range of 3785.58−3785.74 Da. Linear MALDI analysis of enzyme-depleted incubation mixtures showed not only a strong

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MH$^+$ ion (at *m/z* 4138) but also a weaker ion that corresponded to the loss of glycine (at *m/z* 4081). Because this loss was independent of the nature of the matrix, laser power, and buffer, we concluded that the sample contained small concentrations of a truncated peptide. This impurity was also detected by ESI mass spectrometry by HPLC/MS on the unreduced sample.

It was apparent that information on amino acid composition would help to establish the sequence. Immonium ions from PSD experiments provided evidence for the presence of lysine (ions at *m/z* 84 and 129), suggesting that we had not isolated the reported (*2*) PA1b variant.

C-terminal sequencing with carboxypeptidases A, P, and Y showed tyrosine at position 36 whereas the occurrence of lysine (at position 31) was supported by experiments with carboxypeptidase P. N-terminal sequencing with aminopeptidase M indicated alanine at position 1. This enzyme did not give good sequence coverage, likely due to blockage by cysteine at position 3.

Incubation of the peptide isolate with pronase, a proteolytic enzyme mixture containing carboxypeptidases, aminopeptidases, and endopeptidases, gave two main fragments at *m/z* 2353 and 1804. These fragments could be rationalized by the occurrence of amide hydrolysis at arginine-cysteine (positions 21 and 22). Possibilities containing glutamic acid at position 11 were excluded by use of endoproteinase Asp-N, an enzyme that hydrolyzes peptides at the amino side of aspartic acid. With the proposed sequence, the peptide bond of phenylalanine-aspartic acid would be hydrolyzed, leading to expected ions at *m/z* 3058 and 1099. These ions were indeed observed.

The peptide of *m/z* 2353 found in pronase digests was subjected to PSD analysis. This 21 amino acid peptide gave strong C-terminal product ions corresponding to y9, which confirmed isoleucine (rather than methionine) at position 12. The fragmentation efficiency is known (*6*) to decrease considerably at amide bonds on the C-terminal side of proline. Consequently, PSD fragment ions at proline sites (y3, y7, y8, and y12) were very weak. Several of the y ions lost ammonia (y-17). An incomplete set of N-terminally charged b ions (b3−b8) were found and were often accompanied by ions representing the loss of water (b-18). Nearly all of the internal fragments contained proline. The smaller peptide found in pronase digests, representing the 16 amino acid sequence of 1803.2 Da, gave a complete set of y ions with one exception (y15). These data confirmed the sequence (see **Figure 3**).

Sequences of 3757 and 3805 Peptides. We initially considered that these peptides were variants of PA1b, like the 3788 peptide. With their accurate masses available, the variant possibilities from mass calculations showed 10 possible variants of 3752, 10 of 3757, and four of 3805. However, it became apparent that the model of PA1b (see **Figure 3**, top) could not account for the observations from mass spectral data and needed to be modified. For example, the 3752 peptide was shown (both before and after reduction and alkylation) to contain histidine by the appearance of a PSD immonium ion at *m/z* 110, an ion that is characteristic for this amino acid. Although the 3757 and 3805 peptides did not appear to contain histidine, their immonium ion spectra were not definitive with regard to the presence of other possible (according to the model) amino acids, especially methionine, glutamic acid, aspartic acid, and threonine. The oxidized form of methionine, methionine sulfoxide (m), was also considered, but the immonium ion of methionine sulfoxide occurs at the same mass as the immonium ion of phenylalanine (*m/z* 120), the latter of which was always found at position 10 of PA1b and variants.

Before reduction and alkylation, the purified samples of the three peptides when examined by reflectron MALDI mass spectrometry gave not only the expected quasimolecular ions (see **Table 2**) but also ions of lesser intensity that corresponded to the loss of approximately 57−64 Da. Initially, this was a puzzling observation because purified samples of the 3788 peptide (MH$^+$ 3789) were shown under linear MALDI and ESI conditions to contain a truncated species at *m/z* 3732 corresponding to the loss of the C-terminal glycine (3789−57 Da). In linear MALDI experiments with the current peptides dissolved in buffers for incubation (enzyme-depleted treatments), ions that corresponded to both MH$^+$ − 57 and MH$^+$ − 64 were observed, suggesting that these peptides contained not only peptide impurities without a C-terminal glycine but also another structural feature to explain the loss of 64 Da. Because an ion corresponding to MH$^+$ − 64 was not observed in MALDI spectra of 3788, one could conclude that an amino acid, or modified amino acid, was unique to the 3752, 3757, and 3805 peptides. Furthermore, this loss of 64 Da was also observed during MALDI mass spectral analysis of cleavage products from pronase digestions (containing the amino acids 1−21) and represented a predominant ion during MALDI PSD analysis on proteolytic (N-terminal) fragments. In MALDI experiments on a methionine-containing enolase, Larsen and Roepstorff (*11*) showed that the facile loss of 64 Da can be attributed to fragmentation of methanesulfenic acid (CH$_3$SOH) from an oxidized methionine residue. This loss is also diagnostic for oxidized methionine under electrospray MS-MS conditions (*12*). The MH$^+$ − 64 ion is recognized as diagnostic for methionine sulfoxide in current mass spectrometry software packages for peptide sequencing, such as ProteinProspector (http://prospector.ucsf.edu). Identification of oxidized methionine was consistent with that residue, rather than methionine or isoleucine, occurring at position 12 in variants of PA1b.

Mass calculations obtained for the 3757 peptide with methionine sulfoxide at position 12 showed that there were seven variant possibilities within the specified accurate mass range, all with serine at position 36. Ladder sequencing with aminopeptidase M showed alanine as the N-terminal amino acid. Hence, the sequences with isoleucine or valine at position 1 could be eliminated. Other sequence possibilities were easily eliminated on examination of pronase digests. Not only was a C-terminal ladder fragment found at *m/z* 2733.1 corresponding to valine at position 25 but also fragments were observed at *m/z* 2362.7 and 1763.1, corresponding to endoproteolytic hydrolysis at the C-terminal side of arginine at position 21. Supporting evidence for the sequence of 3757 was obtained by PSD experiments on the fragments observed at *m/z* 2362.7 (y1−10, y11−64, y15, and y20) and 1763.1 (y1−8, y10, y13, and y14).

Endoproteinase Glu-C appeared to be a useful enzyme to confirm the presence of glutamic acid at position 11 because it specifically cleaves at the carboxyl side of glutamic acid in ammonium bicarbonate buffer (pH 7.8). With the 3757 sequence, this would be expected to lead to fragments at *m/z* 1228.3 and 2897.4. In experiments with this enzyme, the latter ion was found in relatively small abundance but mostly a complex mixture of N- and C-terminal fragments was observed in the incubation mixtures. A strong molecular ion was still present after 6 h. Glu-C showed little selectivity toward cleavage at glutamic acid in this peptide. This might be related to steric hindrance from the adjacent methionine sulfoxide residue. Substitution of methionine sulfoxide for methionine has also been shown to decrease the rate of proteolysis in methionine-
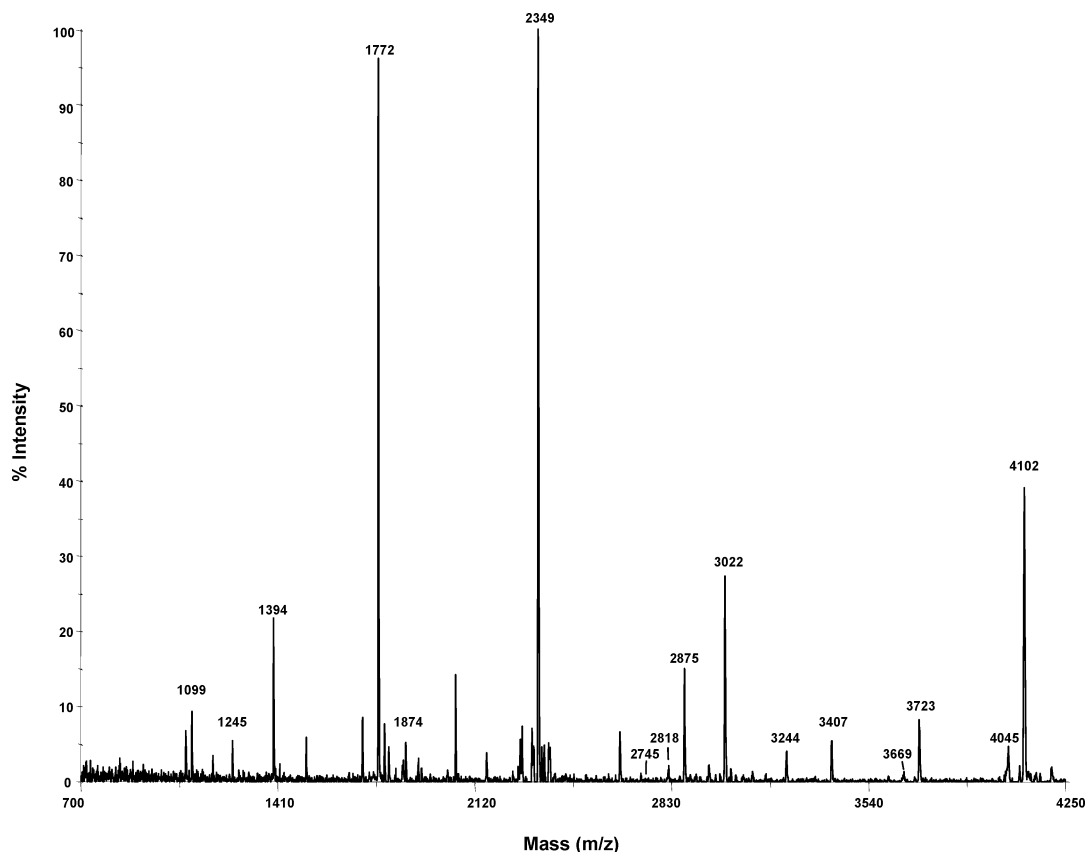
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Figure 2. MALDI mass spectrum obtained from the analysis of a pronase digest from the 3752 peptide. The subsample for analysis was taken after 10 min at pH 4, with an enzyme-to-substrate ratio (w/w) of 1:10.

containing proteins related to Alzheimer's disease (*13*). Nevertheless, a variety of fragments obtained from digestions with Glu-C helped to confirm the sequence of the 3757 peptide (see **Figure 3**). It should be noted that pronase at pH 4 gave an ion at *m/z* 3026, corresponding to cleavage at the N-terminal side of glutamic acid.

Fourteen sequence possibilities were found for the 3805 peptide. All but three were eliminated by experiments with aminopeptidase M (alanine at position 1) and pronase (serine at position 36). Pronase at pH 4 gave an ion at *m/z* 3073, corresponding to cleavage at the N-terminal side of glutamic acid. Hence, the sequence of the 3805 peptide was established and differed from the 3757 peptide in the residue at position 28 (see **Figure 3**). Carboxyl terminal cleavages of the arginine residues at positions 21 and 33 would lead to internal fragments of CIPVGLVIGYCR (*m/z* 1407.7) for the 3757 peptide and CIPVGLFIGYCR (*m/z* 1455.8) for the 3805 peptide. These ions were observed in pronase digests conducted at pH 4.

Sequence of 3752 Peptide. The main pronase hydrolysis products occurred at *m/z* 2348.6 and 1772.1. Although 35 sequence possibilities with methionine sulfoxide at position 12 were found, these were unlikely candidates because none contained histidine. To derive other potential sequences, it was first necessary to locate the position of histidine. Immonium ions from PSD experiments on the main pronase fragments showed that histidine was located in the 1772.1 fragment. We considered that histidine replaced asparagine at the fourth residue from the carboxyl end because a y3 ion (at *m/z* 260.1) was found that supported histidine rather than asparagine at that position. This substitution was also possible from the reported *c*DNA sequence of the pea albumin 1 gene (*2*) and the amino acid sequence of leginsulin (*14*). Additional support was obtained

from the appearance of additional y ions (y4−8, with tyrosine at position 31 and serine at position 36) during PSD analysis on the *m/z* 1772.1 fragment. Despite these considerations, sequences for the 3752 peptide could not be found from the PA1b model that fit the masses of the two main pronase hydrolysis products.

C-terminal cleavages by pronase provided good evidence for the YCRH sequence at positions 31−34, but the remaining sequence (to position 22) of the *m/z* 1772.1 fragment needed additional study. In this regard, a prominent ion at *m/z* 1245.4 was found in pH 8 pronase digests that was considered to represent positions 27−37, supported by the complimentary hydrolysis product at *m/z* 2875.3 representing the remainder of the 3752 peptide. The 1245.4 fragment was selected for PSD analysis, and the appearance of y2−8 ions helped to support the sequence from positions 30−37 (GYCRHPSG). Valine was almost certainly present at position 28 because this fragment showed an immonium ion characteristic of valine, not phenylalanine. Positions 27−29 were therefore considered to be LVV (311 Da) although the isobaric LTP or LPT were possibilities. Support for the former sequence came from the b2 and b3 ions observed during PSD experiments on the *m/z* 1245.4 ion. PSD fragment ions corresponding to b4, b7, b8, and b10 were also observed.

The remainder of the sequence (positions 22−26) for *m/z* 1772.1 was supported by b1−5 ions corresponding to CIPVG. Additional PSD fragment ions attributed to b6−10 and b13 were also found. Carboxyl terminal cleavages of the arginine residues at positions 21 and 33 would lead to an internal fragment of CIPVGLVVGYCR (*m/z* 1393.7). This ion was indeed observed in pronase digests conducted at pH 4 (**Figure 2**), providing

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	1	5	10	15	20	25	30	35 37
PA1b	I S C N G V C S P F	**D** I P P C G **S P L** C R C I P A G L V I G N C R N P Y G						
Variant substitutions	A V		E M		T S A		Y V	F	Y K		S
3788	A		D I		S P L		V	V	K		Y
3752	A		E m		S S A		V	V V Y	H	S
3757	A		E m		T S A		V	V	Y		S
3805	A		E m		T S A		V	F	Y		S
PT (3741)	A		E M		T S A		V	V	Y		S
Leginsulin (3919)	A D	A	E V		R S R D		V I	F V	F	I H	T

Figure 3. Sequence of a PA1b variant (labeled PA1b) with allowed sites of amino acid substitution (boldface letters), possible amino acids of PA1b variants as described by Higgins et al. (*2*), and sequences of the four isolated variants with their indicated molecular masses. The sequence of PT (calculated average molecular mass of 3741 Da) is taken from Delobel et al. (*15*), and the sequence of leginsulin (calculated average molecular mass of 3926 Da) is taken from Watanabe et al. (*14*).

additional evidence for the assigned sequence. Valine at position 25 was conserved in all four peptide samples (see **Figure 3**).

With residues at positions 22−37 established, mass calculations were performed using the allowed substitutions at positions 1, 11, 12, 17, 18, and 19 of PA1b. This resulted in six sequence possibilities for the 3752 peptide, three with an N-terminal alanine. One sequence possibility had alanine rather than valine at position 25, whereas the other possibility with TSA at positions 17−19 (and Dm at 11−12) was unsupported because an immonium ion for threonine could not be found. The derived sequence of the 3752 peptide is shown (see **Figure 3**).

Peptide Interrelations. Sequences of the four peptides identified in C8 extracts are collected in **Figure 3**. For comparison, this figure also shows the reported variant possibilities of PA1b from Higgins et al. (*2*), the reported sequence of PT, an insecticidal variant of PA1b (*15*, *16*), and the reported sequence of leginsulin, a related soybean peptide (*14*). The latter peptide provided a precedent for the occurrence of valine at position 29 and histidine at position 34.

The sequence of PT (3741 average mass) was the same as the deduced sequence of the major 3757 peptide, except for the state of oxidation of methionine at position 12. In that regard, one could sort most of the major and minor peptides of C8 extracts into similar pairs, differing in mass by 16 Da (**Table 3**). Thus, the minor 3736 peptide likely represented the unoxidized form of the major 3752 peptide and the minor 3789 peptide likely represented the unoxidized form of the major 3805 peptide. The conversion of methionine to methionine sulfoxide is known to increase the hydrophilic properties of peptides (*17*), and this was reflected by shorter retention times for the oxidized pea peptides during XTerra HPLC at pH 10.5 (see **Table 3**). The peptide mixtures were never adequately resolved during HPLC at acidic pH (*1*).

With regard to the 3752 peptide, the nucleotide-derived sequence of the unoxidized version of this peptide can be found in a sequence database (*18*) and elsewhere (*19*). However, this report presents the first chemical evidence for that amino acid sequence.

Oxidation of methionine to methionine sulfoxide can occur both in vitro and in vivo (*20*, *21*). The oxidation may be

Table 3. Probable Interrelationships of Peptides Detected in C8 Extracts Containing Methionine (M) and Methionine Sulfoxide (m) and Their HPLC Properties[a]

M Containing		
molecular mass (Da)	retention time (min)	relative peak area (%)
3736[b]	22.7	4.3[c]
3741[b]	28.9	5.7
3789	31.6	5.4
3841	29.8	0.4
3941[b,d]		<0.1

m Containing		
molecular mass (Da)	retention time (min)	relative peak area (%)
3752	17.9	22.4
3757	22.0	29.5
3805	23.9	24.0
3857	22.7	4.3[c]
3957[d]		<0.1

[a] XTerra HPLC (see ref *1*). The 3731 (1.1%) and 3788 (3.2%) peptides did not contain methionine or methionine sulfoxide. [b] Peptides of these masses were reported to occur in the albumin fraction of peas (*15*). [c] Because these peptides coeluted during XTerra HPLC, the peak areas were assumed to be equal. [d] These peptides were not detected with certainty during XTerra HPLC and XTerra HPLC/MS.

promoted by reactive oxygen species (*22*) or other oxidizing agents normally produced by cells (*23*). The presence of methionine sulfoxide residues may lead to significant conformational changes in proteins (*24*) and peptides (*17*). The biological activities of a large number of proteins and peptides are affected by the state of oxidation of methionine residues, and the presence of methionine sulfoxide may cause a loss of function (*20*). Oxidation of proteins and peptides can be reversed by methionine sulfoxide reductase.

Although chemical reagents such as hydrogen peroxide and *t*-butylhydroperoxide are capable of oxidizing methionine-containing proteins (*25*), including heat shock protein from plants (*24*), it was unclear if processing of plant tissues could promote methionine sulfoxide formation. With air-classified peas, the C8 extracts examined by XTerra HPLC showed a similar peptide profile, with the oxidized peptides appearing as the major components in each C8 sample. While some aerial oxidation may have occurred, for example, during hot methanol extraction or during cleanup steps with C8 silica, one would have expected considerable variability in the peak area distribution of the oxidized and unoxidized peptides if the samples underwent partial aerial oxidation in the laboratory. Samples of C8 obtained from different batches of flour were remarkably similar (*1*). Furthermore, Higgins et al. (*2*) and Delobel et al. (*15*) did not discuss any methionine sulfoxide-containing peptides. These authors performed the extractions (with 60% methanol or 0.1 M sodium acetate buffer) on whole or laboratory-ground peas, in contrast to our studies with air-classified protein-rich pea flour from a commercial mill. It was therefore reasonable to conclude that the oxidized peptides were formed in large part during the processing of peas in the commercial mill.

To address this issue further, whole field peas were ground in the laboratory and extracted with 80% methanol, and a C8 extract was obtained (*1*, *8*, *9*). This extract was compared by HPLC/MS to a C8 extract from a fully processed flour sample (**Figure 4**). The chromatograms showed that whole peas gave three main peptides with masses of 3736, 3741, and 3789 Da,
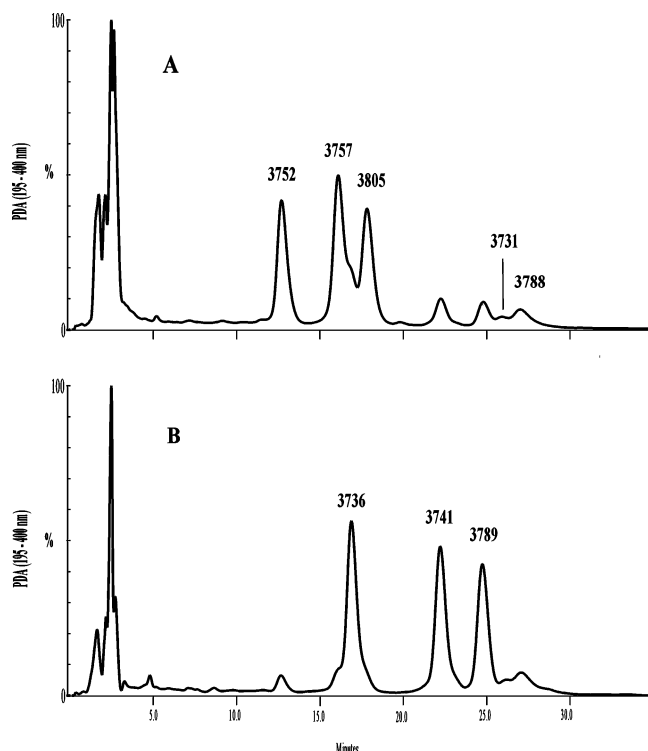
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Figure 4. Comparison by HPLC/MS of pea peptides in C8 extracts (1 mg/mL dissolved in 80% methanol and diluted with an equivolume mixture of 10 mM ammonium hydroxide in water and 10 mM ammonium hydroxide in 80% acetonitrile) with a Waters XTerra C18 column (2.1 mm × 150 mm, 3.5 μm particle size). The traces represent total photodiode array chromatograms (sum of the absorbance from 195 to 400 nm) from (**A**) a C8 extract obtained from air-classified protein-rich (mill processed) pea flour and (**B**) a C8 extract obtained from whole peas processed in the laboratory. Chromatograms are labeled with the molecular ions found by ESI mass spectrometry.

differing by 16 Da from the three main peptides of the air-classified protein-rich flour. These results confirmed that methionine oxidation occurred during mill processing, leading to mixtures containing primarily the oxidized peptides as well as lower concentrations of unoxidized peptides (see **Table 3**). On the other hand, C8 extracts from whole peas and dehulled peas gave primarily the unoxidized peptides. The relatively low concentrations of some of the oxidized peptides in C8 extracts from whole and dehulled peas could arise from aerial oxidation in the laboratory, although their natural occurrence in the seeds could not be excluded. C8 extracts prepared from whole peas and processed pea flour appeared to be equipotent in bioassays with the rice weevil *Sitophilus oryzae*.

ACKNOWLEDGMENT

Supporting Information Available: Figures listing the predicted amino acid sequences of the four experimental peptides and a number of tables showing C- and N-terminal fragments detected by MALDI mass spectrometry following incubation of the reduced and alkylated peptides with various peptidases. This information is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Taylor, W. G.; Fields, P. G.; Elder, J. L. Insecticidal components from field pea extracts: Isolation and separation of peptide mixtures related to pea albumin 1b. *J. Agric. Food Chem.* **2004**, *52*, 7491–7498.

(2) Higgins, T. J. V.; Chandler, P. M.; Randall, P. J.; Spencer, D.; Beach, L. R.; Blagrove, R. J.; Kortt, A. A.; Inglis, A. S. Gene structure, protein structure, and regulation of the synthesis of a sulfur-rich protein in pea seeds. *J. Biol. Chem.* **1986**, *261*, 11124–11130.

(3) Gevaert, K.; Vandekerckhove, J. Protein identification methods in proteomics. *Electrophoresis* **2000**, *21*, 1145–1154.

(4) Marzilli, L. A.; Golden, T. R.; Cotter, R. J.; Woods, A. S. Peptide sequence information derived by pronase digestion and ammonium sulfate in-source decay matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2000**, *11*, 1000–1008.

(5) Brown, R. S.; Lennon, J. J. Sequence-specific fragmentation of matrix-assisted laser-desorbed protein/peptide ions. *Anal. Chem.* **1995**, *67*, 3990–3999.

(6) Chaurand, P.; Luetzenkirchen, F.; Spengler, B. Peptide and protein identification by matrix-assisted laser desorption ionization (MALDI) and MALDI-post-source decay time-of-flight mass spectrometry. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 91–103.

(7) Bonetto, V.; Bergman, A.-C.; Jornvall, H.; Sillard, R. C-Terminal sequence analysis of peptides and proteins using carboxypeptidases and mass spectrometry after derivatization of lys and cys residues. *Anal. Chem.* **1997**, *69*, 1315–1319.

(8) Taylor, W. G.; Fields, P. G.; Sutherland, D. H. Insecticidal components from field pea extracts: Soyasaponins and lysolecithins. *J. Agric. Food Chem.* **2004**, *52*, 7484–7490.

(9) Bodnaryk, R. P.; Fields, P. G.; Xie, Y.; Fulcher, K. A. Insecticidal factor from field peas. U.S. Patent 5,955,082, 1999.

(10) Keough, T.; Youngquist, R. S.; Lacey, M. P. A method for high-sensitivity peptide sequencing using postsource decay matrix-assisted laser desorption ionization mass spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 7131–7136.

(11) Larsen, M. R.; Roepstorff, P. Mass spectrometric identification of proteins and characterization of their posttranslational modifications in proteome analysis. *J. Anal. Chem.* **2000**, *366*, 677–690.

(12) Xiangyu, J.; Smith, J. B.; Abraham, E. C. Identification of a MS-MS fragment diagnostic for methionine sulfoxide. *J. Mass Spectrom.* **1996**, *31*, 1309–1310.

(13) Rapala-Kozik, M.; Kozik, A.; Travis, J. Effect of oxidation of beta-amyloid precursor protein on its beta-secretase cleavage. A model study with synthetic peptides and candidate beta-secretases. *J. Pept. Res.* **1998**, *52*, 315–320.

(14) Watanabe, Y.; Barbashov, S. F.; Komatsu, S.; Hemmings, A. M.; Miyagi, M.; Tsunasawa, S.; Hirano, H. A peptide that stimulates phosphorylation of the plant insulin-binding protein. Isolation, primary structure and cDNA cloning. *Eur. J. Biochem.* **1994**, *224*, 167–172.

(15) Delobel, B.; Grenier, A.; Gueguen, J.; Ferrasson, E.; Mbailao, M. Use of a polypeptide derived from a PA1b legume albumin as insecticide. PCT WO99/58695, 1999.

(16) Jouvensal, L.; Quillien, L.; Ferrasson, E.; Rahbe, Y.; Gueguen, J.; Vovelle, F. PA1b, an insecticidal protein extracted from pea seeds (*Pisum sativum*): ¹H-2-D NMR study and molecular modeling. *Biochemistry* **2003**, *42*, 11915–11923.

(17) Schenck, H. L.; Dado, G. P.; Gellman, S. H. Redox-triggered secondary structure changes in the aggregated states of a designed methionine-rich peptide. *J. Am. Chem. Soc.* **1996**, *118*, 12487–12494.

(18) Domoney, C.; Ellis, N.; Welham, T. Genetic loci controlling albumin synthesis in *Pisum*. Submitted (Mar 2000) to the EMBL/GenBank/DDBJ databases.

Taylor et al.

(19) Yamazaki, T.; Takaoka, M.; Katoh, E.; Hanada, K.; Sakita, M.; Sakata, K.; Nishiuchi, Y.; Hirano, H. A possible physiological function and the tertiary structure of a 4-kDa peptide in legumes. *Eur. J. Biochem.* **2003**, *270*, 1269−1276.

(20) Brot, N.; Weissbach, H. Peptide methionine sulfoxide reductase: Biochemistry and physiological role. *Biopolymers* **2000**, *55*, 288−296.

(21) Manning, M. C.; Patel, K.; Borchardt, R. T. Stability of protein pharmaceuticals. *Pharm. Res.* **1989**, *6*, 903−918.

(22) Sochaski, M. A.; Jenkins, A. J.; Lyons, T. J.; Thorpe, S. R.; Baynes, J. W. Isotope dilution gas chromatography/mass spectrometry method for the determination of methionine sulfoxide in protein. *Anal. Chem.* **2001**, *73*, 4662−4667.

(23) Ciorba, M. A.; Heinemann, S. H.; Weissbach, H.; Brot, N.; Hoshi, T. Modulation of potassium channel function by methionine oxidation and reduction. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 9932−9937.

(24) Gustavsson, N.; Harndahl, U.; Emanuelsson, A.; Roepstorff, P.; Sundby, C. Methionine sulfoxidation of the chloroplast small heat shock protein and conformational changes in the oligomer. *Protein Sci.* **1999**, *8*, 2506−2512.

(25) Liu, J. L.; Lu, K. V.; Eris, T.; Katta, V.; Westcott, K. R.; Narhi, L. O.; Lu, H. S. *In vitro* methionine oxidation of recombinant human leptin. *Pharm. Res.* **1998**, *15*, 632−640.