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Comparison of the α-Amylase Inhibitor-1 from Common Bean (*Phaseolus vulgaris*) Varieties and Transgenic Expression in Other Legumes—Post-Translational Modifications and Immunogenicity

Peter M. Campbell,*[,†] Daniela Reiner,[‡] Andrew E. Moore,[§] Rui-Yun Lee,[‡] Michelle M. Epstein,[‡] and T. J. V. Higgins[§]

[†]CSIRO Ecosystem Sciences, P.O. Box 1700, Canberra, ACT, 2601, Australia

[‡]Department of Dermatology, Division of Immunology, Allergy and Infectious Diseases, Medical University of Vienna, Austria

[§]CSIRO Plant Industry, P.O. Box 1600, Canberra, ACT, 2601, Australia

ABSTRACT: The seeds of peas (*Pisum sativum*) and chickpeas (*Cicer arietinum*) expressing a gene for α-amylase inhibitor-1 (αAI) from the common bean (*Phaseolus vulgaris*) are protected from damage by old world bruchids (pea and cowpea weevils). Here, we used electrospray ionization time-of-flight mass spectrometry to compare the post-translational modifications of αAI from transgenic sources with the processed forms of the protein from several bean varieties. All sources showed microheterogeneity with differences in the relative abundance of particular variants due to differences in the frequency of addition of glycans, variable processing of glycans, and differences of C-terminal exopeptidase activity. The structural variation among the transgenics was generally within the range of the bean varieties. Previously, mice showed allergic reactions following ingestion of transgenic pea αAI but not bean αAI. Here, only minor differences were observed following intraperitoneal sensitization. Both of the transgenic pea and bean forms of αAI elicited Th1 and Th2 antibody isotype responses, suggesting that both proteins are immunogenic and could potentially be allergenic.

KEYWORDS: Transgenic α-amylase inhibitor, post-translational modification of transgenic protein, immunogenicity, glycosylation of transgenic proteins, transgenic pea, transgenic chickpea

INTRODUCTION

Pea seeds (*Pisum sativum*) expressing a gene for α-amylase inhibitor-1 (αAI) from the common bean (*Phaseolus vulgaris*) are protected from damage by old world bruchids (pea and cowpea weevils).[1,2] Protection from these major insect pests also makes this an attractive strategy for other susceptible legume crops. This particular transgene was attractive for use in food crops because humans already consume αAI in beans without any known ill effect. However, Prescott et al.[3] found that the matrix-assisted, laser desorption/ionization, time-of-flight (MALDI-TOF) mass spectrum of purified, pea-expressed αAI was not identical with the spectrum of αAI derived from the same gene in beans. The differences were assumed to reflect variations in post-translational modifications, but the mass accuracy and resolution were not sufficient for precise interpretation. That finding of nonidentity meant that it was not possible to assume that transgenic αAI would be functionally equivalent to the protein from beans in all respects. Indeed, ingestion of the pea form but not the bean form appeared to induce allergic responses in mice.[3]

The αAI protein from various cultivars of *P. vulgaris* has been characterized extensively.[4−8] It is derived from a prepro-protein by removal of a signal peptide, processing through the ER and Golgi where glycans are added, and then processed (Figure 1) followed by transport to protein storage vacuoles, where it is cleaved at two sites C-terminal to asparagine residues to yield α- and β-chains [9] (Figure 2). The resultant C-terminal Asn residue of the α-chain is then removed by a presumed carboxypeptidase.

The dominant form of the α-chain has both of its glycosylation sites occupied almost exclusively by high mannose glycans, $Man_6GlcNAc_2$ (Man6) and $Man_9GlcNAc_2$ (Man9), resulting in a mass of 11647 Da, while minor forms differ by truncations of one to several mannose residues with only trace amounts of any other glycoforms.[5,8] The dominant form of the β-chain has one of its two potential glycosylation sites occupied with $Man_3XylGlcNAc_2$ (MMX), resulting in a mass of 16526, but mass spectra show minor forms with higher and lower masses. MMX accounted for about 70% of the glycan from the β-chain, while various high mannose and fucosylated glycans accounted for the balance.[5] For two *P. vulgaris* cultivars and one of *Phaseolus coccineus* (Scarlet Runner Bean), the degree of glycosylation at the first site was 70−95%, predominantly by MMX.[8] The second site was 50 or 70% occupied in two cultivars, predominantly by MMXF, but one cultivar lacked the glycosylation site due to an amino acid substitution.

Here, we used higher resolution mass spectrometry than Prescott et al.[3] to show that the structural differences between pea and bean forms of αAI can be attributed to subtle differences in glycan and carboxypeptidase processing of both the α- and the β-chains. The immunogenicity of pea- and bean-expressed αAIs was compared by intraperitoneal immunization of mice. The structural comparisons were extended to include forms of αAI from other *P. vulgaris* cultivars and another transgenic *P. sativum* cultivar as well as

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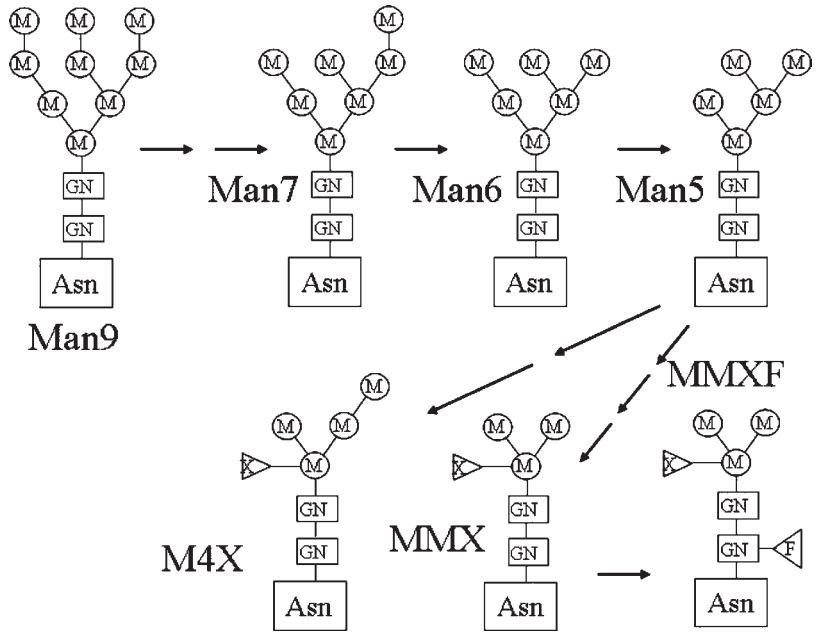

Figure 1. Simplified glycan processing in plants. *N*-Glycosylation of proteins in plants and mammals starts in the endoplasmic reticulum by the transfer of $Glc_3Man_9GlcNAc_2$ to an appropriate asparagine residue. With transport through the endoplasmic reticulum, the Golgi apparatus, and the vacuole, sugars are removed converting the precursor to high mannose-type *N*-glycans ranging from $Man_9GlcNAc_2$ (Man9) to $Man_5GlcNAc_2$ (Man5). Further trimming of mannose (M) and the addition of fucose (F) and/or xylose (X) residues produce some of the plant-specific glycans not found in mammals (M4X, MMX, and MMXF). GN indicates *N*-acetyl glycosamine. Adapted from Gomord and Faye[23] with abbreviations according to the proglycan system (www.proglycan.com).

in chickpeas (*Cicer arietinum*), another legume, to survey the range of variability of processing.

MATERIALS AND METHODS

Isolation of α-Amylase Inhibitors. The transformation of field peas and chickpeas for expression in their seeds of the αAI gene from the common bean (*P. vulgaris*, cv. Tendergreen) has been described previously.[1,10,11] The field pea varieties, Excell and Laura, and the chickpea cultivar are readily available in Australia, the Tendergreen bean was kindly provided by Maarten Chrispeels, University of California (San Diego, CA), and other varieties of common bean (Pinto, Cannellini, and Red Kidney) were purchased from an Australian health food shop.

αAIs from the seeds of the various beans and transgenic legumes were purified as previously described.[12] Briefly, seed meal was extracted with a NaCl solution (1%) followed by a heat treatment (70 °C), dialysis, and centrifugation. The inhibitors were enriched by anion exchange (DEAE-Sepharose CL-6B, Pharmacia) and gel filtration (Sephacryl S-200, Pharmacia) chromatography. Active fractions were determined by inhibition of porcine pancreatic α-amylase (Ceralpha: α-Amylase Assay Kit, Megazyme International, Ireland), and the most pure fractions were determined by inspection of Coomassie-stained sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) gels. Finally, the appropriate pooled fractions were dialyzed against water, lyophilized, and stored at 4 °C. Aliquots (3 μg) from the various preparations were analyzed by SDS-PAGE on 10% NuPAGE gels (Invitrogen) using MES running buffer according to the manufacturer's instructions and staining with Coomassie (Figure 3). The relative amounts of protein in bands within lanes were estimated by densitometry using an ImageScanner with ImagequantTL software (GE Healthcare).

Identification of Protein Bands from SDS-PAGE. Bands from SDS-PAGE were analyzed by in-gel tryptic digestion with C18 reversed phase separation and tandem mass spectrometry of the resultant peptides using an Agilent 1100 capillary LC-ion trap MSD instrument and searching of protein sequence databases with the mass spectral data as previous described.[13,14] The Viridaeplantae subset of the NCBInr protein sequence database (21/1/2011) includes sequences of αAI and other proteins from the seeds of legumes. Protein identifications were accepted with the stringent "autovalidation" settings of the Agilent SpectrumMill software as previously described.[13,14]

Deglycosylation of α-Amylase Inhibitors. Aliquots of purified αAIs were chemically deglycosylated with trifluoromethanesulfonic acid and 10% anisole scavenger using reagents and methods supplied in the GlycoProfile IV kit from Sigma. The method was essentially as used with αAI by Yamaguchi[4] and was reviewed more generally by Edge.[15] The deglycosylated proteins were recovered with a variant of TCA/acetone precipitation (2D Clean-Up Kit, GE Bioscience).

Chromatography and Mass Spectrometry of Intact α-Amylase Inhibitors. Purified αAI protein, with or without deglycosylation, was dissolved in 0.01% trifluoroacetic acid to approximately 1 mg/mL and analyzed with an Agilent 1100 series LC/MSD TOF system. A well-plate autosampler injected 10 μL of each sample to a Waters Symmetry300 C4, 5 μm, 4.6 mm × 50 mm column. The column was eluted with a constant 0.6 mL/min. Washing with 0.1% formic acid in water (2 min) was followed by a linear gradient to 100% acetonitrile/0.1% formic acid over 10 min and then held for 5 min at 100% acetonitrile. Finally, the column was reequilibrated with 0.1% formic acid in water for 5 min prior to the next injection. Material eluting from the column was monitored by UV absorption (220 nm) and the mass spectrometer. In each separation, the column elution profile by UV absorption closely followed the profile of total ion counts.

Electrospray and mass spectrometer instrument settings and the use of an internal mass standard were all as recommended by the manufacturer.[16] Mass spectra from across each chromatographic peak were averaged and deconvoluted using Protein Confirmation Software supplied with the instrument.[16] The software also calculates areas for peaks in the deconvoluted spectra. Peaks in each spectrum were interpreted as matching particular polypeptide chains with or without common plant glycans attached. For clarity, experimental masses are not

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                                                                  Alpha subunit
TG MIMASSKLLSLALFLALLSHANS▼ATETSFIIDAFNKTNLILQGDATVSSNGNLQLSYNSYDS
CN    L
RK                  V  TL          N  G            I
PT       N          V  TL          N  G            I
                                                    Beta subunit
TG MSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQAN▼SAVGLDFVLVPVQPESKGDTVTVE
CN
RK
PT

TG FDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSNNVSTT
CN
RK            A                                  A   L        D
PT                                               A   L        D

TG VELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSN▼IVLNKIL*
CN                                                                *
RK            R                                                   IT*
PT            R                                H                  *
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Figure 2. α-Amylase inhibitors from *P. vulgaris*. The amino acid sequence shown is the translation of the DNA sequence from the gene in the Tendergreen (TG, P02873) variety (and is the sequence that was used for the transformation of the other legumes). Residues that differ in other bean varieties are indicated for Cannellini beans (CN, EF087992), Red Kidney beans (RK, EF087993), and Pinto beans (PT, AY603476). Arrows indicate the cleavage sites that produce the α- and β-chains of the mature protein. Sequons for N-linked glycosylation are boxed but note that the second glycosylation site in the β-chain is lost due to an Asn to Asp substitution in two of the bean varieties. Underlined residues are removed from the C termini of some of the α- and β-chains as indicated in Figures 3−5.

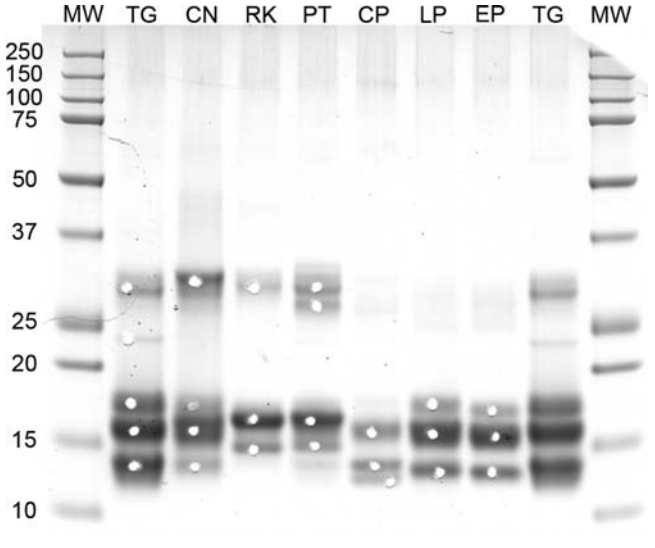

Figure 3. SDS-PAGE of αAI preparations from bean varieties and transgenic legumes. Left to right are molecular mass marker proteins (MW, kDa), αAI from bean varieties, Tendergreen (TG), Cannellini (CN), Red Kidney (RK), and Pinto (PT), transgenic chickpeas (CP), and pea varieties, Laura (LP) and Excell (EP), and then, Tendergreen bean αAI and marker proteins are repeated. Clear circles are visible where gel plugs were excised for identification of the proteins in the bands.

shown for most of the mass spectral peaks in the various figures. However, in each case, they were within 30 ppm or less than 0.5 Da of the theoretical masses of the molecules assigned to them.

Mice. Four to six week old female BALB/c mice were purchased from Charles River Germany and maintained in the Veterinary Medicine University of Vienna animal facility. Mice were provided food and water ad libitum. All experimental protocols were performed in compliance with the appropriate Austrian laws and University guidelines. The protocol for the experiments was approved directly by the Austrian Ministry of Science (68205/12-II/108/209).

Immunogenicity of α-Amylase Inhibitors in Mice. Groups of five mice were immunized intraperitoneally (ip) on days 0 and 21 with 200 μL of phosphate-buffered saline (PBS) or 10 μg of αAI from transgenic Excell pea or Tendergreen bean dissolved in PBS. On day 28, blood was collected by cardiac puncture using a $27Gx^{3/4}$ needle, and after centrifugation, sera were stored at −20 °C. Sera for the measurement of αAI-specific antibodies were tested by standard sandwich enzyme-linked immunosorbent assay (ELISA). For the measurement of antigen-specific IgG1, IgG2a, and IgE, ELISA plates were coated with a solution of dissolved Tendergreen bean or transgenic pea αAI at 10 μg/mL overnight at 4 °C. The plates were washed and blocked with 2% BSA in PBS with 0.05% Tween 20 for 2 h at room temperature. Dilutions of serum from each individual mouse were then incubated in duplicate wells for 24 h at 4 °C. After they were washed, plates were incubated for 2 h at 4 °C with biotinylated monoclonal antibodies to detect IgG1, IgG2a (Southern Biotechnology Associates Inc., Birmingham, AL), or IgE (Becton Dickinson Biosciences, NJ), followed by incubation with streptavidin horseradish peroxidase (Becton Dickinson Bioscience) for 1 h at room temperature. Plates were washed and incubated with a TMB-OptEIATM substrate solution (100 μL) (Becton Dickinson Bioscience) in each well. Following 10 min of incubation in the dark at room temperature, dye development was stopped with the addition of 100 μL of 0.18 M H_2SO_4, and the optical density was measured at 450 nm. Each ELISA was repeated at least twice.

RESULTS

Isolation and Electrophoresis of α-Amylase Inhibitors. The bean αAI gene was transferred previously to two field pea cultivars and chickpeas, where it resulted in the expression of αAI to 2−4% of the total soluble protein in the seeds.[10,11] The various preparations of αAI from peas, chickpeas, and the various beans appeared substantially pure by SDS-PAGE (Figure 3) and similar to each other in their activity as inhibitors. In each case, 100−150 ng of

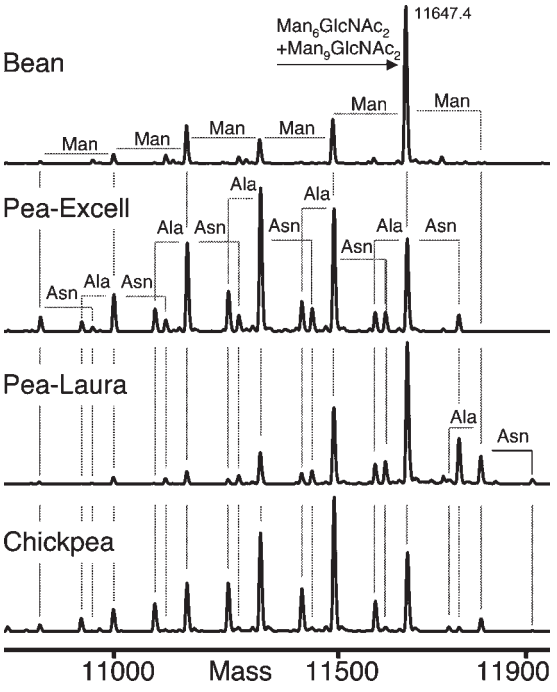

Figure 4. Deconvoluted mass spectra of the α-chain of αAI from *P. vulgaris* cv. Tendergreen (bean) and transgenic peas and chickpeas. The major peak at 11647 Da corresponds to the major form of the α-chain in beans. The major series of mass differences (Man) corresponds precisely to the removal of different numbers of mannose residues from the two high mannose glycans attached to the molecule. Two minor series of mass differences are seen the various transgenics. These are related to the adjacent major glycoform peak by to the removal of an alanine residue (Ala) or the failure to remove an asparagine residue (Asn) from the C terminus of the peptide (Figure 2). Mass spectra for other varieties of *P. vulgaris* are not shown but were virtually identical with the spectrum shown for Tendergreen.

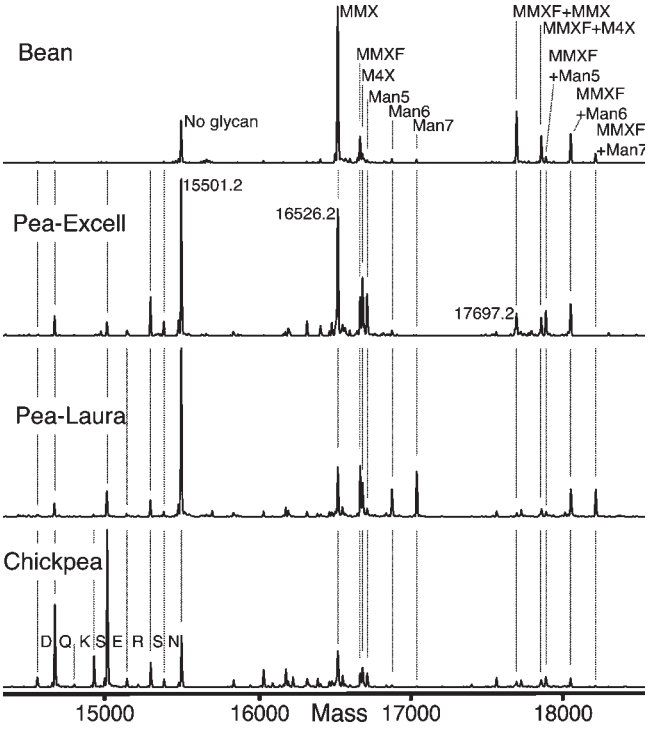

Figure 5. Deconvoluted mass spectra of the β-chain of αAI from *P. vulgaris* cv. Tendergreen and transgenic peas and chickpeas. The major peak at 15501 Da corresponds precisely with the predicted mass of the β-chain without added glycan or other modifications, and the prominent peak at 16526 Da corresponds to the previously demonstrated major glycoform from beans. Peaks in the range up to 17042 are interpreted as indicated by proposing single, alternative glycans, while peaks in the range 17692−18214 are interpreted by proposing glycosylation at both possible sites. In the chickpea β-chain, a series of peaks below 15501 Da show mass separations corresponding to eight residues of the C-terminal sequence of the polypeptide (Figure 2). These truncated forms are also detected to varying degrees in the β-chains from the other legumes. More complex patterns of minor peaks elsewhere in the spectra are consistent with similar C-terminal truncations of the various glycoforms, but for clarity, these have not been annotated. The relative areas of the annotated peaks are shown in Table 1.

αAI preparation caused 50% inhibition of 100 ng of porcine α-amylase (data not shown). Bands from the gel were sampled for identification by mass spectrometry. Across the entire experiment, αAI was identified by 14 distinct tryptic peptides representing 42% coverage of the precursor sequence with a SpectrumMill summed MS/MS search score of 226 where 20 would be sufficient for a confident automatic identification.

The major bands from each sample ran close to each other, between the 10 and the 20 kDa marker proteins, at around 15 kDa (Figure 3). Both subunits of αAI were generally detected in each of these bands. The α-subunit was generally detected only by the peptide, AFYSAPIQIR. The β-subunit was generally detected by 4−9 peptides, generally including the peptide from its N terminus, SAV...ESK. No other protein was detected from these major bands.

The minor bands around 30 kDa also contained peptides from both subunits of αAI. A peptide that spans the site that is cleaved in the mature protein (QANSAV...ESK, Figure 2) was detected from Pinto and Cannellini beans, suggesting that αAI in the 30 kDa bands is not fully processed. The faint band at about 23 kDa from Tendergreen bean yielded two peptides from the β-subunit.

Two contaminating proteins were identified from the minor bands around 30 kDa. Bean phytohemagglutinin (PHA, trEMBL Q8RVX5) together with unprocessed αAI was found in the upper 30 kDa band from Pinto bean. The lower 30 kDa band from Pinto bean was identified as α-amylase inhibitor-like protein (AIL, GenBank BAA86927). PHA and AIL have 50 and 58% amino acid sequence identity, respectively, with the αAI precursor, but they differ from αAI by not being proteolytically processed to form subunits.[17] AIL was not detected from the other beans, and PHA was not detected from Red Kidney bean. The 30 kDa band from Tendergreen bean was mostly unprocessed αAI, and by densitometry on the stained gel, it was about 15% of the total preparation. Comparison of the total ion counts for tryptic peptides suggested that PHA accounted for about 17% of the band or about 2.5% of the Tendergreen bean preparation overall. PHA was much less than 1% of the preparation from Cannellini bean.

Separation of the Subunits of α-Amylase Inhibitors. Purified αAI preparations from peas, chickpeas, and the various beans, with or without deglycosylation, were separated by C4 reversed phase liquid chromatography. This served as a convenient group separation to ensure that the subunits were dissociated from each other and any minor contaminants while being desalted for electrospray ionization and time-of-flight

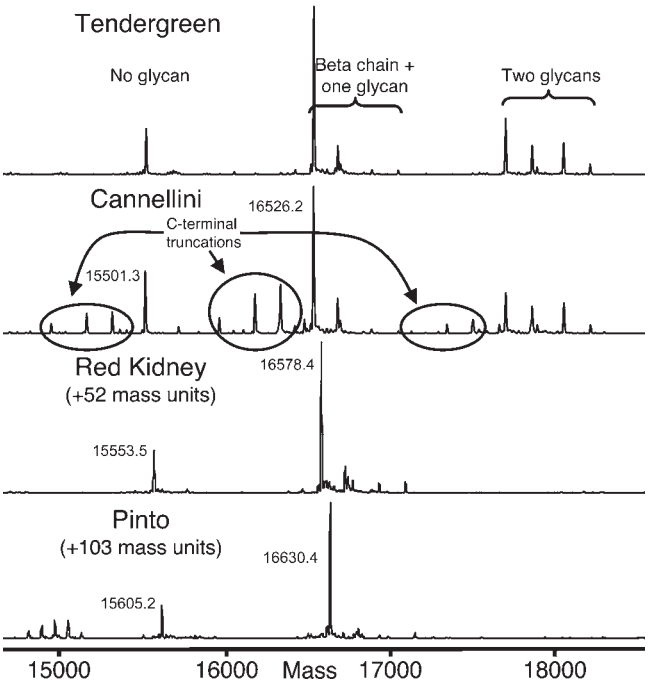

Figure 6. Deconvoluted mass spectra of the β-chain of αAI from *P. vulgaris* varieties. The spectrum for the Tendergreen variety is the same as shown in Figure 5. The amino acid sequence of the β-chain of the Cannellini variety is the same as Tendergreen, while amino acid differences in the Red Kidney and Pinto varieties resulted in mass shifts of +52 and +103, respectively, and the loss of one of the glycosylation sites. Peaks consistent with the removal of residues from the C terminus (like Figure 5) were seen clearly for the Cannellini variety but barely detected for the others. The peaks around 15000 mass units for the Pinto variety appear to be due to a minor contaminant.

mass spectrometry. Generally, three peaks were observed by both UV absorption and a total ion trace from the mass spectrometer. The first contained some low molecular weight contaminants (not shown), the second contained variants of the α-chain of αAI (Figure 4), and the third contained the β-chains (Figures 5 and 6). All samples showed at least some material that eluted in the first peak with acetonitrile concentrations up to 57%. None of the early eluting material was consistent with αAI, and it was not consistent between αAI preparations from different species. The molecular weights of the early eluting material were generally lower than the αAI α-chain. In the case of Tendergreen bean αAI, the mass spectrum of the early eluting material closely resembled the 8−9000 m/z region of the MALDI-TOF mass spectrum shown by Prescott et al.[3] and mentioned by Young et al.[7]

The second peak was eluted sharply from all preparations with 59−63% acetonitrile. Averaging and deconvoluting the mass spectra from across this peak showed that various forms of the αAI α-chain coeluted. In the case of Tendergreen bean, Pinto bean, and Excell pea, the spectra aligned precisely with the previously published MALDI-TOF mass spectra[3] but showed much improved resolution and mass accuracy (Figure 4).

The third peak was broader but consistently eluted from all preparations when the solvent gradient exceeded 65% acetonitrile. This peak contained coeluting variants of the β-chain. As above, the spectra for the β-chain variants aligned precisely with the relevant region of the previously published MALDI-TOF mass spectra[3] (Figures 5 and 6). Furthermore, within each α- or β-chain set, the relative intensities of major and minor peaks were consistent with the corresponding MALDI-TOF spectra. This adds to our confidence that the molecules are ionized in close proportion to their relative abundance within each set of subunit variants. Resolution and mass accuracy were sufficient to allow unambiguous assignment of all of the major peaks in each spectrum.

Mass Spectral Analysis of the α-Subunit of αAI: Transgenics as Compared with Tendergreen Bean. Mass spectra for the α-chain variants from the transgenic sources are shown aligned with the corresponding spectrum for the α-chain from the Tendergreen bean (Figure 4). The same glycoforms were seen for each legume, but they differed in their relative mass spectral intensity. For example, the most abundant glycoform in Excell peas had two fewer mannose residues than the major bean glycoform, whereas the most abundant glycoform in chickpeas had one less mannose residue. On the other hand, the distribution of glycoforms in Laura pea was quite similar to the bean except that it also had a significant amount of a glycoform with one more mannose than the dominant glycoform of the bean. These shifts in the distribution of glycoforms could be due to slight differences in the activity of the enzyme that trims mannose residues in the order Excell pea > chickpea > bean > Laura pea.

Between the peaks corresponding to the glycoforms discussed above were less intense peaks in the mass spectra (Figure 4). For all three transgenic sources, these peaks aligned precisely, but they did not correspond to very minor peaks that can be seen in the bean spectrum. These less intense peaks differed from adjacent glycoform peaks by either +114 or −71 Da. Recalling the known processing of αAI in the bean, the mature α-chain has glycans added and an asparagine residue removed from the C terminus.[7] The +114 peaks are precisely consistent with a subset of molecules retaining the C-terminal Asn residue, while the −71 peaks are consistent with a subset of molecules having both the C-terminal Asn and the next C-terminal residue, alanine, also removed. Thus, it would appear that the presumed carboxypeptidase in the bean is very precise, removing the asparagine residue entirely but not any further residue, while the equivalent enzymes in the transgenics are less precise. In Excell pea, 13 ± 1% of the protein corresponding to any particular glycoform retained the Asn, while 19 ± 2% had both Asn and Ala removed. Laura pea had more protein retaining Asn (24 ± 4%) and less with Ala also removed (10 ± 1%). Chickpea had much less protein retaining Asn (5 ± 2%) and much more with Ala also removed (30 ± 4%). The small standard deviations for all of these data indicated that the extent of C-terminal processing was independent of the extent of processing of the attached glycans.

From the above analysis, deglycosylation would be expected to produce an homogeneous α-chain from the bean, while two further forms (+114 Da, Asn; and −71 Da, Ala) would be produced in the transgenics. Rather than seeing simple peaks following deconvolution, tight envelopes of peaks about 70 mass units wide were seen at approximately the expected masses (not shown). This microheterogeneity was probably due to a complex array of side reactions during deglycosylation involving Ser, Thr, Asp, Cys, Met, and aromatic residues.[15] However, these envelopes of peaks were separated precisely as expected (+114, −71) and with ratios of abundance consistent with the analysis above.

Mass Spectral Analysis of the β-Subunit of αAI: Transgenics as Compared with Tendergreen Bean. Mass spectra for the β-chain variants from the transgenic sources are shown

Table 1. Relative Abundances of Glycoforms and C Terminally Truncated Forms of the α-Amylase Inhibitor β-Chain from Bean Varieties and Various Transgenic Legumes[a]

	no glycan	MMX	MMXF	M4X	Man5	Man6	Man7	one glycan totals	MMXF + MMX	MMXF + M4X	MMXF + Man5	MMXF + Man6	MMXF + Man7	two glycans totals	% C-terminal truncations
bean TG	**11**	42	7	3	1	1	<1	**56**	14	7	2	8	2	**33**	<1
bean CN	**13**	33	9	4	2	2	<1	**51**	9	11	2	12	2	**37**	52
bean RK	**14**	55	9	8	6	5	4	**86**	0	0	0	0	0	**0**	<1
bean PT	**16**	69	2	3	4	1	5	**84**	0	0	0	0	0	**0**	<1
pea Excell	**27**	25	7	12	8	1	<1	**53**	5	3	5	6	<1	**20**	37
pea Laura	**34**	11	11	8	2	7	10	**49**	<1	2	1	7	6	**17**	30
chickpea	**29**	24	8	12	8	<1	<1	**52**	3	5	6	5	<1	**19**	87

[a] The areas of mass spectral peaks annotated in Figures 5 and 6 are expressed as percentages (omitting peaks due to C-terminal truncations). In the rightmost column is the percentage of nonglycosylated forms showing truncations of 1−8 residues from the C terminus. Bean varieties are Tendergreen (TG), Cannellini (CN), Red Kidney (RK), and Pinto (PT).

aligned with the corresponding spectrum for the α-chain from the Tendergreen bean (Figure 5). As with the α-chain, it is apparent that the same glycoforms are seen for each, but they differ considerably in their relative abundances.

Considering first the spectrum for the Tendergreen bean form of the β-chain, the major peak at 16526 Da corresponded to the previously described major glycoform.[7] Peaks in the range of 16672 to 17042 Da were consistent with the mass of the polypeptide with alternative, single glycans attached. A peak at 15501 Da corresponded to the mass of the polypeptide without any glycan or other modification, and peaks in the range 17697−18214 Da were consistent with the polypeptide with various combinations of two glycans. The relative abundances of the various glycoforms (Figure 5 and Table 1) were broadly consistent with the identities and relative abundances of glycans that were cleaved, isolated, and identified from the β-chain of αAI from another bean cultivar.[5] Specifically, a major bean glycoform (42% of the total) had one glycan comprising the mannose core with a xylose residue attached (MMX). Glycoforms with single alternative glycans attached were less abundant: The mannose core with both a xylose and a fucose residue attached (MMXF, 7%), a glycan identical to the major form except for one more mannose residue (M4X, 3%), and high mannose glycans (Man5, Man6, and Man7, each about 1%). In total, 56% of the polypeptide had a single glycan attached. The nonglycosylated polypeptide accounted for about 11% of the total. The remaining 33% of the total consisted of polypeptides with two glycans attached. Their masses were consistent with one MMXF glycan in combination with MMX (14%), M4X (7%), Man5 (2%), Man6 (8%), or Man7 (2%). Other peaks consistent with various C-terminal truncations were only barely detected.

The various transgenic β-chains all differed from the Tendergreen bean β-chain first by showing less overall glycosylation and second by showing significant C-terminal truncations (Figure 5 and Table 1). In comparison with bean (ignoring the C-terminally truncated forms for the moment), the Excell pea and Laura pea showed less polypeptide with two glycans (22 and 17%, respectively), about the same with one glycan (60 and 49%), and more with no glycan (18 and 34%). Similarly, chickpea showed less with two glycans (18%), about the same with one glycan (49%) and more with no glycan (27%). In particular, it can be seen that the major MMX glycoform was more prominent than the nonglycosylated form in the spectrum for the bean β-chain, but the converse was observed in the transgenics (Figure 5).

A series of eight peaks around 15000 Da corresponded precisely with the masses predicted by the sequential removal of amino acid residues from the C terminus of the nonglycosylated polypeptide (Figure 5). These truncated forms were most prominent for chickpea. Corresponding peaks were seen for the other transgenics and Cannellini bean but barely detected in the other beans (Figure 6). Comparing the areas of the peaks for the truncated forms of the nonglycosylated β-chains, we calculate that virtually 100% of the Tendergreen bean β-chain was not truncated, 37 and 30% are C terminally truncated to some degree in Excell pea and Laura pea, respectively, 52% is truncated in Cannellini beans, while only 13% was not truncated in chickpea (Table 1). More complex arrays of minor peaks at masses below the singly and doubly glycosylated peaks (Figure 5) were consistent with the same C-terminal truncations applied to the various glycoforms, but for clarity, we have not attempted to annotate these. As was described above for the α-chain, it would appear that presumed carboxypeptidases in the transgenics act less precisely on this peptide than the bean's enzyme. It is possible that the truncations reflect degradation of the expressed protein rather than differences of processing, but the chickpea αAI is just as active as an inhibitor as the other preparations (data not shown). These results are reminiscent of the variable C-terminal trimming of lectins in the seeds of different legumes, which sometimes has structural consequences and sometimes not.[18]

As with the α-chain, chemical deglycosylation resulted in envelopes of peaks at approximately the masses one would predict from the above analysis and at precisely the expected mass separations between those envelopes, and ratios of relative abundance were also consistent (not shown).

Comparison of α-Amylase Inhibitor from *P. vulgaris* Varieties. The αAIs from several *P. vulgaris* varieties were compared first by DNA sequencing and then by mass spectrometry as above. None had identical sequences (Figure 2), but the amino acid sequences of the Tendergreen, Red Kidney, and Pinto αAIs are, respectively, the same as the TAI, DAI, and MAI αAIs.[8] The Cannellini variety had one predicted amino acid difference from the Tendergreen variety, but this is not in the mature protein. The Red Kidney and Pinto varieties shared three amino acid differences from Cannellini and Tendergreen in the α-chain, resulting in a difference of one mass unit, which was clearly detected in the mass spectra. The α-chain mass spectra of the bean varieties (not shown) were virtually identical to the Tendergreen variety (Figure 4) aside from that one mass unit shift and trace levels of glycoforms with one or two additional mannose residues from the major form.

The spectrum of the β-chain of Cannellini bean resembled Tendergreen very closely (Figure 6 and Table 1) except that 52% of the protein showed truncations of up to five C-terminal residues, thereby resembling the various transgenics (Figure 5). The β-chains of Cannellini and Tendergreen had identical amino

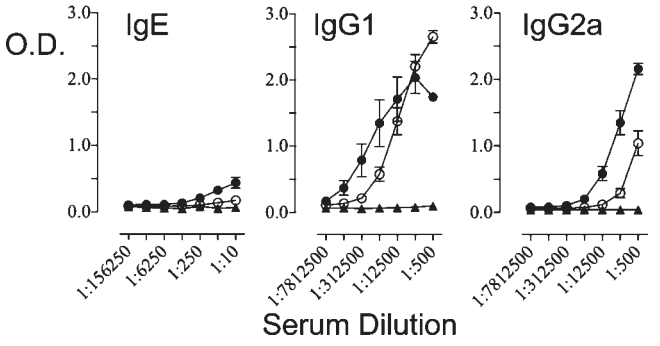

Figure 7. Serum anti-αAI antibody response in mice. BALB/c mice were immunized with 10 μg of Excell pea αAI (○), Tendergreen bean αAI (●), or PBS (▲) ip on days 0 and 21. On day 28, blood was taken, and serum was separated and stored at −20 °C before use. We measured sera for αAI-specific IgG1, IgG2a, and IgE antibodies by standard sandwich ELISA. Data are expressed as the means ± SEMs.

acid sequences, but Red Kidney and Pinto both differed from Tendergreen at five sites (four in common with each other), resulting in mass differences of +52 and +103, respectively (Figure 6). A shared Asn to Asp substitution caused the more C-terminal of the two potential glycosylation sites to be lost in Red Kidney and Pinto beans, and this was reflected in the mass spectra that showed no peaks in the mass range where the other varieties had peaks attributed to the addition of two glycans. However, the relative intensities of peaks attributed to no glycosylation or a single glycan were consistent between the bean varieties (Table 1). For example, the spectrum for the Red Kidney bean β-chain superimposed very well over the Tendergreen spectrum except for the peaks attributed to two glycans after accounting for the shift of 52 mass units (Figure 6).

Immunogenicity of the Transgenic Pea and Tendergreen Bean αAIs. To determine whether there were immunological differences between differentially processed αAIs, mice were immunized intraperitoneally twice with either transgenic pea or Tendergreen bean αAI, and αAI-specific antibody responses were measured (Figure 7). Both forms of αAI induced antibody responses with the titers for IgG1 > IgG2a > IgE. Tendergreen bean αAI generally induced higher titers than the protein expressed in Excell pea, and IgE against the pea αAI was barely detected.

DISCUSSION

Previously, the Excell pea and Tendergreen bean forms of αAI were shown to be nonidentical by MALDI-TOF mass spectrometry and also appeared to be nonidentical in a mouse model for food allergy.[3] Here, we further analyzed the molecules using higher resolution mass spectrometry and compared the immunogenicity of the purified proteins in mice. We describe a wider range of post-translational modifications that occur among edible bean varieties, and we examined an additional pea cultivar and another legume, chickpea, transformed with the same gene. None of the modifications were unusual, or at least all of the observed masses were consistent with typical glycosylation in plants and known modifications of αAI. All of the molecular forms found among the transgenics were also found among the varieties of edible beans.

The α-chains of αAI from the beans and transgenic Excell pea showed the same set of glycoforms albeit in differing proportions. These high mannose glycans are produced by the part of the glycosylation pathway that is common to plants and mammals. It therefore seems unlikely that the tendency to have one or two fewer mannose residues on an otherwise identical molecule would result in a difference of immunogenicity between the transgenic and the native α-subunits. A small proportion of pea αAI had one more amino acid residue or one less at the C terminus. The αAI from transgenic chickpea showed varying proportions of the same set of α-subunit glycoforms as the beans and also showed the more varied C-terminal processing like the Excell peas. While some of the transgenics have a glycoform with one more mannose residue than the major form of αAI in the beans, this was also detected at a trace level in two of the beans.

The variation in the β-chains was more complex. The Excell pea form had (1) relatively more molecules without added glycan, (2) fewer with two glycans, (3) more molecules with the Man5 and M4X glycans although these are minor components, and (4) more molecules with up to eight amino acid residues removed from the C terminus.

A further difference between the beans and the transgenics is that the beans appear to contain variable amounts of unprocessed or incompletely processed αAI precursor seen as bands around 30 kDa by SDS-PAGE (Figure 3).

Mice immunized with isolated αAI proteins from either Tendergreen bean or Excell pea generated Th1 and Th2 isotype antibodies albeit with very low titers of IgE for pea αAI. The Th2 type response (IgG1 and IgE) following intraperitoneal sensitization of BALB/c mice shows that both proteins are immunogenic and potentially allergenic.[19] The bean and pea proteins induced similar immune responses with the bean form minimally more immunogenic, perhaps due to the presence of incompletely processed αAI or about 2.5% PHA. Our data differ from an earlier in vivo study in which αAI from transgenic peas was more immunogenic and allergenic as compared to beans.[3] However, differences between the experimental protocols of the two studies may explain the disparate results. Here, we used isolated Tendergreen bean αAI (Tendergreen being the original source of the gene for peas and chickpeas) and isolated pea αAI for intraperitoneal immunization. In contrast, Prescott et al.[3] initially administered αAIs by the oral route as flours of transgenic pea or Pinto bean. They concluded that the mice were subsequently unresponsive to bean αAI relative to pea αAI. However, the Pinto form of αAI has eight amino acid differences from the Tendergreen αAI that was used for subsequent exposure to bean αAI, including one difference, which removes one of the two glycosylation sites in the β-chain (Figures 2 and 6 and Table 1).

Other animals than mice have been exposed to αAI from transgenic peas. Rats, pigs, and chickens were fed raw, transgenic peas at around 30% or more of their diet in short feeding trials. The only effects on their health could be attributed to dose-dependent reductions of the digestion of starch due to amylase inhibition rather than immunological effects, diarrhea in the case of pigs, and a reduction of weight gain in the case of chickens.[20−22]

We found no evidence for increased immunogenicity of the transgenic αAI, and we note that immunogenicity is not sufficient for allergenicity. Whatever their status as potential allergens, the pure proteins seem to induce similar immune responses, and the ranges of post-translational modifications are similar between the transgenic and the nontransgenic versions. How best to use animal models and other tools to predict the potential allergenicity of novel proteins in transgenic crops is an active area of continuing research and debate.[19] Ongoing work in our

laboratories will compare the immunological consequences of oral exposure of mice to the transgenic and nontransgenically produced proteins.

AUTHOR INFORMATION

Corresponding Author

*E-mail: Peter.M.Campbell@csiro.au.

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