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Transgenic Expression of Bean α-Amylase Inhibitor in Peas Results in Altered Structure and Immunogenicity

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The development of modern gene technologies allows for the expression of recombinant proteins in non-native hosts. Diversity in translational and post-translational modification pathways between species could potentially lead to discrete changes in the molecular architecture of the expressed protein and subsequent cellular function and antigenicity. Here, we show that transgenic expression of a plant protein (α-amylase inhibitor-1 from the common bean (*Phaseolus vulgaris* L. cv. Tendergreen)) in a non-native host (transgenic pea (*Pisum sativum* L.)) led to the synthesis of a structurally modified form of this inhibitor. Employing models of inflammation, we demonstrated in mice that consumption of the modified αAI and not the native form predisposed to antigen-specific $CD4^+$ Th_2-type inflammation. Furthermore, consumption of the modified αAI concurrently with other heterogeneous proteins promoted immunological cross priming, which then elicited specific immunoreactivity of these proteins. Thus, transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants possessing altered immunogenicity.

KEYWORDS: α-Amylase inhibitor; transgenic plant; animal model; Th2 inflammation; mass spectrophotometry

INTRODUCTION

Genetically modified (GM) plants are designed to enhance agronomic productivity or product quality and are being increasingly employed in both agricultural and livestock industries (*1*, *2*). Recently, peas (*Pisum sativum* L.) expressing a gene for α-amylase inhibitor-1 (αAI) from the common bean *(Phaseolus* vulgaris L. cv. Tendergreen) were generated to protect the seeds from damage by inhibiting the α-amylase enzyme in old world bruchids (pea, cowpea, and azuki bean weevils) and are currently undergoing risk assessments (*3*−*6*).

The present study was initiated to (1) characterize the proteolytic processing and glycopeptide structures of αAI when transgenically expressed in peas (pea-αAI) and (2) evaluate in an in vivo model system the immunological consequence of oral consumption of pea-αAI. We demonstrate that expression of αAI in pea leads to a structurally modified form of this inhibitor. Employing experimental models, we show that the structural modification can lead to altered antigenicity. These investigations reveal that expression of proteins in non-native hosts can lead to the synthesis of a protein variant with altered immunogenicity.

MATERIALS AND METHODS

Nontransgenic and Transgenic Plants. Seed meal was obtained from nontransgenic peas, genetically modified peas expressing bean α-amylase inhibitor-1 (αAI) (*5*), genetically modified narrow leaf lupin (*Lupinus angustifolius* L.) expressing sunflower seed albumin protein (SSA) in the seeds (SSA-lupin) (*7*), and from nontransgenic Pinto bean. Seeds were ground into fine flour in liquid N_2 using a mortar and pestle. This seed meal was then suspended in PBS (0.166 g meal/mL), homogenized, sieved through a 70 μm mesh, and stored at −70 °C. In some experiments, seed meal homogenates were cooked at 100 °C for 30 min before administration to mice (indicated in text).

Purification of SSA from Transgenic Lupin and αAI from Common Beans and from Transgenic Peas. αAI was purified from the common beans (Pinto and Tendergreen) and transgenic peas and SSA from genetically modified narrow leafed lupin (SSA-lupin) as previously described (*7*, *8*). Purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 15−

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25% gradient, 1 mm thick, mini-gel format) and MALDI-TOF mass spectrometry.

Western Immunoblot Analysis. αAI polypeptide composition was determined in protein extracts from common bean and transgenic peas as previously described (*3*). Protein was extracted from seeds with 0.5 M NaCl, 1 mM EDTA, and 0.1 M *N*-tris(hydroxymethyl)methylaminoethanesulfonic acid at pH 7.8. Aliquots of reduced protein (20 μg by Bradford assay) were fractionated by SDS-PAGE and electroblotted onto nitrocellulose membrane. αAI polypeptides were detected with an αAI antiserum from rabbit and goat anti-rabbit IgG conjugated to alkaline phosphatase (*3*). The concentration of αAI in transgenic peas was determined as 4% of total protein as previously described (*3*).

Structural Analysis of Purified αAI from the Pinto and Tendergreen Beans and from Transgenic Peas. Purified αAI from the common beans, Pinto and Tendergreen, and from transgenic peas were analyzed by matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS). The proteins were dissolved in water (approximately 1 μg/μL), and then 1 μL was mixed with 1 μL of matrix solution (saturated sinapinic acid in 50% acetonitrile/0.1% trifluoroacetic acid) on the sample plate of a Voyager Elite MALDI-TOF mass spectrometer (Perseptive Biosystems) and allowed to dry. Spectra were collected in linear mode with myoglobin used for close external calibration (Sigma, Cat. No. M-1882, 16952.6 $[M + H]^+$, 8476.8 $[M + 2H]^{2+}$).

Mice and Intragastric Administration of Seed Meal from Nontransgenic and Transgenic Plants. BALB/c mice were obtained from specific pathogen-free facilities at the Australian National University. Mice were intragastrically administered 250 μL of seed meal suspension (~100 mg/mL) containing either transgenic peas, nontransgenic peas, SSA-lupin, or Pinto bean twice a week for 4 weeks. In some experiments, serum was taken from the mice at the start of the third and fifth weeks during feeding. The serum antibody titers were determined as previously described (*9*).

Mice and Delayed Type Hypersensitivity Responses. BALB/c mice were administered seed meal as described above. Seven days following the final intra-gastric challenge, mice were subcutaneously injected with 25 μL of the appropriate antigen [Tendergreen-αAI, pea α-AI, or lupin SSA (1 mg/mL in PBS)] into the footpad. The positive control [(+) control] is mice immunized by i.p. injection of 200 μL containing 50 μg of Tendergreen-αAI dissolved in PBS with Alum (1 mg/mL) and subsequently receiving 25 μL of purified Tendergreen-αAI (1 mg/mL PBS). The negative control [(−) control] is mice immunized by i.p. injection of 200 μL containing 50 μg of Tendergreen-αAI dissolved in PBS with Alum (1 mg/mL) and subsequently receiving 25 μL of PBS. DTH responses were assessed by measuring the specific increase in footpad thickness using a digmatic calliper (Mitutoyo, Kawasaki, Japan) 24 h following the challenge. Serum was collected on day 14, and antibody titers were determined as previously described (*9*).

Murine Model of $CD4^+$ Th2 Cell-Mediated Inflammation. BALB/c WT mice were administered seed meal as indicated in the text. Seven and nine days following the final intra-gastric challenge, mice were anesthetized with an intravenous injection of 100 μL of Saffan solution (1:4 diluted in PBS). Mice were intubated with a 22 gauge catheter needle, through which purified αAI from Tendergreen bean or transgenic pea (1 mg/mL PBS), or vehicle control (PBS), was instilled. Airway responsiveness (AHR), mucus production, and eosinophilia were measured 24 h following the final intra-tracheal challenge. AHR to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, NY) as previously described (*9*). This system yields a dimensionless parameter known as enhanced pause (Penh), reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration, which can be used to empirically monitor airway function. Measurements were performed as previously described (*9*). Lung tissue representing the central (bronchi-bronchiole) and peripheral (alveoli) airways was fixed, processed, and stained with Alcian Blue-PAS for enumeration of mucin-secreting cells or Charbol's chromotrope-Haematoxylin for identification of eosinophils as previously described (*9*).

Intragastric Administration of Purified αAI and OVA. Mice were administered 200 μL of affinity purified Tendergreen- or transgenic pea-αAI (5 μg) with ovalbumin (OVA, 1 mg/mL) in a PBS suspension three times a week for 2 weeks. One week following feeding, the mice were intubated with a 22 gauge catheter needle, through which 25 μL of OVA (1 mg/mL PBS), or vehicle control (PBS), was instilled and the $CD4^+$ Th2-inflammation indices determined as described above. Serum was taken from the mice 1 day after the final intra-tracheal challenge, and serum antibody titers were determined as described (*9*).

Antigen Specific $CD4^+$ T-Cell Response. Peribronchial lymph nodes (PBLN) were subjected to pea-αAI or αCD3/αCD28 stimulation as previously described (*9*). In brief, 5×10^5 PBLN cells/mL were cultured with αAI (50 μg/mL) or αCD3 (5 μg/mL)/αCD28 (1 μg/mL) for 96 h. IL-4, IL-5, IFNγ levels were determined in supernatants from stimulated PBLN homogenates by using the OptEIA Mouse IL-4, IL-5, and IFNγ kits (Pharmingen).

Statistical Analysis. The significance of differences between experimental groups was analyzed using Student's unpaired *t*-test. Values are reported as the mean ± SEM. Differences in means were considered significant if $p < 0.05$.

RESULTS

MALDI-TOF-MS Analysis of αAI. To assess the consequences of transgenic expression of the bean αAI in peas, we initially performed a structural analysis of the transgenically expressed protein (pea-αAI). Pea-αAI was compared by Western blot analysis and MALDI-TOF-MS with natively expressed αAI from the common beans, cvs. Pinto (Pinto-αAI) and Tendergreen (Tendergreen-αAI) (collectively termed bean-αAI). Previous studies have shown that bean-αAI is synthesized as a pre-pro-αAI polypeptide that is cleaved following Asn^{77} to form two peptide chains (α and β), both of which are glycosylated and have one or more amino acid residue(s) removed from their C-termini (*8*). This post-translational processing results in major forms of the α and β chains with masses of 11 646 and 17 319, respectively, and minor forms containing alternative glycans (*10*−*12*). Western immunoblot analysis of Tendergreen-αAI and pea-αAI revealed immunoreactive bands in the 11 000−18 000 mass range consistent with the reported structure (*10*−*13*). Detailed comparison of Tendergreen-αAI with pea-αAI revealed differences in the banding profile, suggesting possible differences in the molecular structure of natively and transgenically expressed αaI (**Figure 1A**).

To better resolve the differences between pea-αAI and bean-αAI, affinity purified αAI was analyzed by MALDI-TOF-MS (**Figure 1B**). The mass spectra of Tendergreen-αAI and Pinto-αAI closely matched a previously published spectrum (*10*) of a bean-αAI (*Phaseolus vulgaris* L. cv. Greensleeves) confirming that both Tendergreen- and Pinto-αAI possess similar well-characterized post-translational modifications and very similar relative abundance of minor processing variants (*10*, *11*). Alignment of our spectra with the previously published data (*10*) allowed identification of peaks in the pea-, Tendergreen-, and Pinto-αAI spectra. The major form of the α-chain (11 646 Da) of bean-αAI contains residues 1−76 by cleavage of the pro-protein following Asn^{77}, removal of Asn^{77}, and the addition of sugar residues ($Man_6GlcNAc_2$ at Asn^{12} and $Man_9GlcNAc_2$ at Asn^{65}). Minor forms of the α-chain of bean-αAI differed by having one to three fewer mannose residues resulting in a series of peaks in the MALDI-TOF spectrum that differ by 162 mass units. In contrast, less heavily glycosylated forms dominated for the α-chain of pea-αAI. In particular, an α-chain with two fewer mannose residues (11 322 Da) was the most abundant for pea-αAI but the least abundant for Tendergreen-αAI (**Figure 1C(i)**). A further difference in the pea-αAI spectrum was a series of minor peaks differing from the main α-chain peaks by either

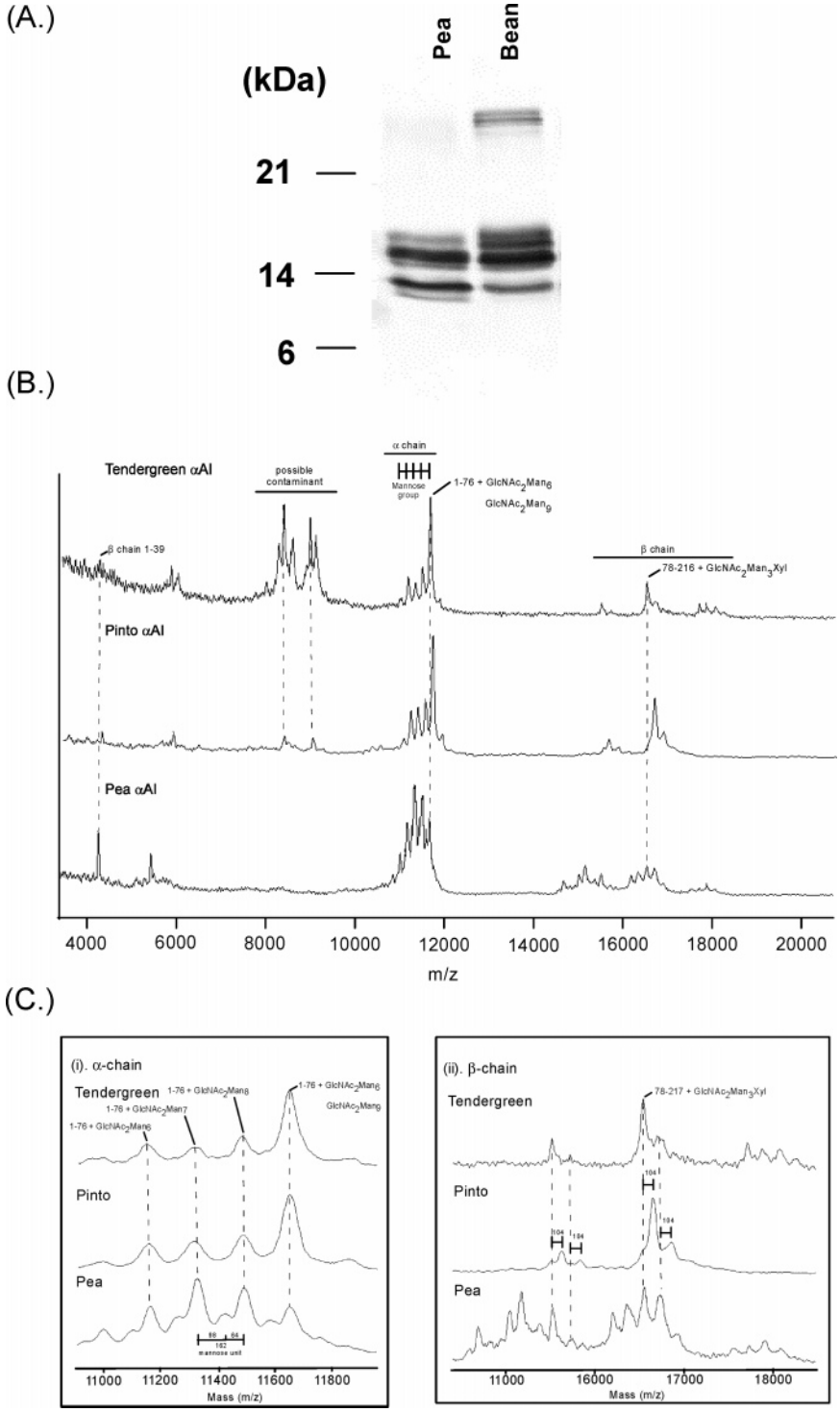

Figure 1. Western immunoblot and MALDI-TOF-MS analysis of common bean-derived-αAIs and αAI from transgenic peas. (**A**) Western blot analysis of αAI protein in extracts of transgenic peas and the Tendergreen variety of common bean. The masses of standard proteins are indicated. (**B**) Aligned MALDI-TOF mass spectra of purified αAI from transgenic pea and the common beans, Tendergreen and Pinto. (**C**) Detail from the spectra in panel B showing the regions of the α-chain (**i**) and the *β*-chain (**ii**).

+98 or −64 mass units, indicating another modification of some of the pea-αAI α-chains (**Figure 1C(i)**).

The major form of the *β*-chain of Greensleeves-αAI (16527 Da) contains residues 78−216 by cleavage of the pro-protein following Asn^{77}, the removal of the seven C-terminal residues following Asn^{216}, and the addition of sugar residues (Man_3-$GlcNAc_2Xyl_1$ at Asn^{140}) (*10*−*13*). The *β*-chain region of the Tendergreen-αAI spectrum closely aligned with that of Greensleeves-αAI (**Figure 1C**). The *β*-chain region of the Pinto-αAI spectrum also closely resembled that of Greensleeves-αAI except that both major and minor peaks of Pinto-αAI were shifted by approximately +104 mass units. This mass discrepancy is consistent with five amino acid residue differences between the *β*-chains of Tendergreen-αAI and Pinto-αAI as predicted by gene sequence comparison (see Supporting Information Figure 1). Further, there are also three predicted residue differences between the Tendergreen-αAI and Pinto-αAI α-chains that result in a difference of +1 mass unit, which would not be

detected by our methods. These sequence differences are consistent with previous reports of αAI polymorphisms among bean cultivars (*12*, *13*). The pea-αAI spectrum showed major peaks corresponding to the two major and minor forms of the *β*-chain found in Tendergreen-αAI; however, the pea-αAI spectrum also showed a number of other peaks (**Figure 1C(ii)**). DNA sequencing of the transgene in pea and comparison with the published sequence (*14*) confirmed that the nucleotide sequences were identical, establishing that the observed further forms of the pea-αAI are related by variations in post-translational modifications including glycosylation (**Figure 1C(ii)**).

Analysis of the spectra of pea- and bean-αAI also revealed several other differences. First, a number of peaks at ~8−9000 and 5824 mass units and below were observed in the bean-αAI spectrum, which are consistent with a previously reported protein that copurifies with bean-αAI (*10*) and doubly charged ($(MH_2)^{2+}$) forms of the α-chain, respectively. Further, a peak at 4223 mass units was detected in the pea-αAI spectrum, which has not been previously reported. While this peak is barely detected in the bean-αAI spectrum presented here, the peak was observed in a number of other bean-αAI preparations (results not shown). The mass of this peak is consistent with the first 39 residues of the *β*-chain, which could be obtained by cleavage following an Asn residue, the same protease specificity that provides the reported processing of αAI at Asn^{77}. Consistent with this hypothesis, a small peak was detected in some preparations at about 12 304 mass units that could correspond to the remainder of the *β*-chain.

While pea-αAI has not yet been characterized as thoroughly as the bean-αAI, it is clear that the transgenic expression of the bean αAI gene in the pea led to differences of glycosylation and possibly other differences in both the α- and the *β*-chains.

Immunological Consequence of Oral Consumption of Beans. Peas are used as a feed component in the livestock industry and also in human diets. Generally, dietary protein antigens undergo gastric digestion leading to the formation of nonimmunogenic peptides and the induction of a state of specific immunological unresponsiveness termed oral tolerance (*15*, *16*). However, the demonstration of structural differences between the transgenic αAI in pea and the natively expressed bean forms raised the concern that the tolerance mechanism may be perturbed, possibly leading to enhanced immunoreactivity.

The induction of oral tolerance results in the failure of the immune system to elicit an active immune response to subsequent exposure to the same antigen in the skin (delayed type hypersensitivity [DTH] response) or lung ($CD4^+$ T-helper [Th_2] cell-mediated inflammation). To examine potential differences in immunological responsiveness following oral consumption, mice were fed Pinto bean, which expresses a native form of αAI and subsequently received purified Tendergreen-αAI in the skin and lung. Most varieties of common beans such as Red Kidney or Tendergreen contain high levels of phytohemagglutinin (PHA), an anti-nutritional factor that induces dietary toxicity in rodents and birds. We therefore used the Pinto variety that contains very low levels of PHA (*17*, *18*) as the appropriate control for oral exposure. Oral consumption of native uncooked Pinto bean seed flour followed by intra-tracheal (i.t.) challenge with Tendergreen-αAI or phosphate buffered saline (PBS) failed to induce an αAI-specific IgG_1 antibody response (**Figure 2A**). Similarly, sub-cutaneous (s.c.) challenge of the footpad or i.t. challenge of Pinto bean-fed mice with Tendergreen-αAI also failed to promote a DTH response (results not shown) or a pulmonary Th_2-inflammatory response [pulmonary eosinophilia, mucus hypersecretion, and enhanced AHR to a bronchoconstrictive agents], respectively (**Figure 2B−D**). While the level of AHR in the Pinto bean-fed αAI-challenged mice was higher than PBS-challenged mice, the level of responsiveness is not significantly different from that of naïve mice i.t. challenged with Tendergreen-αAI (**Figure 2D**). As a positive control, mice were sensitized by intra-peritoneal (i.p.) injection and subsequently challenged via the airways with bean-derived αAI to induce immunological responsiveness (**Figure 2A−D**). Collectively, these data showed that oral consumption of the native bean form of αAI followed by respiratory exposure to bean-αAI did not promote immunological responsiveness or inflammation.

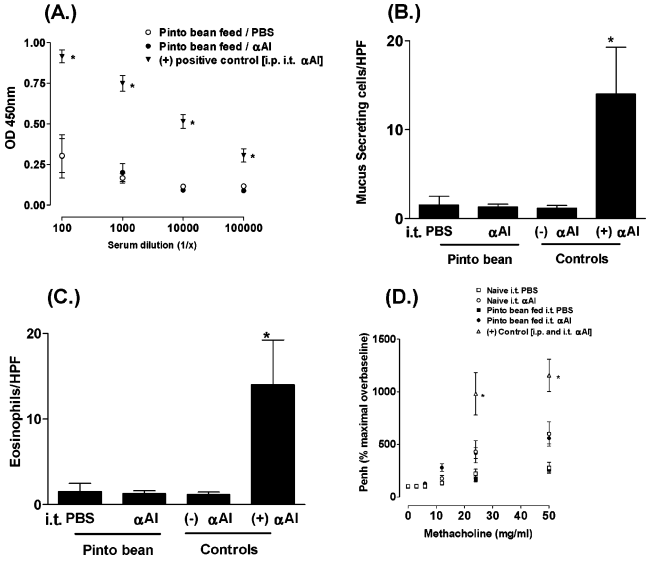

Figure 2. Experimental consumption of bean (cv. Pinto) seed meal does not predispose to inflammation. (**A**) αAI-specific IgG_1 in serum and (**B**) mucus-secreting cell numbers and (**C**) eosinophil levels in lung tissue from Pinto bean-fed mice i.t. challenged with PBS or Tendergreen-αAI. (**D**) AHR in Pinto bean-fed mice i.t. challenged with PBS or Tendergreen-αAI. Data are expressed as the (**A**–**D** and **F**) mean ± SEM and (**E**) mean O.D. of the serum dilution 1/10 ± SEM from 4 to 6 mice per group from duplicate experiments. (**A**–**D**) * $p < 0.05$ as compared to Pinto bean-fed i.t. αAI.

Immunological Consequence of Oral Consumption of Transgenic Peas. To determine whether oral consumption of the transgenic αAI (from pea) elicited an immunological response, mice were orally administered transgenic pea seed meal and αAI; serum antibody titers and DTH responses were examined. Interestingly, in mice that were fed transgenic pea, but not nontransgenic pea, αAI-specific IgG_1 was detected at 2 weeks and at significant levels after 4 weeks of oral exposure (**Figure 3A**). Consistent with the antibody findings, mice fed nontransgenic pea seed meal did not develop DTH responses following footpad challenge with purified pea-αAI (**Figure 3B**). In contrast, mice fed transgenic pea seed meal exhibited a significant DTH response as compared to the nontransgenic pea exposed group when purified pea-αAI was injected into the footpad (**Figure 3B**). As a control for any general effect of genetic modification, we repeated the experiment with material from two other genetically modified plants, lupin (*Lupinus angustifolius* L.) expressing sunflower seed albumin (SSA) [transgenic lupin] (*9*) and chickpeas (*Cicer arietinum* L.) expressing bean derived αAI. Mice were orally administered lupin or transgenic lupin or chickpea or transgenic chickpea seed meal and subsequently footpad challenged with SSA or αAI and DTH responses were examined. In contrast to transgenic pea, mice fed transgenic lupin or chickpea did not develop

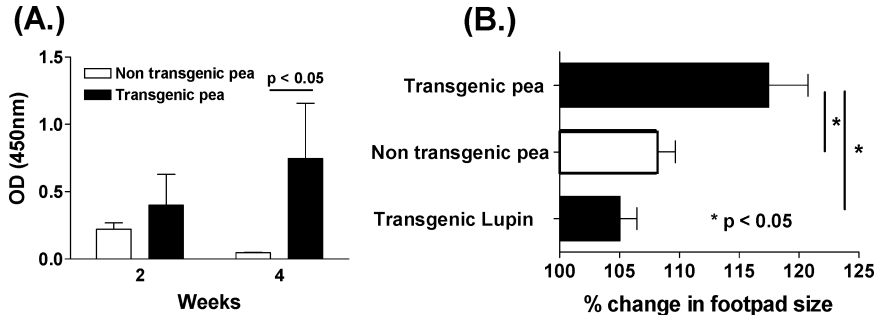

Figure 3. Experimental consumption of transgenic pea seed meal predisposed to antigen-specific IgG_1 and DTH responses. (**A**) Antigen-specific IgG_1 and (**B**) DTH responses in pea nontransgenic and pea transgenic-fed mice. Data are expressed as the (**F**) mean ± SEM and (**E**) mean O.D. of the serum dilution 1/10 ± SEM from 4 to 6 mice per group from duplicate experiments. (**A**–**C**) * $p < 0.05$ as compared to nontransgenic pea or transgenic lupin fed mice i.t. αAI.

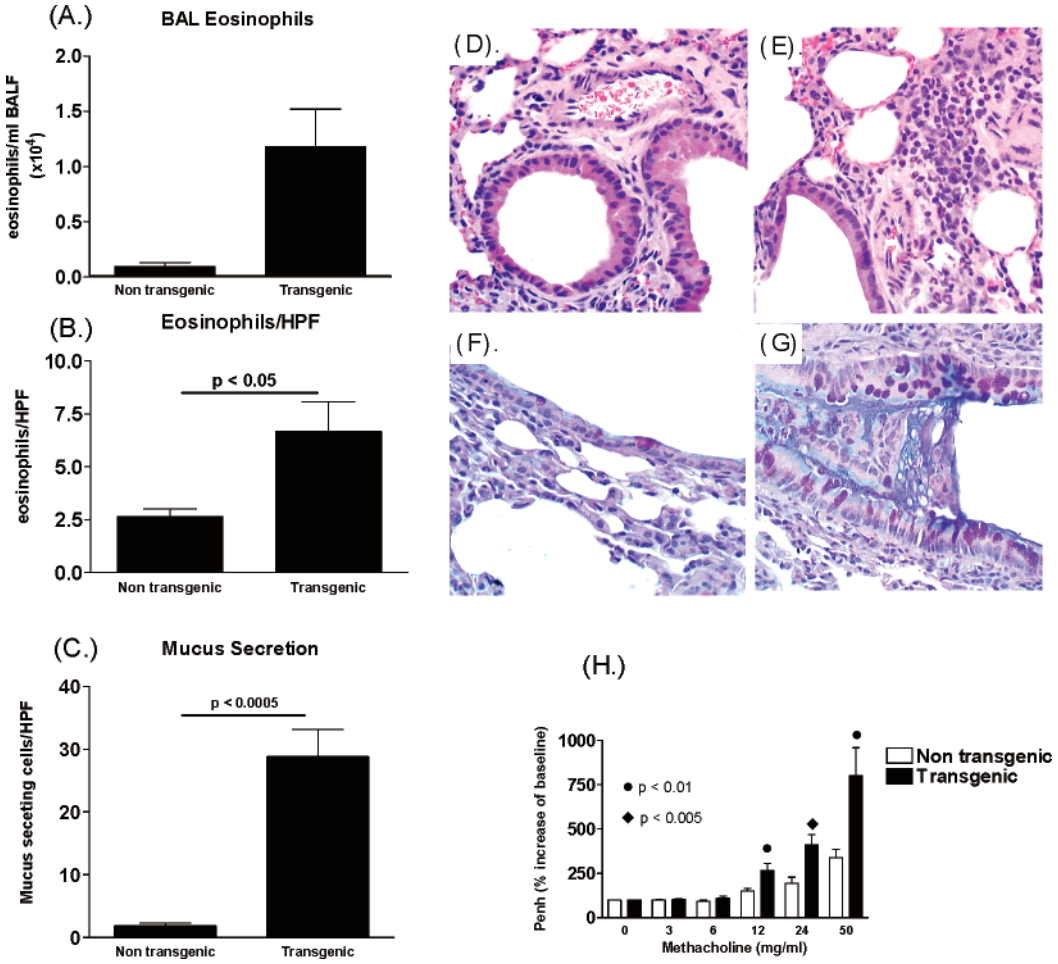

Figure 4. Consumption of transgenic pea seed meal predisposed to $CD4^+$ Th_2-type inflammatory response. Eosinophil accumulation in bronchoaveolar lavage fluid (BAL) (**A**), tissue (**B**), and mucus-secreting cell numbers (**C**) in lung tissue from nontransgenic and transgenic pea-fed mice i.t. challenged with αAI purified from pea. (**D**–**G**) Representative photomicrographs of eosinophil accumulation in lung of (**D**) nontransgenic and (**E**) pea transgenic-fed mice and mucus-secreting cell numbers in lung tissue of (**F**) nontransgenic and (**G**) pea transgenic-fed mice i.t. challenged with αAI from pea. (**H**) Airways hyperresponsiveness (AHR) in nontransgenic and pea transgenic-fed mice i.t. challenged with αAI from pea. Data are expressed as the mean ± SEM from 3 to 6 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$) was determined using Student's unpaired *t*-test. (**D**–**G**) ×400 magnification.

DTH responses following footpad challenge with the transgenically expressed and purified SSA or αAI protein (**Figure 3B**; results not shown). Thus, consumption of transgenic pea containing αAI promoted αAI-specific immunological responsiveness.

To characterize the type of immune response elicited against pea-αAI following oral consumption of transgenic pea, we employed a well-characterized murine model of $CD4^+$ Th_2 cell-mediated inflammation (*19*). Mice were orally administered transgenic pea seed meal and subsequently i.t. challenged with purified pea-αAI, and key features of Th_2-inflammation [pulmonary eosinophilia, mucus hypersecretion, and AHR] were examined. I.t. challenge of nontransgenic pea-fed mice with purified pea-αAI failed to induce features of Th_2-inflammation (**Figure 4A**−**G**). Furthermore, airways responsiveness to the cholinergic spasmogen, methacholine, was not induced in these

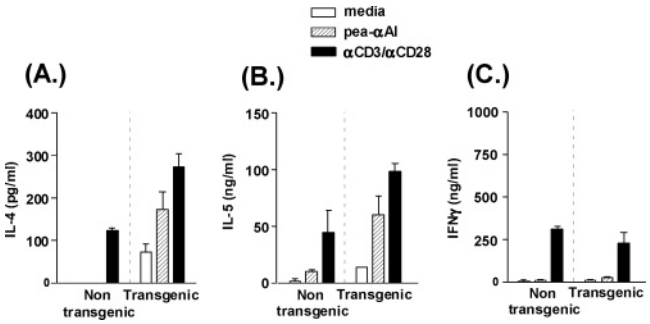

Figure 5. Consumption of transgenic pea seed meal predisposed to $CD4^+$ T-cell derived Th_2-type cytokine production. IL-4 (**A**), IL-5 (**B**), and IFNγ (**C**) levels in supernatants from αCD3/αCD28 or pea-αAI or media alone stimulated PBLN cells from nontransgenic and transgenic pea-fed mice i.t. challenged with αAI from pea. Data are expressed as the mean ± SEM from 3 to 6 mice per group from duplicate experiments. Statistical significance of differences ($p < 0.05$) was determined using Student's unpaired *t*-test.

mice (**Figure 4H**). However, instillation of pea-αAI into the lungs of mice fed transgenic pea induced key features of Th_2-type inflammation including pulmonary eosinophilia, mucus hypersecretion, and AHR (**Figure 4A−H**).

Pulmonary eosinophilia, mucus hypersecretion, and AHR are critically linked to the effector function of the Th_2 cytokines (*20*). To examine whether consumption of transgenic pea promoted a αAI-specific $CD4^+$ Th_2-type T-cell response, $CD4^+$ T-cells in peribronchial lymph node (PBLN) cultures from mice fed nontransgenic pea or transgenic pea seeds challenged with pea-αAI were stimulated with pea-αAI and cytokine profiles determined. Stimulation of $CD4^+$ T-cells in peribronchial lymph node (PBLN) cultures from nontransgenic pea-fed mice challenged with pea-αAI did not elicit Th_2 (interleukin (IL)-4 and IL-5)- or Th_1-type (gamma interferon, IFNγ) cytokine production in response to pea-αAI stimulation (**Figure 5A−C**). By contrast, stimulation of PBLN cultures with pea-αAI from i.t. challenged mice fed transgenic pea resulted in the significant production of Th_2 cytokines (**Figure 5A−C**). Thus, oral exposure of mice to transgenic pea, but not nontransgenic seed meal, predisposed to systemic immunological responsiveness characterized by a Th_2-type immune profile.

Pea-αAI Promotes Immune Responses to Other Oral Antigens. Previous investigations have demonstrated that various plant-derived proteins such as tomatine possess immunomodulatory activity and potentiate and polarize immune responses (*21−23*). We have demonstrated that consumption of transgenic pea in the presence of a large number of potential dietary antigens in the gastrointestinal tract induces an active systemic Th_2-immune response against pea-αAI. In light of these findings, we were next interested in determining whether consumed pea-αAI possessed immunomodulatory activity for Th_2 immune responses and could sensitize mice to heterogeneous nongenetically modified food antigens. Thus, we intragastrically (i.g.) administered purified Tendergreen- or pea-αAI with the well-characterized dietary antigen, chicken egg white protein OVA, or OVA alone and subsequently i.t. challenged mice with OVA. I.g. administration of OVA alone did not systemically sensitize mice to OVA (**Figure 6A**). Further, subsequent OVA challenge in the airways did not promote Th_2-inflammation (mucus hypersecretion, pulmonary eosinophilia, or AHR). Similarly, i.g. administration of bean-αAI and OVA did not systemically sensitize mice or predispose to Th_2-inflammatory processes. However, consumption of pea-αAI and OVA promoted a strong OVA-specific Th_2-type antibody

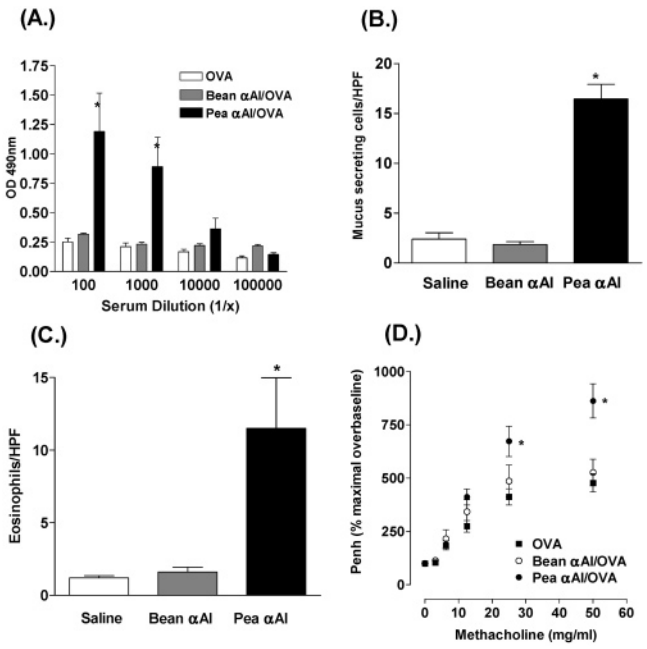

Figure 6. Intra-gastric administration of αAI from pea induces cross-priming of heterogeneous food antigens. OVA-specific IgG_1 levels (**A**) and the Th_2-inflammation phenotype (mucus hypersecretion) (**B**), pulmonary eosinophilia (**C**), and airways hyperreactivity (**D**) in mice that were fed (i.g. challenged) ovalbumin (OVA) alone (the control) or in combination with natively expressed Tendergreen bean-αAI or transgenically expressed (pea) αAI and subsequently intra-tracheal challenged with purified OVA. Data are expressed as the mean ± SEM from 4 to 6 mice per group. * $p < 0.05$ as compared to OVA and bean αAI/OVA.

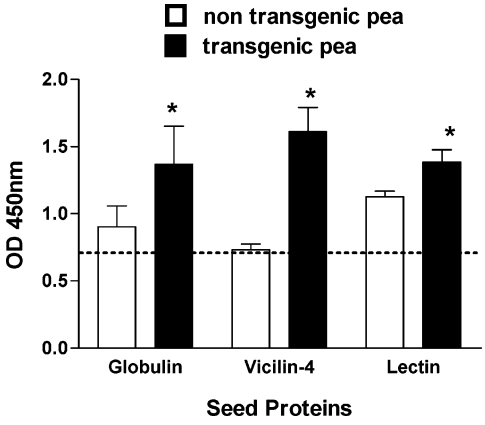

Figure 7. αAI from pea induces cross-priming of pea proteins. Pea globulin-, vicilin-4, and lectin-specific IgG_1 levels in serum from mice that were intragastrically administered 250 μL (~100 mg/mL) of either nontransgenic or transgenic pea seed meal twice a week for 4 weeks. Data are expressed as mean ± SEM from 4 to 5 mice per group. * $p < 0.05$ as compared to nontransgenic pea.

response (**Figure 6A**) and predisposed mice to OVA-induced Th_2-inflammation (**Figure 6B−D**). To support this observation, we examined serum levels of antigen-specific IgG_1 against pea seed proteins (pea globulins, lectin, and vicilin-4) in transgenic pea and nontransgenic pea-fed mice. Interestingly, levels of antigen-specific IgG_1 against pea globulins, lectin, and vicilin-4 in serum of transgenic pea fed mice were significantly higher than those of nontransgenic pea-fed mice, suggesting a heightened immune responsiveness to dietary proteins due to pea-αAI (**Figure 7**). Thus, these studies demonstrate that modified αAI possesses immunodulatory activity and that consumption

of the modified αAI concurrently with heterogeneous proteins can promote immunological cross priming, which predisposes to specific immunoreactivity to these proteins.

DISCUSSION

Recently, peas expressing a gene for αAI from the common bean were generated for protection against field and storage pests (*3*−*6*). Characterization of αAI by structural analysis has demonstrated that transgenic expression of this protein in peas led to the synthesis of a modified form of αAI. Further, we show that the modified form of αAI possessed altered antigenic properties and consumption of this protein by mice predisposed to αAI-specific $CD4^+$ Th_2-type inflammation and elicited immunoreactivity to concurrently consumed heterogeneous food antigens.

Bean-αAI undergoes significant post-translational modification including variable glycosylation and proteolytic processing leading to the synthesis of a mature functional protein (*8*, *11*). We demonstrate that differences in glycosylation and/or other modifications of the pea-αAI lead to altered antigenicity. Consistent with our observations, investigators have previously demonstrated that differential glycosylation of subunits of a cereal α-amylase-inhibitor family (unrelated to legume αAIs) enhances IgE-binding capacity (*24*). Moreover, glycosylated cereal αAI subunits have been shown to possess significantly enhanced IgE-binding affinity when compared to the unglycosylated forms (*24*). These cereal proteins possess identical amino acid sequences and only differ in their carbohydrate moieties, indicating that glycosylation can confer IgE-binding capacity and Th_2-inflammation. In particular, recent investigations have demonstrated that glycan side chains linked to high mannose-type N-glycans on plant-derived glycoproteins can confer immunogenicity and are IgE binding determinants (*25*, *26*). Moreover, α(1,3)-fucose and *β*(1,2)-xylose linkage to high mannose-type N-glycans ($Man_5GlcNAc_2$−$Man_9GlcNAc_2$) promote immunogenicity and IgE binding. The *β*-chain of pea-αAI possesses *β*(1,2)-xylose linked high mannose-type N-glycans, and other complex glycoforms and the α-chain may possess an as yet undefined glycoform variant, and it remains to be determined how these modifications alter pea-αAI immunogenicity.

Functional and structural properties of pea-αAI may contribute to its ability to circumvent immune tolerance and elicit inflammatory responses. Bean-αAI is a potent inhibitor of human α-amylase activity and can induce gastrointestinal dysfunction (*27*). Comparison of bean- and pea-derived αAI activity revealed no difference in enzymatic activity between the two proteins (results not shown). Furthermore, we examined the gastrointestinal tract of pea and transgenic pea-fed mice and observed no histological abnormalities to the gastrointestinal tissue in either group (results not shown). Bean-αAI is also a heat-stable protein and partially resistant to proteolytic degradation (*28*, *29*). Extensive boiling (100 °C for 20 min), while significantly reducing α-amylase inhibitory activity, failed to alter the ability of the transgenic pea to prime for Th_2-inflammation when challenged in the lung [results not shown: see Supporting Information Figure 2]. These findings are consistent with previous demonstrations that cooking of plant material such as lentils and peanuts does not diminish the allergenic potential of certain proteins (*30*, *31*). Furthermore, these studies suggest that the altered immunogenicity of αAI is unrelated to its properties as an amylase inhibitor.

We demonstrate that the immune response elicited against pea-αAI following oral consumption of transgenic pea is characterized by $CD4^+$ Th_2 cell-mediated inflammation, in particular, the presence of IL-4 and IL-5. To examine whether the immune response was dependent on IL-5 and eosinophils, we employed IL-5 and eotaxin-deficient mice. IL-5/eotaxin-deficient mice were i.g. administered nontransgenic and transgenic seed meal and subsequently i.t. challenged with purified αAI. We show that i.t. challenge of transgenic pea fed IL-5/eotaxin-deficient mice induced Th_2-inflammation that was significantly elevated over nontransgenic fed mice (*32*). These investigations suggest that the immune response elicited against pea-αAI following oral consumption of transgenic pea is not dependent on IL-5 and eosinophils.

In this study, we have demonstrated that transgenic expression of αAI in a pea can lead to the synthesis of a modified form of the protein with altered antigenic properties. Furthermore, we show that concomitant exposure of the gastrointestinal tract to modified αAI and heterogeneous food antigens cross primes and elicits immunogenicity. Currently, we do not know the frequency at which alterations in structure and immunogenicity of transgenically expressed proteins occur or whether this is unique to transgenically expressed αAI. These investigations, however, demonstrate that transgenic expression of non-native proteins in plants may lead to the synthesis of structural variants with altered immunogenicity.

ABBREVIATIONS USED

αAI, α-amylase inhibitor-1; pea (*Pisum sativum* L.), transgenic pea; *Phaseolus vulgaris* L. cv. Tendergreen, *Pisum sativum* L. expressing α-amylase inhibitor-1 from the common bean; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry.

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Supporting Information Available: Amino acid sequence of αA1 from common bean and consumption of pea seed meal predisposed to Th_2-type inflammation. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Daniell, H.; Streatfield, S. J.; Wycoff, K. Medical molecular farming: production of antibodies, biophmaceuticals and edible vaccines in plants. *Trends Plant Sci.* **2001**, *6*, 219−226.

(2) Brandt, P. Overview of the current status of genetically modified plants in Europe as compared to the USA. *J. Plant. Physiol.* **2003**, *160*, 735−742.

(3) Schroeder, H. E.; Gollasch, S.; Moore, A. E.; Tabe, L. M.; Craig, S.; Hardie, D. C.; Chrispeels, M. J.; Spencer, D.; Higgins, T. J. V. Bean α-amylase inhibitor confers resistance to the pea weevil (*Bruchus pisorum*) in transgenic peas (*Pisum sativum L.*). *Plant Physiol.* **1995**, *107*, 1233−1239.

(4) Ishimoto, M. Bruchid resistance of transgenic azuki bean expressing seed α-amylase inhibitor of common bean. *Entomol. Exp. Appl.* **1996**, 309−315.

(5) Shade, R. E.; Schroeder, H. E.; Pueyo, J. J.; Tabe, L. M.; Murdock, L. L.; Higgins, T. J. V.; Chrispeels, M. J. Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles. *Biotechnology* **1994**, *12*, 793−796.

(6) Morton, R. L.; Schroeder, H. E.; Bateman, K. S.; Chrispeels, M. J.; Armstrong, E.; Higgins, T. J. V. Bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 3820−3825.

(7) Molvig, L.; Tabe, L. M.; Eggum, B. O.; Moore, A. E.; Carig, S.; Spencer, D.; Higgins, T. J. V. Enhanced methionine levels and increase nutritive value of seeds of transgenic lupins (*Lupins angustifolius* L.) expressing a sunflower seed albumin gene. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8393−8398.

(8) Moreno, J.; Chrispeels, M. J. A lectin gene encodes the α-amylase inhibitor of the common bean. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7885−7889.

(9) Smart, V.; Foster, P. S.; Rothenberg, M. E.; Higgins, T. J.; Hogan, S. P. A plant-based allergy vaccine suppresses experimental asthma via an IFN-gamma and CD4+CD45RBlow T cell-dependent mechanism. *J. Immunol.* **2003**, *171*, 2116−2126.

(10) Martin Young, N.; Thibault, P.; Watson, D. C.; Chrispeels, M. J. Post-translational processing of two α-amylase inhibitors and an arcelin from the common bean, *Phaseolus vulgaris*. *FEBS Lett.* **1999**, *446*, 203−206.

(11) Yamaguchi, H.; Funaoka, H.; Iwamoto, H. Structures of sugar chains of the subunits of an alpha-amylase inhibitor from *Phaseolus vulgaris* white kidney beans. *J. Biochem. (Tokyo)* **1992**, *111*, 388−395.

(12) Sawada, S.; Takeda, Y.; Tashiro, M. primary structures of alpha- and beta-subunits of alpha-amylase inhibitors from seeds of three cultivars of phaseolus beans. *J. Protein Chem.* **2002**, *21*, 9−17.

(13) Ishimoto, M.; Suzuki, K.; Iwanaga, M.; Kikuchi, F.; Kitamura, I. Variation of seed α-amylase inhibitors in the common bean. *Theor. Appl. Genet.* **1995**, *90*, 425−429.

(14) Hoffman, L. M.; Ma, Y.; Barker, R. F. Molecular cloning of Phaseolus vulgaris lectin mRNA and use of cDNA as a probe to estimate lectin transcript levels in various tissues. *Nucleic Acids Res.* **1982**, *10*, 7819−7828.

(15) Mowat, A. M. I. Anatomical basis of tolerance and immunity to intestinal anitgens. *Nat. Rev. Immunol.* **2003**, *3*, 331−341.

(16) Michael, J. G. The role of digestive enzymes in orally induced immune tolerance. *Immunol. Invest.* **1989**, *18*, 1049−1054.

(17) Pusztai, A.; G., G.; Stewart, J. C. A new type of *Phaseolus vulgaris* (cv. Pinto III) seed lectin: isolation and characterization. *Biochim. Biophys. Acta* **1981**, *671*, 146−154.

(18) Banwell, J. G.; Abramowsky, C. R.; Weber, F.; Howard, R.; Boldt, D. H. Phytohemagglutinin-induced diarrheal disease. *Dig. Dis. Sci.* **1984**, *29*, 921−929.

(19) Wills-Karp, M. Murine models of asthma in understanding immune dysregulation in human asthma. *Immunopharmacology* **2000**, *48*, 263−268.

(20) Wills-Karp, M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* **1999**, *17*, 255−281.

(21) Rajananthanan, P.; Attard, G. S.; Sheikh, N. A.; Morrow, W. J. Novel aggregate structure adjuvants modulate lymphocyte proliferation and Th1 and Th2 cytokine profiles in ovalbumin immunized mice. *Vaccine* **1999**, *18*, 140−152.

(22) Rajananthanan, P.; Attard, G. S.; Sheikh, N. A.; Morrow, W. J. Evaluation of novel aggregate structures as adjuvants: composition, toxicity studies and humoral responses. *Vaccine* **1999**, *17*, 715−730.

(23) Morrow, W. J. W.; Wang, Y. W.; Sheikh, N. A. Immunobiology of the tomatine adjuvant. *Vaccine* **2004**, *22*, 2380−2384.

(24) Sanchez-Monge, R.; Gomez, L.; Barber, D.; Lopez-OTIN, C.; Armentia, A.; Salcedo, G. Wheat and barley allergens associated with bakers asthma. *Biochem. J.* **1992**, *281*, 401−405.

(25) Faye, L.; Gomord, V.; Fitchette-Laine, A. C.; Chrispeels, M. J. Affinity purification of antibodies specific for Asn-linked glycans containing a1-3 fucose or b1-2 xylose. *Anal. Biochem.* **1993**, *209*, 104−108.

(26) Van Ree, R.; Cabanes-Macheteau, M.; Akkerdaas, J.; Milazzo, J.; Loutelier-Bourhis, C.; Rayon, C.; Villalba, M.; Koppelman, S.; Aalberse, R.; Rodriguez, R.; Faye, L.; Lerouge, P. b(1,2)-xylose and a(1,3)-fucose residues have a string contribution in IgE binding to plant glycoallergens. *J. Biol. Chem.* **2000**, *275*, 11451−11458.

(27) Layer, P.; Carlson, G. L.; DiMagno, E. P. Partially purified amylase inhibitor reduces starch digestion in vitro and inactivates intraduodenal amylase in humans. *Gastoenterology* **1985**, *88*, 1895−1902.

(28) Marshall, J. J.; Lauda, C. M. Purification and properties of phaseolamin, an inhibitor of α-amylase, from the kidney bean, *Phaseolus vulgaris*. *J. Biol. Chem.* **1975**, *250*, 8030−8037.

(29) Yoshikawa, H.; Kotaru, M.; Tanaka, C.; Ikeuchi, T.; Kawabata, M. Characterization of Kintoki bean (*Phaseolus vulgaris*) alpha-amylase inhibitor: inhibitory activities against human salivary and procine pancreatic alpha-amylases and activity changes by prteolytic digestion. *J. Nutr. Sci. Vitaminol.* **1999**, *45*, 797−802.

(30) Ibanez Sandin, D.; MArtinez San Ireneo, M.; Maranon Lizana, F.; Fernandez-Caldas, E.; Alonso Lebrero, E.; Laso Borrego, T. Specific IgE determinations to crude and boiled lentil (*Lens culinaris*) extracts in lentil-sensitive children and controls. *Allergy* **1999**, *54*, 1209−1214.

(31) Beyer, K.; Morrow, E.; Li, X.; Bardina, L.; Bannon, G. A.; Burks, A. W.; Sampson, H. Effects of cooking methods on peanut allergenicity. *J. Allergy Clin. Immunol.* **2001**, *107*, 1077−1081.

(32) Prescott, V.; Foster, P. S.; Matthaei, K. I.; Hogan, S. P. Mechanistic analysis of food allergy-induced skin and pulmonary inflammation. *J. Allergy Clin. Immunol.* **2005**, *115(2 Suppl)*, S1−360.

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