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# Summary

This report reviews a scientific study by Prescott et al. (2005) which identified a higher immunogenic potential for a bean-derived alpha amylase inhibitor expressed in GM peas compared to the native bean-derived protein using a mouse model. The study implies that the expression of the alpha amylase inhibitor in a foreign organism may lead to modifications of the protein especially to differences in the glycosylation pattern which may increase the immunogenic potential of the protein and hence increase its allergenic potential.

Provided confirmation of these results this study would represent a serious test case for the presently used approach to allergenicity assessment for GM plants and food: i) test proteins for GM risk assessment are normally derived from microbial sources which are usually not capable of glycosylation; ii) animal models in general and the model used by Prescott et al. in particular are not part of GM risk assessment.

This report investigates three main questions. First, are the conclusions in the Prescott paper fully supported by the presented experimental results? Second, is the animal model applied relevant for GM food risk assessment? Third, would the GM pea have alerted risk assessors if undergoing the 'standard' risk assessment procedure?

A thorough review of the Prescott study revealed a number of shortcomings in methodological design and experimental conduct and posed several questions. It appears that the animal model applied is not suitable for the evaluation of IgE-mediated hypersensitivity which is by far the most important type of food mediated hypersensitivities. Rather it is designed for Type IV immunogenic reactions. The injection into the footpad of the mice does not seem to mimic a common and frequent exposure scenario for GM food and plants. Furthermore, the purification protocol used and the measurements mentioned indicate that the authors did not exclude that the effects observed are caused by impurities or co-purified proteins rather than by alterations resulting from transgenic expression.

Furthermore, in the absence of IgE levels measured the induction of IgG1 reported by Prescott et al. is rather an indicator for exposure and an immune reaction to a potential antigen than for its allergenicity. As a logical leap the authors are showing differences between Pinto bean and Tendergreen bean-derived alpha amylase inhibitors in MALDI-TOF mass spectroscopy while at the same time they are priming mice with Pinto bean seed meal and challenging

them with Tendergreen bean-derived alpha amylase – an experimental design anticipating no immunological differences between both protein variants.

Finally, it is difficult to judge the results of several experiments of the Prescott study because proper controls are frequently missing. For example, the immunogenicity of alpha amylase inhibitor from bean and peas was never compared in the same experiment allowing a direct comparison of the two proteins within one experiment. The authors' key claim, that there is a difference regarding immunogenicity of the natural and transgenic protein has not been studied by direct comparison.

On the other hand, the Prescott study clearly shows that a transgenic protein can induce under certain conditions an unwanted immune response leading to organ pathology and certain experiments demonstrate that exposure to the transgenic protein can increase the immunogenicity of other unrelated proteins which are administered together with the transgenic protein, a finding which would not be detected by current risk assessment procedures. In this context the Prescott study indicates a strong need for reconsidering the current approach to GM allergenicity assessment.

Whether the GM pea would have alerted the risk assessors when undergoing a normal GM allergenicity assessment it is difficult to predict for several reasons. The outcome of *in vitro* digestibility tests and homology comparisons depends very much on the experimental conditions chosen and on parameters set respectively. *In vitro* digestibility and *in vivo* digestion experiments may lead to contradicting conclusions about proteolytic stability. Whether homology comparisons provide some positive indications of homology to known allergens entirely depends on the database and algorithm used. In no case striking similarities with known allergens were identified. Finally, the level of scrutiny applied when reviewing the assessment would have been important, especially in case of weak signals or uncertainty.

Given the characteristics of a case-by-case and a weight of evidence approach to allergenicity assessment it is even more difficult to anticipate if risk assessors would have demanded additional tests. It is unlikely that they would have asked for animal testing since animal models are considered by many risk assessing bodies as not yet suitable for routine application. Targeted and/or specific serum screen might have been a further step. However, the results of serum tests will critically depend on the selection of sera for testing. A higher probability of identifying sera containing IgE antibodies against bean-derived alpha amylase inhibitor might be achieved by testing with sera from patients who are allergic to beans but such patients are very rare.

For a routine risk assessment the alpha amylase inhibitor test protein would unlikely be produced from *E.coli* or other microbes. It is well established in the scientific literature that the bean alpha amylase inhibitor is a complex protein consisting of two subunits which are undergoing extensive posttranslational proteolytic processing and glycosylation. More likely, the protein would have been purified from the transgenic peas. Whether and how the equivalence of the natural bean and the pea-derived protein would have been investigated remains difficult to predict as there is very little experience in GM risk assessment with complex and/or glycosylated proteins.

Although not all conclusions of the Prescott study are supported by the experimental results, the study has merits as it contains some unexpected finding such as the augmentation of immunogenicity of other proteins when they are fed together with the transgenic protein. For this reason and because of the uncertainties whether the GM pea would have passed the normal GM risk assessment procedure without additional testing the authors of this report reiterate their earlier proposal to apply serum screens and animal models to compare the allergenic activity of transgenic with wild-type (i.e., parent) organisms as well as their de-novo sensitizing potential to obtain the crucial information whether the transgenic plant has a higher allergenic potential than the wild-type. The basic principles and rationale for such an approach have already been described elsewhere by the authors of this review.

# Acronyms

| | |
|---|---|
| aAI | Alpha amylase inhibitor |
| CSIRO | Commonwealth Scientific and Industrial Research Organisation |
| DTH | Delayed type hypersensitivity |
| ELISA | Enzyme-Linked Immune Sorbent Assay |
| EPA | Environmental Protection Agency |
| FAO/WHO | United Nations Food and Agriculture Organization/ World Health Organisation |
| FSANZ | Food Standards Australia New Zealand |
| GM | Genetically modified |
| GMO | Genetically modified organism |
| GMP | Genetically modified plant |
| i.p. | Intraperitoneal |
| IFN | Interferon |
| MALDI-TOF MS | Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry |
| NMR | Nuclear magnetic resonance spectroscopy |
| PHA | Phytohemagglutinin |
| SAXS | Small-angle X-ray scattering |
| SDS-PAGE | Sodium dodecylsulfate polyacrylamide gel electrophoresis |
| SGF | Simulated gastric fluid |
| SIF | Simulated intestinal fluid |
| SSA | Sunflower seed albumin |
| TSP | Total soluble protein |
| USP | United States Pharmacopeia |

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# 1 Terms of References

The research article of Prescott et al. (2005) "Transgenic expression of bean alpha-amylase inhibitor in peas results in altered structure and immunogenicity" (subsequently referred to as Prescott et al. or Prescott study) basically implies that the transgenic expression of a given protein (alpha amylase inhibitor (aAI) from bean) in another host (i.e. pea) may lead to the expression of a protein which exhibits altered structure as compared to the protein expressed in the natural source and that this altered structure may affect the protein's immunogenicity so that it becomes allergenic. The abstract of the study even concludes that the modified protein resulting from the transgenic expression, but not the natural protein, induces an allergic inflammation (CD4+ Th2-type) upon consumption in an animal model.

The study may be of particular relevance for GM risk assessment if it would have detected relevant immunogenic effects that would not have been identified using the presently applied allergenicity assessment approach. The assessment of genetically modified organisms (GMOs) applies sequence homology comparisons of the target protein to known allergens and *in vitro* digestibility tests with a bacteria-derived protein (which are usually not glycosylating proteins). Weaknesses of this approach were recently pointed out by the authors of this report (Spök et al. 2004, 2005). For instance bacteria-derived test proteins are undergoing a different posttranslational processing and are usually lacking any glycosylation. Therefore, native and bacteria-derived aAI could have passed the currently applied allergenicity assessment – including possible IgE reactivity studies.

In a subsequent publication Prescott & Hogan (2006) explicitly draw such conclusions from their previous work:

> "Studies with aAI support the usage of animal models to assist in the assessment of GM plants for allergenicity. These studies highlight the need for the weight of evidence approach to not only consider the source of the gene but also to take into consideration the potential for post-translational modifications to alter the structure and antigenicity of a protein when expressed in a new host. Full characterization of the protein of interest expressed in the native and transgenic state might also be warranted for assessment of allergenicity. Furthermore, these studies suggest that sequence comparison analysis with known allergens may have limited predictive ability." (p. 379).

The Prescott study represents a rare case where scientists could show evidence of possible harm associated with immunogenic effects of the novel protein in GM

plants. The impacts, thus, resemble those of Nordlee et al. (1996). Nordlee et al. described cross-reactions of sera from patients allergic against nuts with a GM soybean that expressed a gene from a Brazilian nut. This nut is a well known allergenic food but not all allergens had been identified. Thus, a targeted serum screen was perhaps an obvious task to do and in fact revealed positive results indicating that by coincidence one of the nut allergens had been transferred to the soybean.

Similar to the study of Nordlee et al. (1996) the research was conducted and results were published by those who were developing the GM plant in a peer reviewed scientific journal. This enhanced the credibility and impact of both studies.

Both studies have swiftly been taken up by promoters and critics of GM crop technology and quoted as evidence for both that the established GM risk assessment works well to protect man and the environment and also that it points to holes in the safety net (for the pea: Editorial 2006; EFSA 2006; FoE 2006; ISIS Press Release 2005; Smith 2005).

This report provides an extended review of the study of Prescott et al. and of topics linked to this paper. Chapter 2 describes the technological objectives to develop the GM peas and briefly summarises the safety studies conducted. Chapter 3 critically reviews the Prescott study, scrutinizes the methodology applied and the experiments conducted and to what extent the conclusions drawn are backed up by the empirical evidence. Chapter 4 investigates whether the GM pea would have been identified as a putative allergenic crop applying the standard allergenicity assessment approach for GM crops. Based on this review and analysis, Chapter 5 draws conclusions for GM risk assessment and provides suggestions for further research. Figures referred to in the text are those used in the original paper of Prescott et al. which are included in Annex D.

# 2 Context[1]

Field peas are an important crop for animal feed in Australia. But field peas are threatened by a pest called pea weevil which can reduce yields up to 30%. Australia's Commonwealth Scientific and Industrial Research Organisation (CSIRO) developed GM peas harbouring an aAI from common beans. This protein inhibits the activity of alpha amylase and causes the weevils feeding on the GM pea to starve before they cause any damage.

Various strains of GM pea have been subjected to safety and nutritional testing from the mid 1990s onwards. From the very outset it was clear to the developers that the heterologous pea aAI could not be produced from *E.coli* or other microbes as the aAI is a more complex protein compared to many other novel proteins in commercially available plants. The aAI consists of two subunits that need to undergo a more complex post translational processing including proteolytic cleavage of C-terminal amino acid residues and glycosylation. Thus, safety studies were conducted on the aAI purified from the GM pea. As the heterologous protein is expressed in significant amounts this did not pose technical problems.

Feeding studies with the GM pea were conducted by Pusztai et al. (1999) in rats and later on pigs (Collins et al. 2006) and broiler (Li et al. 2006). According to CSIRO comparative compositional analysis using non-modified parent strains of the pea was conducted and did not reveal any statistically significant differences (interview CISRO) although the data of Collins et al. suggested otherwise.[2] The rat study did not report any striking differences between GM and conventional peas in terms of nutritive value, including starch digestion but did reveal changes to the heterologous aAI. The GM pea aAI had a different banding behaviour on SDS-PAGE and was much more susceptible to digestion in *in vivo* studies compared to the native bean equivalent. The GM pea aAI was shown to be quickly degraded in the terminal ileum of the rats. Preliminary studies also suggested differences in posttranslational processing. The study concluded that it might be possible to use the peas in the diet of farm animals (Pusztai et al. 1999; Pusztai pers. comm.).

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[1] Unless otherwise referenced this section is based on CSIRO (2006) and on interviews with three researchers, one of which is a member of CSIRO.

[2] See for instance crude protein and arginine in Table 2 of Collins et al. (2006). However, no statistical analysis was conducted and/or shown in the paper. Thus, it cannot be excluded that in fact there was no statistically relevant difference.

In contrast to the rat study a subsequent pig study revealed remarkably reduced illeal digestibility of the GM pea starch (Collins et al. 2006). This was confirmed by the broiler study (Li et al. 2006) which found a reduction of more than 60% in the apparent metabolizable energy (AME)[3] content. According to the latter studies the aAI effectively inhibits the alpha amylase of farm animals. None of these subsequent animal studies did provide a convincing explanation for the differences found to the rat study. However, it has to be mentioned that these feeding studies as well as the Prescott study were apparently conducted with different strains of GM peas.[4] Furthermore, there are apparent methodological differences between the pig study and the rat study. Hence, it is difficult to relate all this studies to each other.

Given the results of Collin et al. and Li et al. the peas would need to undergo a heat treatment in order to inactivate the aAI before it can be used for animal feed (Collins et al. 2006). As this would not be economically feasible CSIRO decided to drop the original idea of applying the GM peas as animal feed.

Collins et al. (2006) also proposed an alternative use of the GM pea as functional food component for humans in controlling weight gain and obesity allowing normal protein digestion but drastically reducing energy. While this has never been made explicit it appears that this option was indeed considered by CSIRO targeting also medial application for disorders like diabetes. In fact an extract from beans has already been marketed at that time as a slimming agent.[5] These products consist of crude bean extracts which also contain phytohemagglutinins (PHA) and trypsin inhibitor which are considered major anti-nutrients. The GM pea would be devoid of these substances and therefore might have had an advantage. The Prescott study on the immunogenicity, however, eventually led to the termination of the whole project after ten or more years of research and development.

A main question is why the particular approach described by Prescott et al. was applied. Routine allergenicity assessment conducted for GM crops basically

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[3] Apparent ME (AME) is a widely used measure of food energy. AME is defined as intake energy minus excretory (fecal and urinary) energy losses. True metabolizable energy may be obtained by correcting the estimate for the fraction of excretory energy that originates from non-dietary sources.

[4] From these papers it is not clear whether Collins et al. (2006) and Li et al. (2006) did use the same GM pea transformation events. Clearly they were using the same transformed cultivar cv. Excell. Pusztai et al. (1999) used a different transformation event, GF-10 from cv. Greenfeast. Prescott et al. (2005) did not specify the transformation event but according to one interviewee it was at least different to the one used in the Pusztai study.

[5] E.g. Carb Blaster (http://www.herbsofgold.com.au/Carb%20Blaster%20Phase%202.htm).

comprises homology comparisons to known allergens and *in vitro* digestibility studies. However, no indication of such studies could be found in published documents with the GM pea.

In fact homology comparisons and *in vitro* digestibility studies had been conducted by the developers but never been published. From these studies the aAI appeared to be "suspicious" (homology studies using the FAO/WHO threshold revealed a soybean allergen and in the pepsin studies the aAI was stable for about one hour (interview CSIRO). From these results and on the basis of the FAO/WHO decision tree it was anticipated that the regulators would like to see some additional tests. The Australian regulators Food Standards Australia New Zealand (FSANZ) were consulted for their overall requirements for the risk assessment but they did not advise on any particular studies beyond the fairly general recommendations provided in their guidelines (Higgins 2006). Co-authors of the then Prescott study at the Australian National University in Canberra had already developed a mouse model for the assessment of allergenicity and offered to use this model for the GM pea.

The focus on the aAI from the GM pea and the differences between the native and the GM pea aAI might not have come as a surprise since research in the mid 1990s had already provided some evidence of these differences (differences in *in vivo* digestibility in rats, differences in posttranslational proteolytic processing and glycosylation). However, the latter data were generated by methods less reliable than the presently used MALDI-TOF and were only partly reported (Pusztai, pers. comm.).

# 3 Review of the Prescott et al. study

## 3.1 Approach

The study contains in principle two experimental approaches, *in vitro* and *in vivo* studies.

### 3.1.1 *In vitro* studies

In the first part of the study aAI is purified from common beans (Pinto and Tendergreen) and from transgenic peas. For control purposes, sun flower seed albumin, another transgenic protein is also purified.

The proteins are subjected to biochemical characterization to provide insight into their biochemical and biophysical properties.

### 3.1.2 *In vivo* studies

The second part of the study comprises *in vivo* experiments carried out in mice with the aim to obtain information regarding the development of immune responses against these proteins or other proteins (i.e. bystander effect) following administration of the proteins or extracts containing these proteins.

## 3.2 Reagents, test substances

### 3.2.1 Seed meals

Many of the experiments in the study were not carried out with purified proteins but only with crude extracts which were obtained from non-transgenic peas, transgenic peas expressing bean aAI, non-transgenic Pinto beans and genetically modified narrow leaf lupin expressing sunflower seed albumin protein.

Crude extracts were prepared by grinding of the materials, re-suspension in a physiological buffer and removing insoluble materials. These extracts were stored at –70°C until use and in some experiments heated to 100°C before use. A fundamental problem for all following experiments, especially the *in vivo* sensitizations, is that the authors have not determined the precise amounts of

aAIs in the different crude extracts[6] and it is therefore easily possible that differences in the development of aAI-specific immune responses are simply due to presence of different amounts of the proteins in the extracts used for feeding of the various mouse groups.

It is not clear why seed meals were not used from Tendergreen beans because aAI was purified from both types of beans (see below). It is also not clear whether there might be differences between Pinto and Tendergreen beans and the aAIs from the two bean species.

### 3.2.2 Protein purification

Alpha amylase inhibitor was purified from non-transgenic Pinto and Tendergreen beans and transgenic peas using immobilized porcine pancreas alpha amylase as described by Moreno and Chrispeels (1989).

According to Prescott et al. sunflower seed albumin was purified as described by Molvig et al. (1997) but this paper contains no detailed purification protocol for this protein. Instead it says that the protein is simply extracted using a certain buffer (0.5 M NaCl, 0.1M Tris pH 7.8, 1 mM EDTA). In the absence of a reference to a protein purification protocol it is therefore not clear whether the authors used a pure protein. [7]

Methods for the *in vitro* characterization

The authors say that they have analyzed the purified aAIs from bean and transgenic peas by SDS-PAGE but these results are not shown in the paper. The demonstration of purity of proteins would have been crucial because no conclusion can be drawn regarding structural differences of two proteins if the homogeneity and purity has not been demonstrated. In this context it should be noted that the purification procedure as cited by the authors (Moreno & Chrispeels 1989) gave rise to multiple protein bands of which the amino acid composition was shown to be not fully identical to the amino acid sequence deduced from the DNA sequence of the aAI cDNA. Also differences in the amino

[6] The amount of aAI in extracts was determined by Western immunoblot analysis which is not an accurate method to measure protein concentration. Furthermore, the wide range of 3 to 4% of protein found in the analysis (Hogan, pers. communication) could itself lead to differences in immune response.

[7] Affinity chromatography using the porcine pancreas alpha amylase as described in Moreno and Chrispeels (1989) can be expected to be less specific compared to affinity chromatography using monoclonal antibodies. Other proteins could co-purify with the aAI.

acid composition of the purified material from black and red kidney bean were noted already by Moreno & Chrispeels (1989). Accordingly Prescott et al. even admit in their paper that there have been previous reports of "other proteins" which co-purify with aAIs using their purification protocol which according to information obtained from the authors contained contaminants and did not deliver a homogenous pure protein preparation.

The putatively purified proteins are then subjected to MALDI-TOF spectrometry, a method which is suitable to determine the molecular mass of a given component but which does not allow to determine the purity of a protein. The MALDI-TOF method applied by Prescott et al. only allows assessing the molecular mass and hence one may draw conclusions about the primary structure, i.e., the sequence of a protein, but this method is not suited for the analysis of the three-dimensional structure or the fold of a protein.

The authors furthermore use Western immunoblot analysis of the aAI in crude protein extracts from common bean and transgenic peas using a polyclonal rabbit antiserum. The Western immunoblot experiments are carried out without showing the usual control experiments, i.e., the demonstration of lack of reactivity of a non-specific rabbit antibodies with the preparation. The control was performed with the detection reagent without addition of the specific rabbit antibodies and the inclusion of Western-blotted proteins from other sources, in particular non-transgenic peas. A final conclusion regards the accurateness and specificity of these experiments can therefore not be drawn from the data shown by the authors.

## 3.3 Experimental animal model

The experimental animal model used by the authors is a mouse model. BALB/c mice were intragastrically administered seed meal suspensions from transgenic, non-transgenic peas, Pinto bean or lupin SSA. From the authors' description of the experiment it is not completely clear whether lupin seed meal suspension or purified sunflower seed albumin was fed. The intragastric administration was done for four weeks twice a week. It is not said precisely how many mice were included in each of the groups and how often the experiments were repeated. Seven days after the final intragastric challenge 25 microgram of purified aAIs from Tendergreen bean, transgenic pea or lupin SSA were injected into the animals footpads. It should be noted in this context that this protocol is commonly used for the induction of Type IV hypersensitivity but not for Type I, i.e., IgE-mediated allergies. It is quite surprising that the authors have used a murine model for delayed type hypersensitivity because they must have been

aware of the fundamental difference between murine models for IgE-mediated allergies and delayed type hypersensitivity (i.e., Type IV hypersensitivity) analysed in a previous publication co-authored by T.J. Higgins and S. Hogan who are corresponding authors of the Prescott study (Smart et al. 2003). In the paper by Smart et al. the authors clearly define in Figure 1A a murine model for IgE-mediated allergy and in Figure 1B the murine model for delayed type hypersensitivity as used in the Prescott study. It should also be noted that this protocol does not mimic a possible exposure scenario in man. While gastric exposure might be considered it is not expected that individuals will be injected into the footpad with purified proteins. The results therefore do not allow drawing conclusions that the transgenic pea bears an increased allergenicity risk and only showing that the protein, as many other proteins administered under the given conditions, can induce an immune response.

It is therefore totally unclear why the authors choose this model because the fundamental differences between Type I (i.e., IgE-mediated) and Type IV allergies are well established in immunology (Kay 1997):

IgE-mediated allergy (i.e., immediate type hypersensitivity) describes that an individual produces IgE antibodies against antigens (i.e., allergens) when the individual is exposed to mostly low doses of the allergens. The IgE antibody production in the sensitization event is supported by T helper cells (CD4+ cells) of the Th2 phenotype which are characterized by the production of Th2 cytokines such as IL-4, IL-13 or IL-5. The pathology in IgE-mediated allergy is due to the fact that the IgE antibodies bind with high affinity to FcεRI receptors on mast cells, basophils and other effector cells and upon repeated allergen contact the allergen induces cross-linking of effector cell-bound IgE and the subsequent release of inflammatory mediators such as histamine, leukotriens, proinflammatory cytokines and proteases. The degranulation process of effector cells occurs within few minutes and the reactions have therefore been referred to as immediate type hypersensitivity. IgE-mediated allergies are the most common hypersensitivity reactions in man and affect more than 25% of the population. The most common manifestations of IgE-mediated allergies are allergic rhino conjunctivitis (hay fever), allergic asthma bronchiale, allergic dermatitis (urticaria and atopic dermatitis), food allergy (ranging from oral allergy syndrome, a swelling of the mouth, lips and tongue upon ingestion of certain food to more severe and systemic forms of food allergy such as allergic gastroenteritis, diarrhoea, vomiting, urticaria, food-induced anaphylaxis) and the life-threatening anaphylactic shock which can be induced by systemic allergen contact for example after food intake or insect stings.

Delayed type hypersensitivity (i.e., Type IV hypersensitivity) is much rarer than IgE-mediated allergy. It normally occurs after exposure to high doses of antigen and primarily is based on the recognition of the antigen by CD4+ T cells of the Th1 phenotype secreting IFN-gamma but little or no Th2 cytokines or CD8 positive T cells. Antigen is presented by antigen presenting cells to these types of T cells which produce in concert with the antigen-presenting cells a cocktail of inflammatory cytokines and various cytotoxic factors which are responsible for inflammation. The inflammatory reaction normally occurs after considerable time (more than 12 hours) and does not involve antibodies to a major extent. Classical manifestation of delayed type hypersensitivities are contact eczema, reaction to mycobacteria and certain autoimmune diseases. Mechanisms of delayed type hypersensitivity may play a role in celiac disease, a hypersensitivity reaction of the gastrointestinal tract to certain wheat proteins (gluten fraction) and affects approximately 1:1000 persons. Celiac disease and IgE-mediated food allergy are diseases with fundamentally different pathomechanisms and also the spectrum of recognized antigens is different (Constantin et al. 2005).

The mouse model used by Prescott et al. therefore is a model for a hypersensitivity reaction to antigens (i.e., delayed type hypersensitivity) which may in principle occur in man after antigen contact but is much rarer (CD in 1:1000 patients) than IgE-mediated Type I allergies (250 in 1000 patients).

In fact, for positive control purposes mice were sensitized with aluminium hydroxide-adsorbed Tendergreen aAI by i.p. injection followed by footpad challenge. It should be noted that injection of aluminium hydroxide-adsorbed protein would have been the typical protocol for induction of IgE-mediated allergies, whereas the subsequent footpad injection would favour another type of hypersensitivity reaction (i.e., Type IV hypersensitivity). It is unclear why the authors have mixed two different sensitization protocols in their experiments.

Lung inflammation was induced in mice approximately one week after final intragastric challenge by intubation and administration of highly concentrated antigen preparations (1 mg/ml) of aAI from Tendergreen beans, transgenic peas or ovalbumin. It is unclear why the authors now switch from the gastric exposure to the lung exposure because in doing this they mix two possible exposure scenarios, i.e., sensitization via food versus sensitization via inhalation. Their model is therefore neither representative for food allergy nor for respiratory allergy but only for mixed pathologies. Furthermore they apply extremely high concentrations of the proteins as they would hardly ever occur in reality into the lung. In case of IgE-mediated allergy, patients are exposed to more than thousand-fold lower concentrations of allergens. Such high amounts

of antigen would be rather used for the set up of animal models for Type III or Type IV hypersensitivity. Again it is unclear why these conditions were chosen.

The following read outs are then used in the mice:

1. Assessment of DTH (delayed type hypersensitivity)[8]
2. Measurement of IgG1 antibodies by ELISA
3. Airway hyper-responsiveness using methacholine challenge
4. Determination of mucus secreting cells and eosinophils in lung tissue
5. Lymphoproliferation and cytokine secretion in peribronchial lymph node cells.

Another open question is why the authors did not show the results for IgE antibody responses as would be expected for the development of IgE-mediated allergy. The Prescott et al. (2005) study does not even mention IgE. However, according to a subsequently published paper IgE has been measured (Prescott and Hogan 2006) and it might be interfered that the fact that it has not been mentioned means that no IgE has been detected. The fact that DTH responses were assessed even demonstrates that the applied murine model is indeed not a model for IgE-mediated, i.e., Type I allergy but rather for Type IV hypersensitivity.

## 3.4 Results

### 3.4.1 Western blot analysis

Figure 1A of the Prescott study shows a Western blot comparison of aAIs from Tendergreen bean and transgenic peas. The authors describe immunoreactive bands which are identified with a polyclonal anti-aAI rabbit antiserum. They conclude that “differences in the banding profile suggest possible differences in the molecular structure of the native and the heterologous aAI”. This conclusion is not fully supported by the results because

[8] The authors measure the increase in footpad thickness using a digmatic calliper 24 hours following the challenge.

- The Western blots were performed only with crude extracts containing the proteins instead with the purified protein.
- The gels containing the purified proteins were not shown to allow the reader to judge their purity and integrity.
- The usual controls in the experiments were not performed (see comments for methods).
- Normally it is not possible to conclude from subtle differences in the banding pattern of a Western blotted crude protein extract that there are structural differences between the proteins.

Moreover, the authors did not consider more likely explanations for the differences in the binding pattern.

The following criticisms can be made regarding the Western blots:

1. A Western blot as a denaturing assay is not a suitable method to assess structural features of a protein, especially not features of three dimensional structure.
2. The missing controls do not allow to make conclusions about the specificity of the binding pattern.
3. There are several more likely explanations for the subtle differences in binding pattern. The differences observed in the binding pattern, i.e., the weaker bands above 20 kDa and at approximately 18 kDa in the pea extract and the additional band below 14 kDa may more likely result from degradation of the aAI caused by proteases present in the crude pea extract or from differences in the overall protein composition between the pea and bean extracts. Controls of the possible binding profile of the antibodies with extracts from non-transgenic peas to exclude the presence of immune-reactive material in native peas have not been shown.

### 3.4.2 MALDI-TOF-MS analysis

The authors perform a MALDI-TOF mass spectrometry (MS) analysis to assess the molecular masses of the purified protein preparations. A major problem is that MALDI-TOF-MS is not suited to make definitive conclusions about the purity of a protein preparation and there is no result shown in the paper which demonstrates the purity of the analyzed materials. The authors even label in the mass spectra of Tendergreen and Pinto aAI two peaks as possible contaminants.

It is therefore not clear whether the differences described for the spectra of bean and transgenic pea aAIs are the result of different posttranslational modifications or of the presence of further contaminants in the protein preparation. It is also possible that the aAIs have been modified in different ways by other proteins from pea and bean, e.g. proteolysis.

The differences in the mass spectra between the aAIs from bean and transgenic peas are not so striking and it is unclear why these results have prompted the authors to conduct extensive animal studies before doing more thorough biochemical and structural *in vitro* studies to solve the many open questions and to confirm the results from the biochemical characterization.

Finally, it has to be stressed that differences in mass spectra may at best indicate differences in sequence or posttranslational modifications which may have little or no influence on the structure of a given protein. In this context it is not clear what the authors mean with structural differences. Differences in the three dimensional structure of proteins can only be assessed by suitable methods such as circular dichroism spectroscopy, NMR or crystallization followed by x-ray analysis, SAXS or other methods (see Table in Ferreira et al., 2005). In fact there have been several studies published long before the Prescott study which provide clear evidence that structural differences can indeed affect the allergenic activity of proteins (e.g., Vrtala et al. 1998), but the authors do not cite any of these studies and they also fail to apply the proper methods for the structural assessment of proteins.

### 3.4.3 *In vivo* experiments

In the first *in vivo* experiment (Figure 2) the authors investigate whether feeding with bean alpha-amylase containing bean extract and subsequent footpad challenge or intratracheal administration with the purified bean aAI would cause a specific immune response and inflammation. The authors argue against the inconsistency of their experimental approach (i.e., the fact that they feed with Pinto bean extract and then challenge with Tendergreen aAI instead with the Pinto protein) by saying that Tendergreen is rich in PHA and therefore Pinto extracts containing less of this anti-nutritional factor were used for feeding. However, they could have used purified[9] Pinto aAI for subsequent challenge to maintain a consistent experimental approach. The inadequate description of the

[9] In fact the PHA could have been removed from the Tendergreen bean seed meal prior to administration, e.g., by affinity chromatography.

experimental design also leaves the question open if the mice were fed and then intratracheally challenged without doing the footpad injection or whether the footpad injection was done before the intratracheal challenge. This is critical because the first type of experiments in Figure 2 does not include the direct comparison with the transgenic pea. In fact the second type of experiments conducted with transgenic peas (Figures 3 and 4) also do not contain the direct comparison with bean. These pea experiments were in fact done by feeding, footpad challenge and subsequent intratracheal challenge and it is possible that all three treatments were necessary to yield positive results. The major problem is therefore that the first experiments from Figure 2 done with beans and the second type shown in Figure 3 and 4 for transgenic peas cannot be compared at all.

The authors claim that the experiment carried out with bean aAI in mice shows that there is no immune response induced in mice but in Figure 2A some IgG1 reactivity of mice which have been fed with bean aAI at the 1:100 serum dilution is documented and this IgG1 reactivity can be diluted out when sera are subjected to higher dilutions. The legend to Figure 2A seems to be incorrect because it says that the "mean O.D. of the serum dilution 1/10 is shown, but the x-axis shows several serum dilutions from 1:100 to 1:10.000 but no 1:10 dilution. This is very important because the IgG1 response for the transgenic pea experiment in Figure 3A is shown only for a 1:10 serum dilution. It is quite possible that if the serum in the pea experiment would have been diluted also 1:100 as in the bean experiment, no or weak IgG1 reactivity would have been obtained.

In the bean experiment there are some effects of this treatment seen when mice received bean aAI intratracheally (Figure 2D) even without a prior feeding with bean extract leaving the impression that bean alpha amylase can have effects on airway inflammation. The authors include as a positive control an experiment which is based on the classical induction of IgE-mediated allergies which shows that sensitization with aluminium hydroxide-adsorbed aAI from bean induces IgG1 antibody responses, mucus secretion, eosinophilia and airway hyper-responsiveness. It is not clear how the authors finally can conclude from these experiments that bean-derived aAI does not induce immune responses and inflammation. The authors merely say: "Collectively, these data showed that oral consumption of the native bean form of aAI followed by respiratory exposure to bean-aAI did not promote immunological responsiveness or inflammation".

Other major criticisms of this experiment are that the concentrations of aAI in the bean extract used for feeding have not been determined and that no direct

comparison with the transgenic peas has been performed in these experiments. No IgE reactivity data are shown and therefore no conclusion can be made whether the bean treatment induced an IgE-mediated allergy or not. The results from the footpad challenge are not shown at all for the first type of experiments.

In the second type of experiments the authors compare transgenic and non-transgenic peas regarding the induction of alpha amylase-specific immune and inflammatory responses. Since bean has not been included in this experiment and the levels of aAI in the transgenic peas have not been determined it is not possible to make a comparison of the experiments carried out with bean (Figure 2) and peas (Figures 3, 4). As mentioned above, the measurement of IgG1 responses was carried out with a 1:10 serum dilution which is very unusual because significant responses in the IgG1 class in mice should be detectable up to dilutions of 1:1000 at least. No IgE data are shown. A comparison with the bean experiment is also not possible because the lowest serum dilution used there is 1:100 and the experiments were carried out independently and thus do not allow a comparison for reasons of experimental variation and plate to plate variability.

The results from the footpad experiments show an approximately 17% increase of footpad swelling for the mice having received transgenic materials compared to an approximately 7% increase in the mice treated with non-transgenic material leaving the question if this is a relevant result. Again the crucial comparison between bean-derived alpha amylase inhibitor with the transgenic, pea-derived protein is missing. Thus no statement can be made that the transgenic protein induces stronger footpad swelling than the bean-derived protein. The finding that alpha amylase inhibitor from pea induces stronger swelling in the mice which were sensitized with the extract from transgenic peas than in mice having received the non-transgenic pea is not surprising because when mice have not been sensitized against the protein no footpad reaction is expected. One must therefore assume that the 7% increase in the non-transgenic group represents the fluctuation/background of the applied method and one therefore wonders if another plus of 10% is really a meaningful result. Again, the crucial question, whether there is a difference between bean- and transgenic pea-derived alpha amylase inhibitor has not been studied at all. Intratracheal instillation of pea-derived aAI causes lung inflammation in the second type of experiments but this cannot be directly compared with results from the bean experiments. It is unclear why the transgenic lupine was included as control in the footpad experiments but not in the antibody measurements and the lung experiments.

The authors perform an analysis of peribronchial lymph node-derived cells regarding cytokine secretion in the pea experiment. They find secretion of IL-4 and IL-5 but not interferon gamma in the transgenic pea group but this experiment has not been performed in the previous bean experiment and no bean group was included in the pea experiment to allow any comparison between transgenic peas and beans.

In the third type of *in vivo* experiments the authors study whether intragastric instillation of aAI from bean and transgenic peas has effects on the development of sensitization to an unrelated antigen, i.e., ovalbumin (OVA). This again shows the inconsistency regarding the experimental design because the authors switched again the design and fed purified aAIs and not protein extracts as in the previous two types of experiments. They find that alpha amylase inhibitor from transgenic pea but not from bean induced or instillation of OVA alone induced OVA-specific IgG1 antibody responses, mucus production and eosinophilia. These results are clear but unusual because OVA per se represents a strong immunogen in mice. One would have expected an OVA-specific immune response when OVA is fed alone such as was observed for the feeding of globulin, vicilin and lectin in Figure 7. It would be interesting to know why globulin, vicilin and lectin, but no OVA induced antibody responses in mice without pea-derived alpha amylase inhibitor (compare Figures 6 and 7).

In an obviously unrelated experiment the authors show that intragastric instillation of transgenic pea extracts induces higher IgG1 responses to three other antigens which were co-administered (globulin, vicilin, lectin; Figure 7) with extracts from non-transgenic and transgenic peas but this experiment again does not include bean feeding to allow a comparison with bean-derived aAI. There is also a detectable IgG1 response when non-transgenic pea extract was used for feeding which shows that the proteins alone also induce IgG1 responses. This finding calls into question the results obtained for OVA, because the mice feed with OVA alone (Figure 6A) showed no OVA-specific IgG1 response. Again no IgE responses have been studied in any of the latter experiments.

## 3.5 Summary of open questions and problems related to the experiments

The first major concern relates to the use of materials for the subsequent *in vivo* experiments where it is either not clear how much of the relevant proteins (aAI) was actually present and also the possibility that the purity of the "purified proteins" has not been shown or is doubtful. It is therefore possible that the

differences in the *in vivo* studies are not caused by different posttranslational modifications of the aAI from the wild-type and GM organisms but rather by the presence of different amounts of aAI in the seed meal preparations or due to contaminations in the "purified proteins" or their different degradation.

It is also unclear why the authors have used a methodology for the structural assessment of the purified proteins which is not suited for the analysis of the fold or three-dimensional structure of a given protein. This is particular relevant because it has been reported that allergens after genetic modification can alter their fold and hence may induce different types of immune responses (Vrtala et al. 2000; 2001). Furthermore, the presence and nature of oligosaccharides may impact glycoprotein folding, stability, trafficking and immunogenicity as well as its primary functional activity (Kobata 1992; Willey 1999).

The first crucial question is therefore how they have assessed the contents of aAI in the crude extracts and how they can exclude the presence of impurities in the protein preparations? In fact, the authors confirmed that they cannot exclude the presence of contaminants in the protein preparations (Hogan, person. communication).

The second question is why they have used a methodology for the purification and characterization of the aAI which is only partly suitable to justify their conclusions?

The authors do not provide any rationale why they performed *in vivo* allergenicity assessment by using a murine model which does not reflect the most common forms of hypersensitivities, i.e., IgE-mediated allergies. IgE-mediated allergies are relevant for food allergy and allergic asthma and would be the most likely manifestations according to the possible exposition scenarios to the genetically modified plants (GMPs). Instead they use a model of delayed type hypersensitivity which may play a role in certain more rare manifestations of food hypersensitivity. From personal communication it became clear that the authors did not consider their model as mimicking IgE-mediated allergies. The experimental system was developed to assess whether consumption provoked an immune response. It was used as part of an overall assessment of pea aAI. The relevance of these investigations for GM food has not yet been assessed (Hogan, person. communication).

However, this latter statement contradicts somehow the authors' conclusion in the abstract of their study saying: "…we demonstrated in mice that consumption of the modified alpha amylase inhibitor and not the native form predisposed to antigen-specific CD4+ Th2-type inflammation". In fact CD4+

Th2-type inflammation is the same as IgE-mediated allergy. Especially important, the *in vivo* experiments were performed in such a way that the direct comparison of wild-type aAI with the GMP-derived protein has never been performed within one experiment so that no conclusion can be made whether one of the proteins is more allergenic than the other. The crucial statement in the abstract is therefore not fully supported by the results of the study.

The third question is therefore why an *in vivo* model was chosen that is not suitable to mimic IgE-mediated allergies and that does not take into account a realistic exposure scenario as it may happen in man? No one will be immunized in the footpad with GMP-derived material.

The fourth question is why no *in vivo* experiments were conducted which would allow for a direct comparison of the wild-type and GMO-derived aAI so that their *in vivo* allergenic activity can be truly compared?

## 3.6 Discussion

In the discussion the authors say that they have shown that "transgenic expression of this protein (i.e., bean aAI) in peas led to the synthesis of a modified form of alpha amylase inhibitor".

According to the analysis of the experiments made and the results obtained by the authors this statement is not supported by the results. As mentioned above there are several other possibilities for the differences observed in the western blot analysis and MALDI-TOF-MS experiments. Furthermore the experiments lack important controls and the methodology used is not suited for the assessment of true (i.e., three-dimensional) structural features of the proteins.

The authors fail to provide a full biochemical characterization of the proteins and they did not study the fold of the proteins which could be done by circular dichroism or NMR. They do not investigate the aggregation behaviour of the proteins and no information regarding the putative post-translational modifications is given.

The second statement of the authors "Further, we show that the modified form of aAI possessed altered antigenic properties and consumption of this protein by mice predisposed to alpha amylase-specific CD4+ Th2 type inflammation and elicited immunoreactivity to concurrently consumed heterogeneous food antigens" is also not fully supported by the results. Preceding experiments were carried out with extracts of unknown alpha amylase protein contents. Other components in the extract may have triggered the phenomenon and finally no

direct comparison was made between the wild-type and transgenic proteins in the first two experiments. The only piece of data which remain is the fact that concomitant feeding with the bean and pea-derived protein appeared to have different effects on the development of an immune response to another antigen, i.e., OVA, but again this could be related to impurities in the protein preparations as is indicated by the MALDI-TOF-MS analysis in Figure 1B.

In fact, a recent conference paper - published after this review was completed - identified differences in the proteomes of GM peas and conventional comparators including accumulation of a number of proteins as well as two new proteins associated with the GM pea (Chen et al. 2007).

The authors' argument that altered glycosylation and or other modifications may be the reason for the obtained results is therefore on weak grounds. It should also be noted in this context, that glycosylated proteins and in particular carbohydrates may react with IgE antibodies but these structures normally have a very low allergenic activity (Mari 2002; van Ree et al. 2002).

However, if the results were indeed correct, which cannot be judged at present, our earlier suggestion to compare the full wild-type organisms with the transgenic organism regarding allergenic activity (i.e., ability to elicit an allergic reaction in allergic individuals) and allergenicity (i.e., ability to induce an allergic sensitization in a not yet sensitized individual) would be fully supported (Spök et al., 2005).

One result of the Prescott study may however raise justified concerns about the current practice of allergenicity assessment. The authors provide some evidence that co-administration of transgenic aAI may promote the immunogenicity of another immunologically unrelated protein. This finding basically implies that the insertion of a foreign protein into a host organism may have influence on the immunogenicity and perhaps allergenicity of other proteins from this organism by pleiotropic effects which cannot be predicted with the current technologies of allergenicity assessment. Spök et al. (2005) have in fact brought up this point in their recent opinion paper where they point to the possibility that the insertion even of a per se non-allergenic protein may have effects on other proteins of the host organism and eventually increase the allergenicity of one of these unrelated components. As a possible experimental approach they suggest two rather simple types of experiments. For the testing whether insertion of a foreign protein into a host increases the presence of allergenic proteins, they suggest to compare the complete wild-type organism with the GMP and not only the isolated foreign protein in an IgE-reactivity screen using sera from allergic patients or eventually investigations which analyse the allergenic

activity of the organisms (e.g., basophil activation tests or *in vivo* provocation testing (van Hage-Amsten & Pauli 2004; Valenta et al. 2004).

Regarding the assessment of allergenicity (i.e., whether a certain substance can induce *in vivo* a new allergic immune response) Spök et al. (2005) suggest to compare the whole GMP (i.e., plant extracts) with the parental plants or non-modified counterparts rather than investigating the isolated protein. Comparisons should rather be made *in vivo* (e.g., murine models for IgE-mediated allergies) compared to relying on indirect and *in-vitro* evidence only.

In summary the Prescott study fails to provide a convincing case but in principle it points to substantial wholes in the current procedure for allergenicity assessment and calls for more adequate methods which are also suitable to detect pleiotropic effects. There is thus a need for the development of methods which allow the comparison of the allergenic activity and allergenicity of the whole plant.

# 4 The GM pea in standard GMO risk assessment

The conclusions implied in the Prescott paper and more explicitly drawn in the subsequent paper (Prescott & Hogan 2006) sparked a fierce debate among and between risk assessors and environmental organisations on two main questions:

- Would the particular type of study have been required in the GM risk assessment?

- (Assuming a negative answer to the first question): Would possible allergenic effects also have shown up using the standard approach for assessment possible allergenic risks for GM crops and food?

The pea story is sometimes told in ways implying that the particular approach for allergenicity testing has been chosen in consultation with the Australian regulators (e.g., www.gmo-compass.org). This is, however, being denied by most commentators (AFSSA 2006; ISP 2006; Young 2005; interview senior scientist reviewing the pea study). Moreover FSANZ did never advise on the particulars of the allergenicity assessment (FSANZ 2005; interview CSIRO; interview senior scientist reviewing the pea study, FSANZ pers. comm.).

The second question is more difficult to answer and requires a more extensive discussion.

EU and other international risk assessment guidance require some standard data and testing (FAO/WHO 2001). From 2003 onwards the originally proposed decision tree approach was replaced by what was then called "weight of evidence" approach. Instead of a tiered testing system the next step of which is depending on the outcome of the former, these recent guidance propose several tests that could be conducted without specifying which tests should be conducted in what particular case. Weight of evidence means to consider the total information available including data not specific for allergenicity assessment, e.g., on chemico-physical aspects and functional properties of the protein (EFSA 2004). Allergenicity testing in practice was comprising and still comprises homology comparisons to known allergenic proteins, *in vitro* digestibility studies and any data on potential allergenicity of the donor organism. Furthermore, expression levels, glycosylation, history of safe use of protein or source organism in food, and the allergenic potential of the host is occasionally considered (Spök et al. 2005; 2004; unpublished results).

Whether allergenicity assessment would also go beyond these tests is likely to be case-dependent. The EFSA and FAO/WHO Guidance documents propose

additional tests, e.g., targeted and specific serum screens. The use of animal models is rather considered as a future option once these models have become validated (EFSA 2004). Hence, in the first place, it would be on the applicant to decide what data would be considered appropriate and second it would depend on the risk assessors reviewing the application case-by-case. This approach makes it much more difficult to anticipate additional data or testing requirements.

Whether the peas would have alert the risk assessors would depend on one or more of the following aspects:

- Results from the tests and data mentioned above. According to EU practice it can be expected that homology comparisons, digestibility studies, information about donor organism and use of the protein in food production might have been provided. Glycosylation might have also been required because it has already been documented in the literature that the bean aAI is glycosylated (Young et al. 1999). No such information has, however, been published to date.

- Scientific rigour of the testing conducted.

- Even if these studies and information would have provided evidence of possible immunogenic properties the question remains what kind of additional studies would have been provided by the applicant or requested by the risk assessors.

This chapter attempts to anticipate the results of such studies and discusses possible requirements for additional data by drawing on information available from the scientific literature and on homology testing conducted by the authors of this report.

## 4.1 *In vitro* digestibility tests

By referring to Yoshikawa et al. (1999) Prescott et al. indicate that aAI is "partially resistant" against proteolytic attack. This paper investigates the stability of an aAI from Kintoki bean against pepsin, chymotrypsin and trypsin. It was shown that aAI is fairly resistant against pepsin and trypsin but susceptible to chymotrypsin. These results correspond to an earlier study conducted by Andriolo et al. (1984) on another bean variety (lingot blanc).

However, there are several important methodological differences between these two studies and routine *in vitro* digestibility tests described in the scientific literature and in GMO risk assessment dossiers (Table 1).

***Table 1: Methodological differences between in vitro digestibility studies applying stomach pepsin.***

| | **Yoshikawa et al. (1999)** | **Andriolo et al. (1984)** | **FAO/WHO** | **SGF normal practice[a]** |
|---|---|---|---|---|
| Ratio pepsin/test protein | 1:10 | 1:10 | 1.28:1 | 1:1 – 1,600:1 |
| Units Pepsin/µg test protein | N.sp. | $7x10^{-4}$ | Units should be assessed | $10^{-2}$ - $4x10^{3}$ |
| Methods for tracing protein degradation | Indirect: Decrease in alpha amylase activity (activity of aAI inhibitor) | | N.sp. | SDS-PAGE |

*a) Ranges calculated from scientific literature and risk assessment dossiers (Spök et al. unpublished data). Acronyms: n.sp…. not specified; SGF… Simulated gastric fluid.*

First, the amount of pepsin applied by Andriolo et al. (1984) is magnitudes lower compared with what is being normally applied. Second, aAI degradation is being monitored in both cases by indirect methods only. The decline or non-decline of alpha amylase activity is used as a measure of degradation of the aAI protein whereas normally an SDS-PAGE would provide information on the structural intactness of the protein. The activity based methods would mask any inactivation of the protein without degradation. Furthermore, there might be a risk to confuse a decline by alpha amylase activity caused by other reasons with a degradation of the aAI, e.g. inactivation of amylase in the test conditions.

According to CSIRO a digestibility study with pepsin actually had been conducted and the aAI found to be relatively stable could be tracked for one hour (CSIRO interview, results not published). Nevertheless, given what is said above it is still possible that at higher pepsin activity levels which would correspond to normal practice aAI might appear to be more susceptible to pepsin degradation.

A similar picture emerges from the tests conducted to conclude resistance against trypsin and susceptibility against chymotrypsin (see Table 2). Both groups have applied these enzymes in isolation. Whereas the routine testing of protein degradation in simulated intestinal fluids (SIF) uses mixtures containing both enzymes and other substances. This is in accordance to the US Pharmacopeia (USP 22 and 23). It is quite likely that such a mixture applied to aAI would result in a rapid degradation due to the action of chymotrypsin. Again the degradation was monitored by the indirect activity-based method, allowing for possible errors as described above.

*Table 2: Methodological differences between in vitro digestibility studies applying proteases from the intestine.*

| | **Yoshikawa et al. (1999)** | | **Andriolo et al. (1984)** | | **SIF normal practice**[a] |
|---|---|---|---|---|---|
| SIF cocktail | N.a. | N.a. | N.a. | N.a. | USP 23 |
| Enzyme | Trypsin, units/mg n.sp. | Chymotrypsin units/mg n.sp. | Trypsin, 11,000 Units/mg | Chymotrypsin, 45 units/mg | USP 23 |
| Ratio enzymes/test protein | 1:10 | 1:10 | 1:50 | 1:50 | 2500:1 – 1:2 |
| Units enzyme/µg test protein | N.sp. | N.sp. | 0,2 U trypsin | 0,0009 U chymotrypsin | 5000-13 U peptidase |
| pH | 8 | 8 | 8.1 | 8.1 | 7.5 |
| Reaction terminated | 10 min/70°C | 10 min/70°C | N.sp. | N.sp. | 5 min/100°C |
| Methods for monitoring protein degradation | Indirect: Decrease in alpha amylase activity (activity of aAI) | | | | SDS-PAGE |
| Results/conclusion | Inhibitor activity within 2 h are almost equal and high | | | | n.a. |

*a) Ranges calculated from scientific literature and risk assessment dossiers (Spök et al. unpublished data)*

*Acronyms: USP: United States Pharmacopeia; N.a.: not applicable; N.sp.: not specified; SIF: simulated intestinal fluid.*

In any case it is not clear how SGF and SIF would provide a realistic model for *in vivo* digestion. Yoshikawa et al. (1999) and Andriolo et al. (1984) reported about susceptibility of the native aAI to chymotrypsin. Pusztai et al. (1999; Pustzai pers. comm.) showed that the GM pea derived aAI is readily degraded in *in vivo* studies in rats whereas the native aAI is much more stable *in vivo*. Moreover, results taken from such *in vivo* studies might not have alerted the risk assessors of possible allergenic properties. Nevertheless, these results might have pointed to imported differences between the bean-derived and the GM pea-derived aAI.

## 4.2 Homology comparisons

Homology comparison of the deduced amino acid sequence to (suspected) allergenic proteins are included in virtually all allergenicity assessments of heterologous proteins expressed in GMPs. In the absence of a publicly available comparison conducted by the plant developers homology comparisons were conducted by the authors of this report using different databases and algorithms.

According to FAO/WHO (2001) an allergenic potential can be expected if homology comparisons are exceeding a 35% identity in an 80 amino acid stretch or, if resulting in at least 6 consecutive identical amino acids.

By the time of the FAO/WHO consultation specialised sequence databases for allergenic proteins were under construction and thus comparisons were suggested to a derived subset from protein database such as PIR, SwissProt and TrEMBL. Meanwhile a number of specialised databases have become available some of which offer an allergenicity prediction using the FAO/WHO requirements. These databases and algorithms were used for comparing the deduced amino acid sequence of the bean aAI (sequence derived from Prescott et al. 2005; Pubmed supplements) to potential allergens. The results are summarised in Table 3, more details are shown in Annex B.

Most significantly the alignments identified homologies to a minor allergen from soybean (Gly m lectin) and to two allergens from peanut (Ara_h_F042 and Ara_h_F043). Only the homology to the soybean agglutinin exceeded the 35% threshold (51.2%) whereas the peanut proteins were slightly below (33.7%). A stretch of 8 homologous amino acids was identified in case of the soybean allergen and for another peanut allergen (Ara_h_F044, Ara_h_F045). Soybean and peanuts are both important food allergens.

Also striking are the different results obtained when using different databases and algorithms (BLAST, FASTA). If the FAO/WHO approach is applied positive results would have only been obtained from the ADFS and SDAP databases whereas a search of ALLERMATCH and ALLERDB would not have identified any positive matches. Moreover an normal FASTA search in ALLERMATCH would not have revealed any homologies exceeding 22%. The homology to the peanut allergens was only revealed using the ADFS database and did not show up from the SDAP database.

According to EFSA homology comparisons from of the bean aAI to known allergens conducted by a Swedish group in fact resulted in a "weak" signal without specifying the particular allergens (EFSA 2006). However, the references provided (Bjorklund et al. 2005; Soeria-Atmadja et al. 2004) are referring to an homology comparison approach which might not be representative. EFSA also pointed out a "moderate sequence similarity to agglutinin from peanut (*Arachis hypgaea*), a suspected allergen" (Burks et al. 1994). This was in fact confirmed by the homology comparisons conducted by the authors of this report using the ADFS database. No homologies to the peanut protein where found with the databases ALLERDB, ALLERMATCH, and SDAP. Interestingly the EFSA document did not mention any homologies to soybean allergens.

*Table 3: Results of allergenicity prediction on the basis for sequence homologies to specialised databases.*[b]

| Results | Protein/ sequence reference | Identity length [%] | E-score | Identical stretch | Full FASTA/ BLAST | Database/source |
|---|---|---|---|---|---|---|
| Agglutinin (soybean) | ADFS\|P05046 \|Gly_m_F091<br>ADFS\|P05046 \|Gly_m_lectin | 39.8 (251) | 2.7e-11 | 6mer (1) | 39.8 (251)<br>2.7e-11 | ADFS, http://allergen.nihs.go.jp/ADFS /index.jsp?pagen=top |
| | Gly m lectin, sequence: AAA33983 | 51.2 (41 of 80 aa) | N.i. | 8mer (1) | 41.06 (101/246)<br>6.7e-14 | SDAP - Structural Database of Allergenic Proteins, http://fermi.utmb.edu/SDAP/index.html |
| Kunitz trypsin inhibitor (soybean) | Gly m TI, sequence: CAA56343 | N.a. | N.a. | 6mer (1) | N.i. | SDAP - Structural Database of Allergenic Proteins, http://fermi.utmb.edu/SDAP/index.html |
| Agglutinin (peanut) | ADFS\|P02872 \|Ara_h_F043<br>ADFS\|P02872 \|Ara_h_F042 | 33.7 (253) | 1,9e-11 | N.d. | 33.7 (252)<br>1.9e-11. | ADFS, http://allergen.nihs.go.jp/ADFS /index.jsp?pagen=top |
| Mannose/glucose binding lectin precursor (fragment) (peanut) | ADFS\|Q43376\|Ara_h_F044<br>ADFS\|Q43377\|Ara_h_F045 | 34.3 (105) | 7.6e-9<br>1.5e/8 | 8mer (1) | 34.3 (105)<br>7.6e-9<br>1.5e-8 | ADFS, http://allergen.nihs.go.jp/ADFS /index.jsp?pagen=top |
| No homologies detected according to FAO/WHO criteria | | | | | N.i. | ALLERDB, http://sdmc.i2r.a-star.edu.sg/Templar/DB/Allergen/ |
| a | a | No homologies detected according to FAO/WHO criteria | | | a | ALLERMATCH, http://allermatch.org/ |

*a) A full BLAST search resulted in numerous homologies of less than 22% (results not shown).*

*b) Default settings for all searches.*

Furthermore, EFSA pointed to literature research which would have revealed aAIs as allergenic components of cereals (James et al. 1997). Drawing on this evidence EFSA concluded that "it is clear that these indications would have triggered additional investigations on the possible allergenicity of the GM pea aAI" (EFSA 2006).

CSIRO researchers in fact did conduct some homology comparisons which never had been published. These comparisons showed homologies to a soybean

allergen – but according to the researchers – it did not appear to be very relevant (interview CSIRO).

Given results it is likely but not entirely clear if the homologies to the soybean allergen would have been readily identified. It is less clear if the homologies to the peanut proteins would have been found – especially as the percentage of homology is below the FAO/WHO threshold of 35% identity in 80 amino acids.

## 4.3 Test Protein/serum screen

In risk assessments for heterologous proteins test proteins are normally derived from microbes rather than the GM plant itself (Spök et al. 2004; Spök et al. unpublished results). Applicants generally argue that purification from the GM plant would be very difficult and very expensive given the low concentrations of these kinds of proteins in plant tissue. This is so far routine practice although criticized by some (Freese & Schubert 2005; Spök et al. 2004).

If the aAI from bean would have been produced by microbes it would not have been glycosylated in the same way as in the plant or – more likely and depending on the microbes used – not glycosylated at all. This would only be relevant if the microbial protein would have been applied in serum screen or animal testing. However, no examples of animal tests conducted in GMP risk assessment have come to the attention of the authors. Serum screenings are also rarely provided. In case of the soybean 260 Novel Food application a serum screen was conducted with soybean extracts. However, in this case no novel protein was expressed, thus, homology comparisons and digestibility tests would not have been applicable anyway (see Spök et al. 2003).

Overlooking GM crops authorised in the EU, the USA and elsewhere, there have been only a few cases of plant proteins being expressed in other plants, e.g., flax CDC-FL001-2, canola 23-198 and 23-18-17, a few carnations and one tobacco variety. As far as it can be revealed from publicly available documents, even in these cases the test protein was of microbial origin and no serum screen or any other direct allergenicity testing was conducted. The carnation varieties and the tobacco are not intended to be used as food or feed and the canola contains a plastid protein from California laurel. Plastid proteins are not known to be glycosylated. In case of the ALS protein in flax which originates from Arabidopsis thaliana no allergenicity study has been conducted with the purified protein.

In the case of the aAI it is difficult to anticipate what would have been required for a normal GMO risk assessment. Clearly, it would have been evident from the

literature that the aAI is glycosylated in beans (Young et al. 1999). A microbial expression system might have even abandoned in early stages if it would not lead to a functional protein, e.g., in the case it does not pursue the complex proteolytic processing or the propter folding to yield a functional aAI. If so, the aAI might have been purified from the GM pea. Different to most heterologous proteins of first generation GM crops the aAI is expressed in GM peas at high levels. The low expression levels in the range of ng and µg of TSP has always been motioned as the major disincentive to purify the heterologous protein from the GM plants.

# 5 Discussion and Conclusions

## 5.1 Relevance of the experimental design for allergenicity assessment in humans

The experimental animal approach used by Prescott et al. in animals is not suitable for allergenicity assessment for several reasons:

1. The model mixes various forms of sensitization and priming which are unlikely scenarios. The exposure scenario used (priming via intestinal tract and subsequent injection into the footpad or intratracheal instillation using highly concentrated protein) is not relevant to GM exposure scenarios of food/feed.

2. The model is neither a clear model for IgE-mediated allergy nor for delayed type hypersensitivity (i.e., Type IV hypersensitivity) or for immunecomplex diseases (i.e., Type III hypersensitivity).

3. The model is performed in inbred mice and it is therefore difficult to say how the effects will be reproduced in an outbreed population with different genetic backgrounds.

The fundamental problem with allergenicity assessment methods is that they cannot answer the question whether something is allergenic or not. Instead we have suggested earlier comparing wild-type and transgenic organisms to find out whether the transgenic material has a higher likelihood to induce sensitizations. In addition, exposure scenarios need to be considered when the animal studies are planned.

## 5.2 Application of "standard" allergenicity assessment to GM peas

Risk assessors and regulators in many countries have considered the Prescott study. Reassuring statements have been published that the presently applied approach to allergenicity assessment would have alerted them to possible immunogenic properties implying that the GM never would have made it through the regulatory procedure (e.g., ACNFP 2005a, b; EFSA 2006). The Australian regulators did not make such reassuring statements but pointed out that the presently available animal models are not considered to be sufficiently well developed or validated to be used at present (FSANZ 2005).

On the other hand ACNFP Members also concluded that the Prescott study "illustrated the importance of using plant derived proteins in the safety assessment rather than a microbial equivalent" (ACNFP 2005a). It appears that in fact the Prescott study did encourage the UK Food Standard Agency (FSA) to launch a project on post-translational modification of transgenic proteins compared with their native equivalents that can be applied in the safety assessment of GM organisms (FSA 2006).

Regulators at the Irish Environmental Protection Agency (EPA) appear to have changed their risk assessment requirements as they were asking a company to conduct the tests done by Prescott et al. for a deliberate release of a GM potato (EPA 2006, interview Irish EPA). Risk assessors in Taiwan are considering to ask for similar studies for market authorisation of GM food (Spök et al., unpublished).

In fact it is not entirely clear cut whether this case would have alerted risk assessors. If, nevertheless, further studies would have been required a request for animal studies is quite unlikely.

From homology comparison studies and *in vitro* glycosylation studies as well as from the fact that the aAI is a glycoprotein risk assessors might have concluded that additional studies would be needed to further investigate the allergenic properties of the novel protein. However, as shown in preceding sections, depending on database and algorithm used and on the particular experimental approach to the digestibility studies there is a chance that the aAI might also have passed these tests unrecognised.

The use of specific and targeted serum screening is proposed for allergenicity assessment by Codex Alimentarius, FAO/WHO and EFSA (CODEX ALIMENTARIUS COMMISSON 2003; EFSA 2004; FAO/WHO 2001). A homology search or in case of a protein from an allergenic source a specific serum screen should be conducted, e.g., according to Codex. For targeted serum screening the situation is more complex. Whereas a negative homology search would be followed by a targeted serum screening according to FAO/WHO (2001), it is not clear what would trigger a targeted serum in the Codex and EFSA Guidance. This type of these experiments is normally not included in the GM risk assessment dossiers. On the other hand, serum screens using, e.g., either sera of patients allergenic against beans, peanuts and soybeans or a range of legume or even employing a broader range of sera might well have been required as additional tests. From the Prescott et al. paper and from the homology comparisons it cannot be anticipated whether this serum screens would have resulted in any additional evidence supporting an immunogenic potential.

Given the high expression level of the aAI in peas and the known glycosylation of the protein it might well be expected that aAI would have been purified from the GM peas. It is therefore likely that in case of serum screens, they might have been conducted with purified plant protein.

## 5.3 Overall conclusions

Despite the shortcomings of the Prescott study, the many open questions and the concerns regarding the relevance of the animal model and experimental design used it cannot be excluded that different posttranslational processing of the GM pea aAI compared to the native bean protein has changed the immunogenic properties. This could and should be clarified by further research. Any such research should however consider the relevance of the experiments conducted for human exposure. With the possible exception of testing of the adjuvant effects of the aAI the overall experimental design used by Prescott et al. does not seem to be relevant for a scenario of introducing such a plant for food and/or feed purposes. Furthermore, the conclusions drawn by Prescott et al. are not supported by sound data and do not allow for an exclusion of other possible causes such as pleiotropic effects.

Whether the pea aAI would have alerted the risk assessors in any case is difficult to tell as digestibility tests and homology comparisons along with data from literature lead to contradicting conclusions.

Given the exclusive focus of GM allergenicity assessment on the heterologous protein it is, however, not likely that allergenicity testing of the whole crop would have been required in order to search for allergenic properties other than of the novel proteins.

The uncertainties associated with the actual cause for the immunogenic effects in mice once more point to the need to go beyond the introduced proteins, and the standard homology comparisons and digestibility tests. Assessment should consider possible pleiotropic effects when evaluating the allergenic properties. In such a way it would be possible to determine whether the insertion of the gene induces expression of other allergens. Such an evaluation could answer the question of whether the GMP has a greater, similar or perhaps reduced allergenic potential than the wild type. A proposal for a procedure that would evaluate the allergenic properties including the potential to sensitize has been outlined elsewhere (Spök et al. 2005) by the authors of this report and will therefore not be repeated here.

## 5.4 Recommendations for further research

The conclusion of the Prescott study was to demonstrate that the aAI from GM peas is more allergenic and better promotes the development of allergic immune responses against other proteins than the wild-type bean-derived protein. Certainly it should be avoided to commercialise GMOs containing new proteins which have already a kind of basic allergenicity.

As discussed above, the more relevant question is whether the allergenic potential of the GM plant as a whole increases, i.e., either via expression of a new allergenic protein or by increasing the allergenicity caused by the newly inserted protein through pleiotropic effects compared to the parental wild-type organism.

In order to answer this question one could compare the GM pea to the parental wild-type plant regarding IgE reactivity and *in vivo* allergenicity. The first may be achieved by testing large numbers of sera from allergic patients from different populations and various broad sensitivities to find out whether the IgE reactivity of the GMP is substantially increased. It is also suggested that extracts from different tissues of the GM pea and the wild type be tested, taking into consideration possible exposure scenarios. For example, large-scale industrial processing of the plant creates other exposure scenarios.

Second, one could attempt to induce an allergic immuneresponse in animals with these preparations to find out whether the GM pea is more allergenic. Parameters to consider are the choice of sensitization protocols which induce indeed allergies, mimic natural exposure scenarios and the use of various inbred or better outbreed animals.

The latter two types of experiments are relatively easy to perform and give a clear answer whether the GM pea is more allergenic than the parental wild-type organism, which is perhaps the most relevant question.

The difficulties to find and agree on suitable models could be settled by consensus meetings of experts and launching of research programs addressing the suitability and feasibility of the various models.

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# Appendix A: Amino acid sequence of aAI used for sequence comparisons

P02873|LEA1_PHAVU Alpha-amylase inhibitor 1 - Phaseolus vulgaris (Kidney bean) (French bean).

MIMASSKLLSLALFLALLSHANSATETSFIIDAFNKTNLILQGDATVSSNGNLQLSYNSYDS
MSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQANSAVGLDFVLVPVQPESKGDTVTVE
FDTFLSRISIDVNNNDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSNNVST
TVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSKFINLKDQKSERSNIVLNKIL

# Appendix B: Sequence homology comparisons

## B.1 ADFS

**Allergenicity Prediction : FAO / WHO Method**

**Allergenicity Prediction Result**( open fasta output)

| Match | Description( from swissprot ) | Score | E value | Identity( Length ) |
|---|---|---|---|---|
| ADFS\|P02872\|Ara_h_F043 | Galactose-binding lectin precursor (Agglutinin) (PNA). | 295.6 | 1.9E-11 | 33.7(252) |
| ADFS\|P02872\|Ara_h_F042 | Galactose-binding lectin precursor (Agglutinin) (PNA). | 295.6 | 1.9E-11 | 33.7(252) |
| ADFS\|P05046\|Gly_m_F091 | Lectin precursor (Agglutinin) (SBA). | 292.7 | 2.7E-11 | 39.8(251) |
| ADFS\|P05046\|Gly_m_lectin | Lectin precursor (Agglutinin) (SBA). | 292.7 | 2.7E-11 | 39.8(251) |
| ADFS\|Q43376\|Ara_h_F044 | Mannose/glucose-binding lectin precursor (Fragment). | 248.8 | 7.6E-9 | 34.3(105) |
| ADFS\|Q43377\|Ara_h_F045 | Mannose/glucose-binding lectin precursor (Fragment). | 243.4 | 1.5E-8 | 34.3(105) |

> ADFS|P02872|Ara_h_F043 Galactose-binding lectin precursor (Agglutinin) (PNA).

Length = 252 Identity = 33.7 E-Value = 1.9e-11
Query = 8-228 Subject = 7-253

```
Query   : LLSLALFLALLSH-ANSATETSFIIDAFNKTN--LILQGDATVSSNGNLQLS-YNSYDSMSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQANSAVGLDFVLVPVQPE----------------SKGDT
Subject : FLTFFLLLAASSKKVDSAETVSFNFNSFSEGNPAINFQGDVTVLSNGNIQLTNLNKVNSVGRVLYAMPVRIWSSATGNVASFLTSFSFEMKDIKDYDPADGIIFFIAPEDTQIPAGSIGGGTLGVSDTKGAGHFVGVEFDTYSNSEYN
```

To the top of the window

> ADFS|P02872|Ara_h_F042 Galactose-binding lectin precursor (Agglutinin) (PNA).

Length = 252 Identity = 33.7 E-Value = 1.9e-11
Query = 8-228 Subject = 7-253

```
Query   : LLSLALFLALLSH-ANSATETSFIIDAFNKTN--LILQGDATVSSNGNLQLS-YNSYDSMSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQANSAVGLDFVLVPVQPE----------------SKGDT
Subject : FLTFFLLLAASSKKVDSAETVSFNFNSFSEGNPAINFQGDVTVLSNGNIQLTNLNKVNSVGRVLYAMPVRIWSSATGNVASFLTSFSFEMKDIKDYDPADGIIFFIAPEDTQIPAGSIGGGTLGVSDTKGAGHFVGVEFDTYSNSEYN
```

To the top of the window

> ADFS|P05046|Gly_m_F091 Lectin precursor (Agglutinin) (SBA).

Length = 251 Identity = 39.8 E-Value = 2.7e-11
Query = 9-227 Subject = 17-264

```
Query   : LSLALFLALL-SHANSATETSFIIDAF--NKTNLILQGDATVSSNGNLQLSYNSYD------SMSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQANSAVGLDFVLVPV--QPESK----------G
Subject : LTLTLVLVLLTSKANSAETVSFSWNKFVPKQPNMILQGDAIVTSSGKLQLNKVDENGTPKPSSLGRALYSTPIHIWDKETGSVASFAASFNFTFYAPDTKRLADGLAFFLAPIDTKPQTHAGYLGLFNENESGDQVVAVEFDTFRNSW
```

To the top of the window

> ADFS|P05046|Gly_m_lectin Lectin precursor (Agglutinin) (SBA).

Length = 251 Identity = 39.8 E-Value = 2.7e-11
Query = 9-227 Subject = 17-264

```
Query   : LSLALFLALL-SHANSATETSFIIDAF--NKTNLILQGDATVSSNGNLQLSYNSYD------SMSRAFYSAPIQIRDSTTGNVASFDTNFTMNIRTHRQANSAVGLDFVLVPV--QPESK----------G
Subject : LTLTLVLVLLTSKANSAETVSFSWNKFVPKQPNMILQGDAIVTSSGKLQLNKVDENGTPKPSSLGRALYSTPIHIWDKETGSVASFAASFNFTFYAPDTKRLADGLAFFLAPIDTKPQTHAGYLGLFNENESGDQVVAVEFDTFRNSW
```

To the top of the window

> ADFS|Q43376|Ara_h_F044 Mannose/glucose-binding lectin precursor (Fragment).

Length = 105 Identity = 34.3 E-Value = 7.6e-09
Query = 125-228 Subject = 142-240

```
Query   : FDTFLSRISIDVNN-NDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSSKFI
Subject : WDPNYQHIGIDVNSIKSAATTKWERRN--GQTLNVLVTYDANSKNLQVTASYPD-GQRYQVSYVVDLRDHLPEWGRVGFSASSGQ---QYQSHELQSWSFTSNLL
```

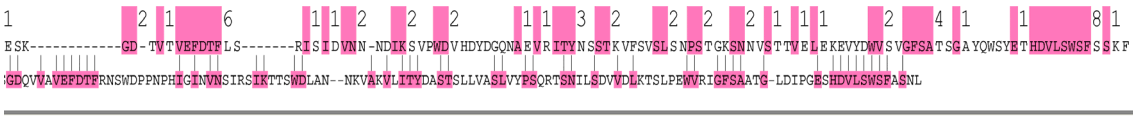
To the top of the window

> ADFS|Q43377|Ara_h_F045 Mannose/glucose-binding lectin precursor (Fragment).

Length = 105 Identity = 34.3 E-Value = 1.5e-08
Query = 125-228 Subject = 142-240

```
Query   : FDTFLSRISIDVNN-NDIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVSLSNPSTGKSNNVSTTVELEKEVYDWVSVGFSATSGAYQWSYETHDVLSWSFSSSKFI
Subject : WDPNYQHIGIDVNSIKSAATTKWERRD--GQTLNVLVTYDANSKNLQVTASYPD-GQRYQLSYRVDLRDYLPEWGRVGFSAASGQ---QYQSHELQSWSFTSTLL
```

To the top of the window



## B.2 Exact wordmatch result: 6mer

| Match | Description (from UniProt) | No of exact Wordmatch |
|---|---|---|
| ADFS\|Q39869\|Gly m TI | Kunitz trypsin inhibitor precursor. | 1 |
| ADFS\|Q43376\|Ara h F044 | Mannose/glucose-binding lectin precursor (Fragment). | 2 |
| ADFS\|Q43377\|Ara h F045 | Mannose/glucose-binding lectin precursor (Fragment). | 2 |

## B.3 SDAP

### B.3.1 Exact match for contiguous amino acids: 6mer

Alignment 1
Sequence 1: aAI (bean)
Sequence 2: Allergen Gly m TI, Sequence: CAA56343

```
Sequence 1 -----SLALFL---------------------------------------
Sequence 2 MKSTTSLALFLLCALTSSYQPSATADIVFDTEGNPIRNGGTYYVLPVIRG

Sequence 1 ------------------------------------------------
Sequence 2 KGGGIEFAKTETETCPLTVVQSPFEGLQRGLPLIISSPFKILDITEGLIL

Sequence 1 ------------------------------------------------
Sequence 2 SLKFHLCTPLSLNSFSVDRYSQGSARRTPCQTHWLQKHNRCWFRIQRASS

Sequence 1 ------------------------------------------------
Sequence 2 ESNYYKLVFCTSNDDSSCGDIVAPIDREGNRPLIVTHDQNHPLLVQFQKV

Sequence 1 --------
Sequence 2 EAYESSTA
```

## B.3.2 Full FASTA alignment

The FASTA alignments between the query sequence and all SDAP allergens have an E score higher than 0.010000.

Search performed in the SDAP allergens database.

| No | Allergen | Sequence Link in SwissProt/NCBI/PIR | View Sequence | Sequence Length | bit score | E score |
|---|---|---|---|---|---|---|
| 1 | Gly m lectin | AAA33983 | Go! | 285 | 69.6 | 6.7e-14 |

Alignment made with FASTA 3.45

As explained in the FASTA manual, the bit score is equivalent to the bit score reported by BLAST. A 1 bit increase in score corresponds to a 2-fold reduction in expectation, and a 10-bit increase implies 1000-fold lower expectation. Sequences with E values < 0.01 are almost always homologous.

```
Alignment 1
Sequence 1: aAI (bean)
Sequence 2: Allergen Gly m lectin, Sequence: AAA33983
Sequence identity: 41.06 (101/246)
Sequence 1 --------MIMASSKLLSLALFLALL-SHANSATETSFIIDAF--NKTNL
           -------------S--L-L-L-L-LL-S-ANSA---SF----F-----N-
Sequence 2 MATSKLKTQNVVVSLSLTLTLVLVLLTSKANSAETVSFSWNKFVPKQPNM

Sequence 1 ILQGDATVSSNGNLQLSYNSYD------SMSRAFYSAPIQIRDSTTGNVA
           ILQGDA-V-S-G-LQL------------S--RA-YS-PI-I-D--TG-VA
Sequence 2 ILQGDAIVTSSGKLQLNKVDENGTPKPSSLGRALYSTPIHIWDKETGSVA

Sequence 1 SFDTNFTMNIRTHRQANSAVGLDFVLVPV--QPESK------------GD
           SF---F------------A-GL-F-L-P----P--------------GD
Sequence 2 SFAASFNFTFYAPDTKRLADGLAFFLAPIDTKPQTHAGYLGLFNENESGD

Sequence 1 -TVTVEFDTFLS-------RISIDVNN-NDIKSVPWDVHDYDGQNAEVRI
           --V-VEFDTF----------I-I-VN----IK---WD--------A-V-I
Sequence 2 QVVAVEFDTFRNSWDPPNPHIGINVNSIRSIKTTSWDLAN--NKVAKVLI

Sequence 1 TYNSSTKVFSVSLSNPSTGKSNNVSTTVELEKEVYDWVSVGFSATSGAYQ
           TY--ST-----SL--PS---SN--S--V-L------WV--GFSA--G---
Sequence 2 TYDASTSLLVASLVYPSQRTSNILSDVVDLKTSLPEWVRIGFSAATG-LD

Sequence 1 WSYETHDVLSWSFSSKFINLKDQKSERSNIVLNKIL--
           ---E-HDVLSWSF-S----------------------
Sequence 2 IPGESHDVLSWSFASNLPHASSNIDPLDLTSFVLHEAI
```

# Appendix C: Interviews and contacts

In the course of this study extended phone interviews were conducted with and/or phone or e-mail inquiries were answered by a number of scientists and regulators:

Regulator, Food Standard Agency Australia and New Zealand

Regulator, Food Standards Agency, UK

Senior project manager, CSIRO, Australia

Senior researcher of the Prescott team

Senior scientist working on protein glycosylation

Senior regulator, EPA, Ireland

Two senior scientists, reviewing the Prescott study

# Appendix D: Abstract and Figures of Prescott et al. (2005)

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

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**Abstract:**

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.

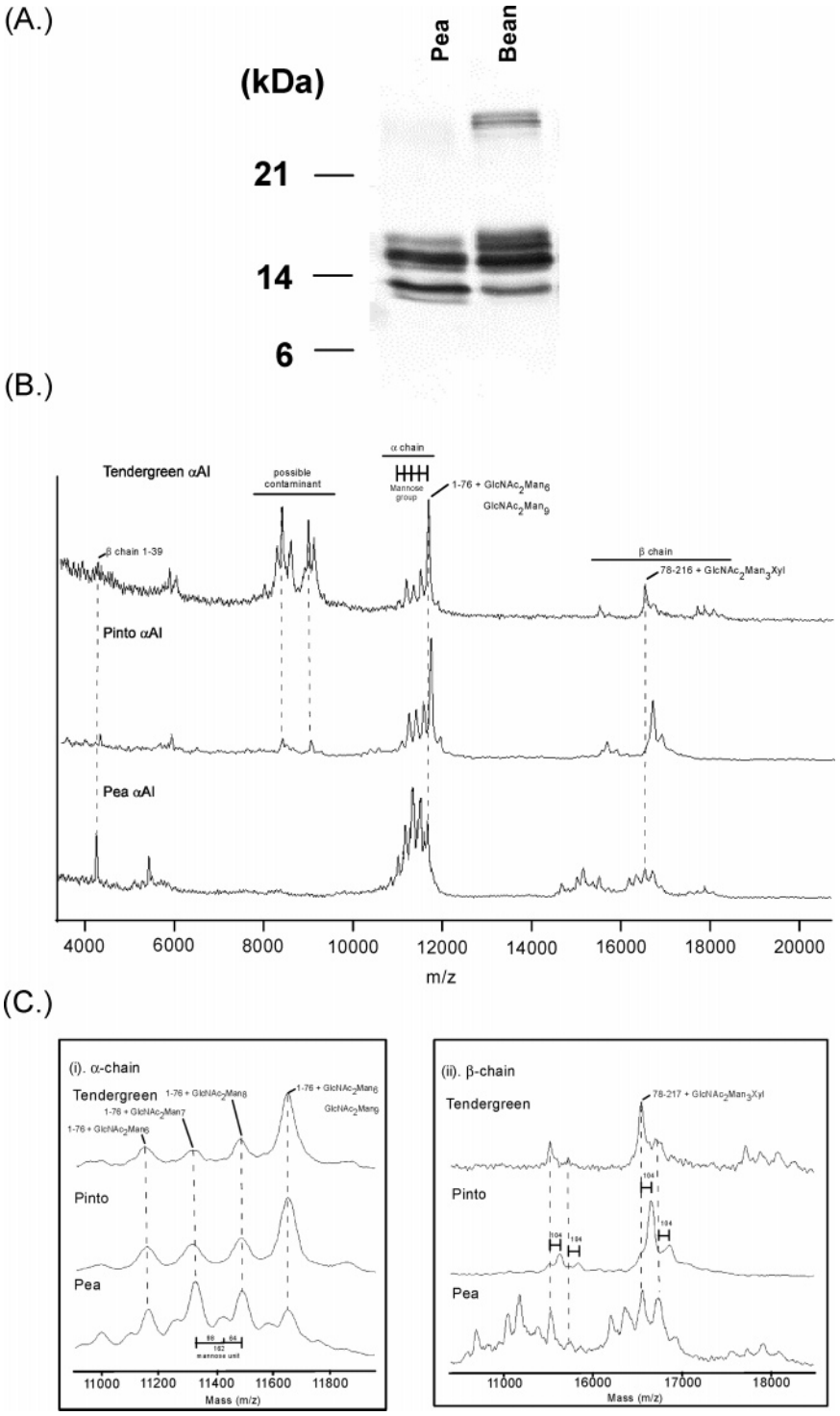


**Figure 1.** Western immunoblot and MALDI-TOF-MS analysis of common bean-derived-αAIs and RAI from transgenic peas. (**A**) Western blot analysis of αAI protein in extracts of transgenic peas and the Tendergreen variety of common bean. The masses of standard proteins are indicated. (**B**) Aligned MALDI-TOF 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**).

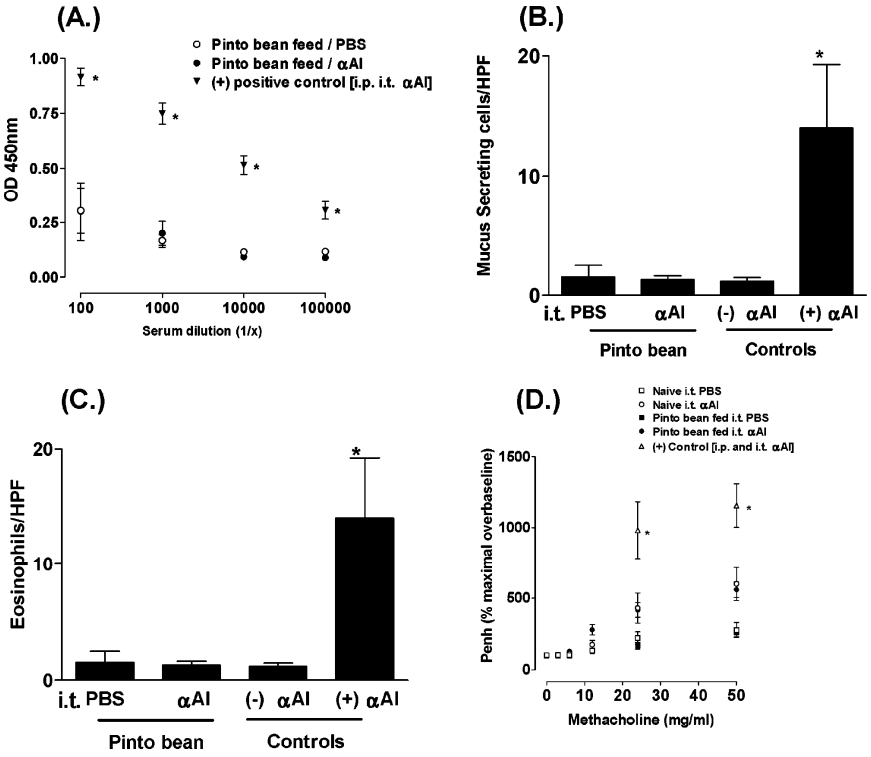


**Figure 2.** Experimental consumption of bean (cv. Pinto) seed meal does not predispose to inflammation. (**A**) αAI-specific $IgG_1$ in serum and (**B**) mucus-secreting cell numbers and (**C**) eosinophil levels in lung tissue from Pinto bean-fed mice i.t. challenged with PBS or Tendergreen-αAI. (**D**) 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. RAI.

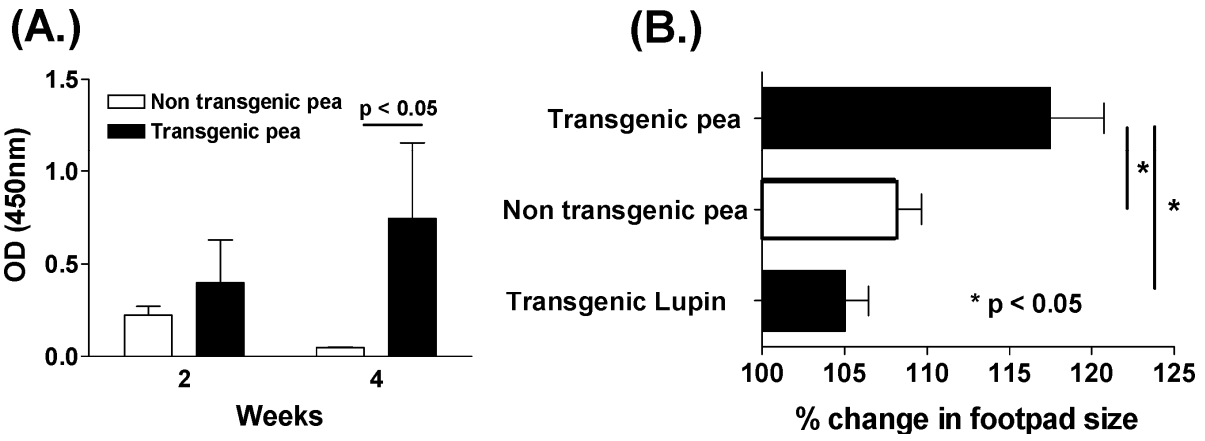


**Figure 3.** Experimental consumption of transgenic pea seed meal predisposed to antigen-specific $IgG_1$ and DTH responses. (**A**) Antigen-specific $IgG_1$ and (**B**) DTH responses in pea nontransgenic and pea transgenic-fed mice. Data are expressed as the (**F**) mean ± SEM and (**E**) mean O.D. 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.

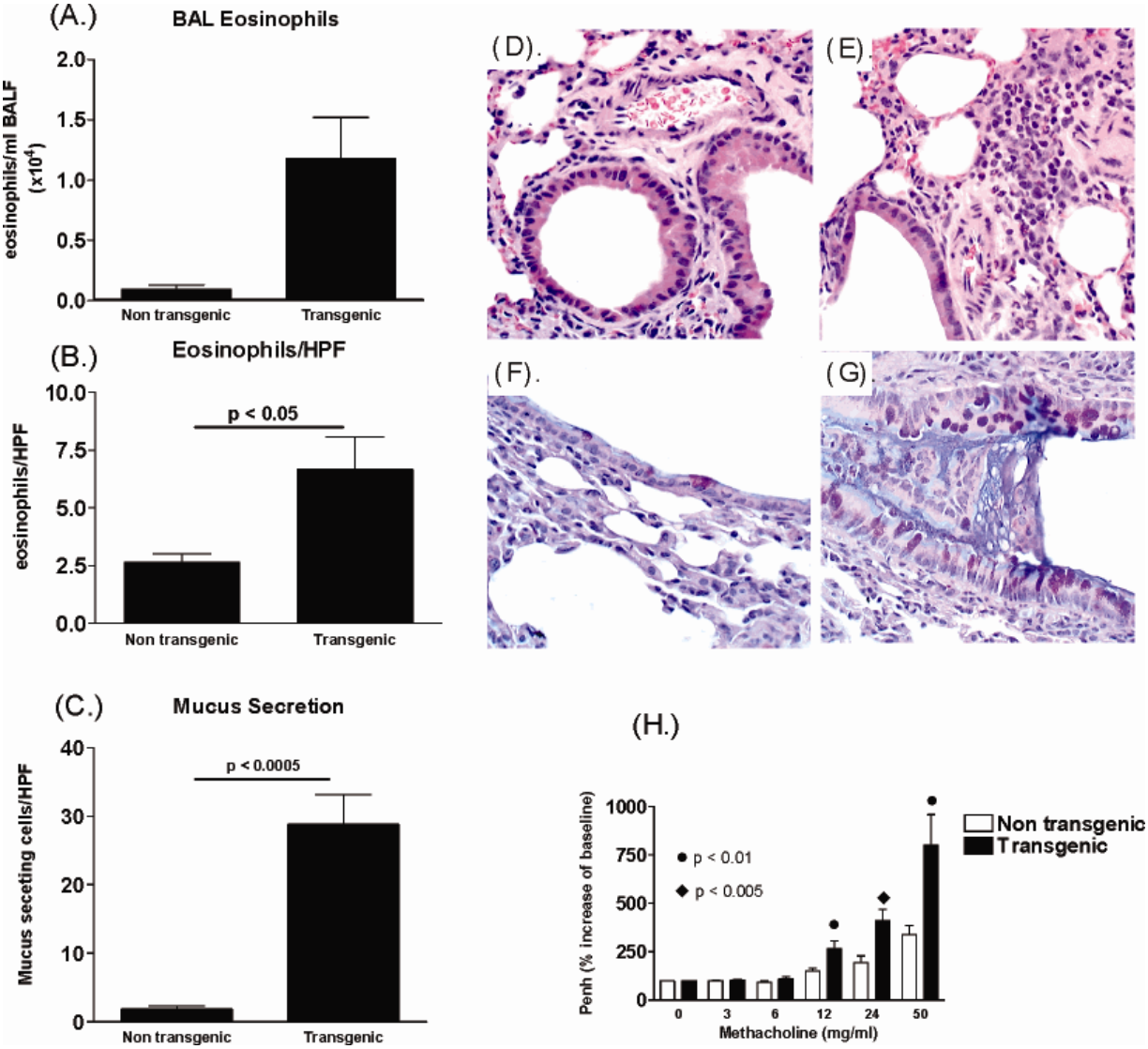


**Figure 4.** Consumption of transgenic pea seed meal predisposed to $CD4^+$ $Th_2$-type inflammatory response. Eosinophil accumulation in bronchoaveolar lavage fluid (BAL) (**A**), tissue (**B**), and mucus-secreting cell numbers (**C**) in lung tissue from nontransgenic and transgenic pea-fed mice i.t. challenged with RAI 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.

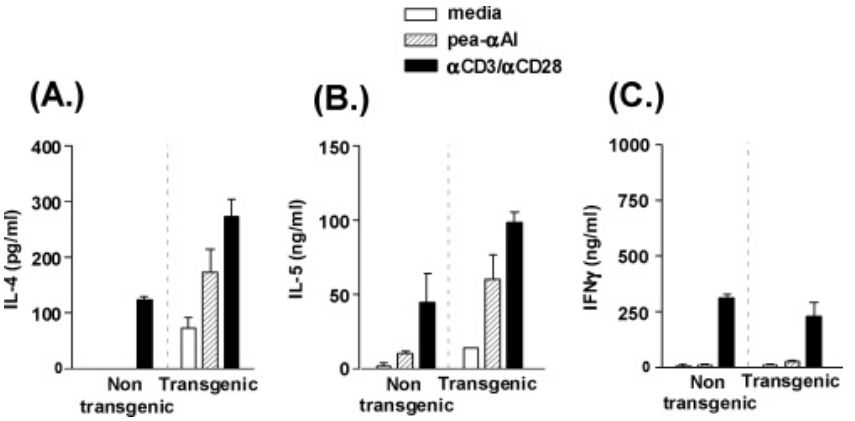


**Figure 5.** Consumption of transgenic pea seed meal predisposed to $CD4^+$ T-cell derived $Th_2$-type cytokine production. IL-4 (**A**), IL-5 (**B**), and IFNγ (**C**) levels in supernatants from RCD3/RCD28 or pea-αAI or media alone stimulated PBLN cells from nontransgenic and transgenic pea-fed mice 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

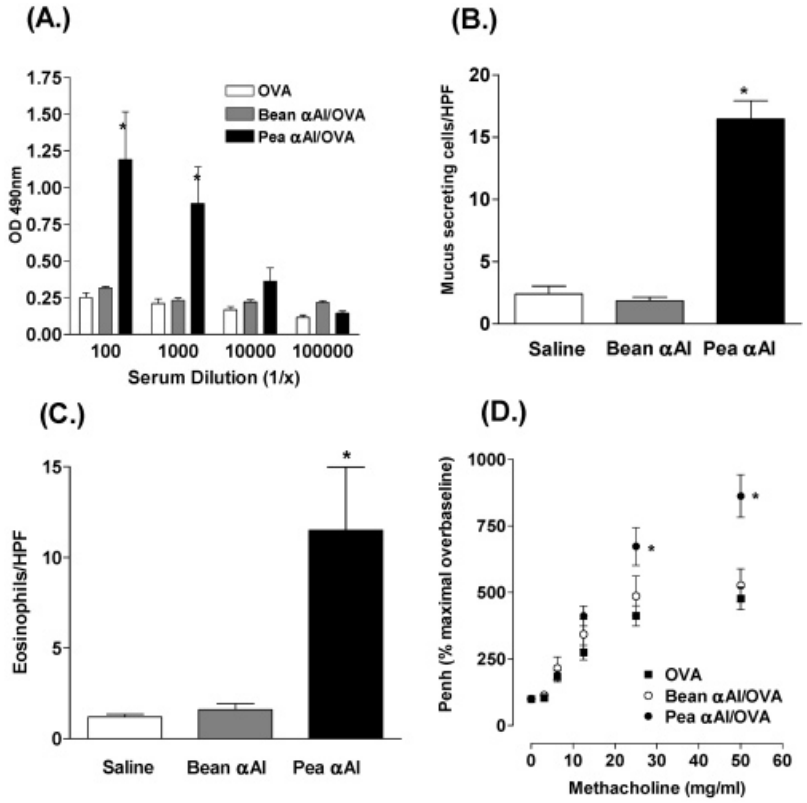


Figure 6. Intra-gastric administration of αAI from pea induces crosspriming of heterogeneous food antigens. OVA-specific IgG1 levels (A) and the Th2-inflammation phenotype (mucus hypersecretion) (B), pulmonary eosinophilia (C), and airways hyperreactivity (D) in mice that were fed (i.g. challenged) ovalbumin (OVA) alone (the control) or in combination with natively expressed Tendergreen bean-RAI or transgenically expressed (pea) RAI 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.

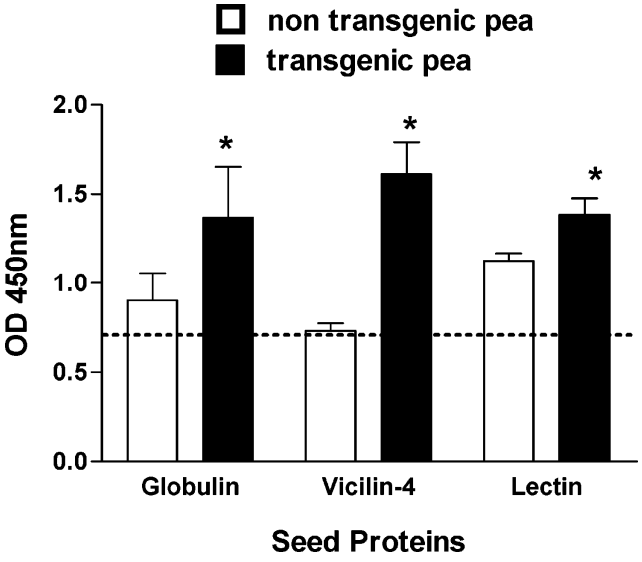


Figure 7. αAI from pea induces cross-priming of pea proteins. Pea globulin-, vicilin-4, and lectin-specific IgG1 levels in serum from mice that were intragastrically administered 250 µl (~100 mg/mL) of either nontransgenic or transgenic pea seed meal twice a week for 4 weeks. Data are expressed as mean ± SEM from 4 to 5 mice per group. * $p < 0.05$ as compared to nontransgenic pea.