

Characterization of the allergenic potential of proteins: an assessment of the kiwifruit allergen actinidin

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ABSTRACT: Assessment of the potential allergenicity (IgE-inducing properties) of novel proteins is an important challenge in the overall safety assessment of foods. Resistance to digestion with pepsin is commonly measured to characterize allergenicity, although the association is not absolute. We have previously shown that specific IgE antibody production induced by systemic [intraperitoneal (i.p.)] exposure of BALB/c strain mice to a range of proteins correlates with allergenic potential for known allergens. The purpose of the present study was to explore further the utility of these approaches using the food allergen, actinidin. Recently, kiwifruit has become an important allergenic foodstuff, coincident with its increased consumption, particularly as a weaning food. The ability of the kiwifruit allergen actinidin to stimulate antibody responses has been compared with the reference allergen ovalbumin, and with the non-allergen bovine haemoglobin. Haemoglobin was rapidly digested by pepsin whereas actinidin was resistant unless subjected to prior chemical reduction (reflecting intracellular digestion conditions). Haemoglobin stimulated detectable IgG antibody production at relatively high doses (10%), but failed to provoke detectable IgE. In contrast, actinidin was both immunogenic and allergenic at relatively low doses (0.25% to 1%). Vigorous IgG and IgG1 antibody and high titre IgE antibody responses were recorded, similar to those provoked by ovalbumin. Thus, actinidin displays a marked ability to provoke IgE, consistent with allergenic potential. These data provide further encouragement that in tandem with analysis of pepsin stability, the induction of IgE after systemic exposure of BALB/c strain mice provides a useful approach for the prospective identification of protein allergens. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: allergenicity; IgE; genetically modified; kiwi fruit; animal models

Introduction

In common with other IgE-mediated allergic diseases, the prevalence of food allergy appears to have been increasing over the last decade or more. Indeed, it has been suggested that the most recent increases in food allergy represent a 'second wave' of the allergy epidemic, that has lagged behind the rise in asthma and allergic rhinitis that has been witnessed during the last 40 or 50 years (Prescott and Allen, 2011). Although it had been previously estimated that 3–4% of adults and 4–6% children in the USA suffer from a food allergy (Sicherer and Sampson, 2009), more recent studies suggest that the incidence may be even higher. Thus, it is estimated that a challenge-proven IgE-mediated food allergy affects up to 10% of infants in Australia (Prescott and Allen, 2011), and a recent study in the USA suggested that the prevalence in children is 8% (Gupta *et al.*, 2011). IgE-mediated allergies are of particular relevance to toxicology. With the development of novel foods and food products, including those that are produced by genetic modification (GM), there is an important need for the safety assessment of transgene products, and in particular for the characterization of potential allergenic activity (Lack *et al.*, 2002; Selgrade *et al.*, 2009). This is a legitimate concern given that there is strong evidence that changing patterns of food consumption and dietary habits can result in alterations in the demographic profile of those foods identified as major allergens in a population.

As an example, allergy to sesame, an important allergen in countries such as Israel, has become much more common in the UK in line with increasing consumption (Aaronov *et al.*, 2008; Derby *et al.*, 2005). It is clear that a spectrum of allergenic potential and potency exists among food proteins. Most cases of food allergy are associated with a relatively limited number of foods and food products; most commonly peanuts, tree nuts, hens' egg, cows' milk, wheat, soybeans, fish and shellfish (Gupta *et al.*, 2011; Sicherer and Sampson, 2009).

Current recommendations for the assessment of the potential allergenicity of novel (GM) food proteins incorporate consideration of the source of the gene, bioinformatics search comparisons for amino acid sequence or structural homology with known allergenic proteins, measurement of the resistance or stability of the protein to digestion by pepsin and, if appropriate (source of the transgene is allergenic/homology with known

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allergen is confirmed), *in vitro* IgE binding tests (Goodman and Tetteh, 2011). The inclusion of pepsin digestibility results from the observation that there is an association between stability to pepsin and allergenic potential, with many confirmed human allergens being stable, whereas common plant proteins that apparently lack allergenic potential are digested very rapidly (Astwood *et al.*, 1996). However, this relationship is not absolute, with reports of both pepsin resistant non-allergens (Thomas *et al.*, 2004) and pepsin sensitive allergens (Fu *et al.*, 2002; Herman *et al.*, 2007).

The various investigations described above provide useful information as part of the overall safety assessment of novel food proteins, and are particularly appropriate for identifying whether there is sequence homology (and potential cross-reactivity) or other shared biophysical features with known human allergens (Sharma *et al.*, 2011). However, these tests do not individually or collectively provide a direct evaluation of inherent sensitizing potential and are therefore not able to identify *de novo* protein allergens that do not have homology with known allergens and to which there has been no previous exposure. This is of particular importance when considering appropriate strategies for evaluating the safety and allergenic potential of proteins from unusual sources. Among such are, for example, thermoresistant proteins from thermophilic microorganisms and the ice structuring protein originating from an arctic fish for activity at low temperatures (Bindslev-Jensen *et al.*, 2003; Bruins *et al.*, 2001). For this reason, there has been interest in the development of more holistic approaches, including the use of appropriate animal models (Dearman and Kimber, 2009; Lehrer and McClain, 2009). The general consensus is that experimental animals may not be able to model accurately all aspects of the likely prevalence, persistence and severity of food allergy among human populations exposed to a novel allergen in the diet, however, they may have value in the identification of allergenic potential.

Such experimental models have utilized a variety of animals [rodents (rats and mice), dogs or pigs], different routes of exposure (oral or systemic exposure) and the presence or absence of adjuvants such as cholera toxin (Dearman and Kimber, 2009; Ladics *et al.*, 2010; Lehrer and McClain, 2009). One experimental system that has shown some promise with respect to differentiating between allergenic proteins and those proteins that apparently lack allergenic potential employs systemic (intraperitoneal) exposure of BALB/c strain mice to the test protein in the absence of adjuvant (Dearman *et al.*, 2003). Protein-specific IgG and IgE antibody production are measured as markers of immunogenicity and allergenicity, respectively. To provide further evidence of the utility of the standardized animal model approach for the identification of potential allergens, it is important to examine a wider range of protein allergens, including important plant allergens.

To this end, the ability of the kiwifruit allergen actinidin (Act d 1), a proteolytic enzyme, (Palacin *et al.*, 2008; Pastorello *et al.*, 1998) to stimulate antibody responses has been compared with bovine haemoglobin, a protein that lacks known allergenic properties. In the past 10 years, kiwifruit has become an important allergenic foodstuff in the UK, coincident with its increased consumption, particularly as a weaning food. From the first case report in 1981 (Fine, 1981), kiwifruit allergy is now recognized as a common allergen that can cause severe life-threatening reactions (Lucas *et al.*, 2004; Palacin *et al.*, 2008). In addition, the resistance to digestion with pepsin has been characterized.

Materials and Methods

Animals

Young adult (8–12 weeks old) female BALB/c strain mice (Harlan Olac, Bicester, UK) were used throughout these studies. Mice were housed on sterilized wood bedding with materials provided for environmental enrichment. Food (Beekay Rat and Mouse Diet No. 1 pellets; B&K Universal, Hull, UK) and water were available *ad libitum*. The ambient temperature was maintained at 21 ± 2 °C and relative humidity was 55 ± 10% with a 12-h light/dark cycle. All procedures were approved by the UK Home Office and carried out in compliance with the Animals (Scientific Procedures) Act, 1986 under a Home Office granted Project Licence.

Test Materials

Commercial preparations of ovalbumin (OVA; Grade V, 98% pure) and bovine haemoglobin (98% pure) were obtained from Sigma Chemical Co., St Louis, MO, USA. Actinidin was a gift from the New Zealand Pharmaceuticals (Central Palmerston North, New Zealand). The protein content of preparations was determined using a modification of the Lowry assay, the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), a colorimetric assay for determination of protein concentration using a standard reference curve of bovine serum albumin (BSA; Sigma). The endotoxin content of protein preparations was measured chromatographically by limulus amebocyte (LAL) assay according to the manufacturer's instructions (Cambrex BioSciences, Wokingham, UK). Aliquots of test proteins were reduced by incubation at 20 mg mL⁻¹ in water with 2.4 M 2-βmercaptoethanol (BME) and 8 M urea (Sigma) for 2 h at 37 °C. Reduced protein solutions were dialysed extensively against 0.05% acetic acid followed by distilled water; native protein solutions were dialysed extensively against water alone.

Analysis of Protein Digestibility

Pepsin digestion assay

The method used was based upon the multi-laboratory study published by Thomas *et al.* (2004). For each protein, a single tube containing 1.52 ml of simulated gastric fluid [SGF; 0.084 N HCl, 35 mM NaCl, pH 1.2, and 4000 units of porcine pepsin (Sigma)] and 0.08 ml of test protein solution (5 mg mL⁻¹) was prepared. The tube contents were mixed by mild vortexing and the tube was immediately placed in a 37 °C water bath. Aliquots of 200 µl were removed after 0, 0.5, 2, 5, 10, 20, 30 and 60 min of incubation and immediately quenched with 70 µl 5× Laemmli buffer [40% glycerol, 5% BME, 10% sodium dodecyl sulphate (SDS), 0.33 M Tris, 0.05% bromophenol blue, pH 6.8] and 70 µl 200 mM NaHCO₃ pH 11 and placed in a heat block at > 75 °C for approximately 10 min. Controls for pepsin auto-digestion (pepsin without test protein) and controls to show test protein stability under test conditions in the absence of pepsin (reaction buffer with test protein but without pepsin) were included. Aliquots of these control samples were taken at 0 and 60 min only. Samples were stored at –20 °C prior to analysis.

SDS-PAGE electrophoresis

Samples from each time point and control reactions were subjected to SDS-PAGE electrophoresis in reducing conditions

using 15-well, 0.75-mm thick, 10% polyacrylamide peptide gels. Resolving gels were made with 5 ml of water, 3 ml of lower gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4% SDS), 4 ml of N,N'-methylenebisacrylamide 37.5:1 (acrylamide; Sigma), 18 µl N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma) and 43.2 µl ammonium persulfate (APS; Sigma, 10% solution in water). Stacking gels were made using 3.25 ml of water, 1.25 ml of upper gel buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS), 0.5 ml of acrylamide, 7 µl TEMED and 15 µl APS. Mark10 standard markers (Bio-Rad) containing 10 proteins ranging in size from 10 to 250 kDa were included on all gels. A tricine buffer (100 mM Tris, 100 mM Tricine, 0.1% SDS pH8.2; Bio-Rad) system was used for resolving small polypeptides. Samples were loaded at 10 µl per well and markers at 5 µl per well and run at 125 Volts.

Gel staining

Gels were fixed for less than 5 min in 5% trichloroacetic acid, followed by washing in 45.5% methanol/9% glacial acetic acid for 1 h. Gels were stained in Coomassie biosafe brilliant blue solution (Biorad, G-250) for 10 min and destained in water.

Gel analysis

Gels were scanned using a Li-cor gel scanner (Li-cor Biotechnology, Lincoln, NE USA) and densitometric analysis carried out using Odyssey[®] software (Li-cor). Integrated intensity (intensity and area of band after subtraction of background values) was calculated to facilitate between gel comparisons. The percentage change in band density compared with the time zero value was calculated.

Sensitization procedure

Groups of mice ($n = 5$) received 0.25 ml of various concentrations (w/v) of protein in phosphate-buffered saline (PBS) by intraperitoneal (i.p.) injection. Seven days later the treatment was repeated. Mice were exsanguinated 14 days after the initiation of exposure. Individual and pooled serum samples were prepared and stored at -20°C until analysis.

Antibody Analyses

Anti-Protein IgG and IgG subclass antibody analysis

Protein-specific IgG, IgG1 and IgG2a antibodies were detected using an enzyme-linked immunosorbent assay (ELISA). Plastic microtitre plates (Nunc, Copenhagen, Denmark) were coated with $100\text{ }\mu\text{g ml}^{-1}$ of protein in PBS overnight at 4°C . The plates were blocked with 2% BSA in PBS at 37°C for 30 min. Serial doubling dilutions of pooled or individual mouse serum samples in 1% BSA in PBS were added and plates incubated for 3 h at 4°C . Plates were incubated for 2 h at 4°C with horseradish peroxidase labelled sheep anti-mouse IgG (1 in 4000), rat anti-mouse IgG1 (1 in 1000) or rat anti-mouse IgG2a (1 in 2000) antibody; all antibodies were from Serotec, Kidlington, UK. Enzyme substrate (*o*-phenylenediamine and urea hydrogen peroxide) was added and the reaction stopped by the addition of 0.5 M citric acid. Substrate conversion at optical density (OD) 450 nm was measured using an automated reader. Titre was determined as the maximum dilution of serum at which an OD450 nm reading of greater than 0.5 was achieved. For negative control serum (untreated mouse sera) readings never exceeded this OD, even at the maximum concentration tested. Data are displayed according to logarithmic transformation as mean and SD $\log_2$ antibody titres.

Measurement of Anti-Protein IgE antibody

The presence in mouse serum of IgE antibodies was detected by homologous passive cutaneous anaphylaxis (PCA) assay. In order to determine the number of IgE responder animals per treatment group, PCA assays were performed with individual serum samples (neat or diluted 1 in 2). Individual serum samples were injected (30 µl) into the dermis of the ears of naïve recipient mice ($n = 2$). Pooled serum samples diluted to various extents with PBS were injected (30 µl) into the dermis of the ears of naïve recipient mice ($n = 3$ or 4). Concurrent negative control serum samples (derived from naïve animals) diluted to an equivalent extent with PBS were incorporated into every PCA assay. Two days later, 0.25 mg of relevant protein, together with Evans Blue dye (1.25 mg), in 0.25 ml of PBS were injected intravenously. Thirty minutes after the challenge, mice were killed and the diameter of cutaneous reactions measured. A positive (IgE) response was recorded if the challenge resulted in a $> 3\text{-mm}$ blue lesion in the skin in the majority of recipient animals (pooled serum samples), with antibody titre recorded as the highest dilution of serum resulting in a positive PCA reaction. For individual serum samples, the mean diameter of the cutaneous reaction in the two recipient animals was recorded, and the serum sample was identified as positive for IgE antibody if the mean diameter of the challenge-induced cutaneous reaction was $> 3\text{ mm}$.

Statistical Analyses

For IgG antibody responses, the reciprocal IgG titres for the individual serum samples were considered by ANOVA after logarithmic transformation, separately for each concentration of protein. Differences between individual group means were compared using Student's *t*-test based on the error mean square of the analysis of variance. The statistical significance of differences in the number of IgE and IgG responder animals was considered by comparison of each pair of groups using Fisher's exact test. The statistical significance of differences in IgE titres between pairs of proteins was considered by comparison of the whole dose response using the Sign test. The statistical significance of effects of pepsin on protein stability was analysed by ANOVA followed by Dunnett's post-hoc test.

Results

The stability of bovine haemoglobin and actinidin to digestion with pepsin was examined. Aliquots of protein were incubated for various periods of time (0 to 60 min) with pepsin, the reaction quenched and the samples analyzed by SDS-PAGE electrophoresis (Fig. 1; representative gels). In each gel, lanes 11 and 12 contain pepsin alone (after 0 and 60 min, respectively) and illustrate that the molecular weight is approximately 35 kDa (as expected) and that there is no autodigestion of pepsin under these conditions. Lanes 1 and 2 contain the protein alone (after 0 and 60 min), with the major band for haemoglobin at approximately 15 kDa as reported previously (Sun and Palmer, 2008) and the major band for actinidin at 25 kDa. There was little effect of incubation of either protein in SGF buffer for 60 min in the absence of pepsin. However, haemoglobin was very unstable in the presence of pepsin, with a marked loss of full-length protein after 0.5-min incubation, whereas the main actinidin band was visible throughout the time course. In

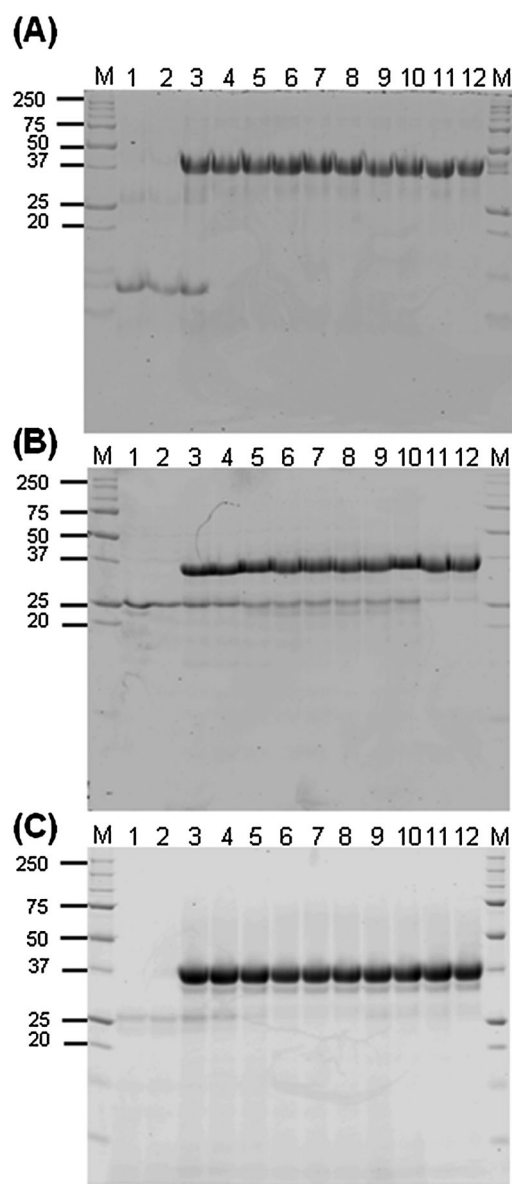


Figure 1. Pepsin digestion of haemoglobin and actinidin using simulated gastric fluid (SGF): representative images. Native haemoglobin (A), native actinidin (B) and reduced actinidin (C) were incubated at 37 °C with SGF for 60 min. At 0.5, 2, 5, 10, 20, 30 and 60 min samples were removed and the reaction quenched. Controls were prepared concurrently and quenched at 0 and 60 min (the positive control contained test protein alone in reaction buffer, to show stability of the test protein in the absence of pepsin; the negative control contained pepsin and no test protein to show autodigestion of pepsin). The samples were analysed using SDS-PAGE (10% Tris-HCl gel) with a tricine buffer system for resolving small peptides. Bands were stained with Coomassie brilliant blue. Representative gels for native haemoglobin (A), native actinidin (B) and reduced actinidin (C) are shown. Molecular weight markers are indicated (M), with molecular weights ranging from 10 to 250 kDa. Lanes 1 and 2 are protein alone incubated for 0 and 60 min, lanes 3–10 are for protein incubated with pepsin for 0, 0.5, 2, 5, 10, 20, 30 and 60 min, lanes 11 and 12 are pepsin alone incubated for 0 and 60 min.

addition to native actinidin, the stability of chemically reduced actinidin to pepsin was examined (Fig. 1C). This material was considerably less stable than the native protein, with reduced band intensity observed after 5 min. The effect of pepsin on

protein stability was quantified using densitometric scanning using a Li-cor scanner (Fig. 2; three independent experiments). Within 0.5 min of incubation with pepsin, the full length haemoglobin band intensity was reduced by 80% ($P < 0.01$), significant fragments were not detected at any time point and there was no detectable full length protein after 10 min. In contrast, native actinidin was considerably more resistant to digestion. Although there was a gradual loss of band intensity with time, with $> 50\%$ loss recorded after 60 min, the reduction did not reach statistical significance and was paralleled by increased expression of fragments with a slightly lower molecular weight (Fig. 1B). After chemical reduction, however, actinidin was more rapidly degraded and to a greater extent, with $< 20\%$ of the protein remaining after 10 min ($P < 0.01$) and no detectable fragments.

After systemic exposure to BALB/c strain mice, the ability of actinidin and bovine haemoglobin to induce immune (IgG) and allergic (IgE) antibody responses was examined. Antibody production was compared with that provoked by OVA, a known human food and inhalant allergen (Escudero *et al.*, 2003; Tay *et al.*, 2007) that is used frequently as a reference allergen in experimental systems (Dearman *et al.*, 2003; Dearman and Kimber, 2007; Untersmayr *et al.*, 2010). Prior to immunization, proteins were tested for their endogenous endotoxin content by LAL assay. It has been reported previously that such levels can vary from 1 to 2000 endotoxin units (EU) mg^{-1} of protein and levels of > 100 EU mg^{-1} of protein may have adjuvant effects on immune responses to proteins (Dearman and Kimber, 2007). Indeed, endotoxin may have a complicated relationship with the induction of antibody responses, with relatively low doses reported to have an IgE-inducing adjuvant effect whereas high doses have an inhibitory effect on IgE (Eisenbarth *et al.*, 2002; Ormstad *et al.*, 2003). The results of the LAL assay demonstrated that all three proteins had acceptable levels of endogenous endotoxin, with bovine haemoglobin expressing the highest levels at 15 EU mg^{-1} and somewhat lower levels of 8 and 10 EU mg^{-1} recorded for OVA and actinidin, respectively. Mice received i.p. injections of 2%, 1% or 0.25% OVA, 0.25%, 0.5% or 1% actinidin or of 1% or 10% haemoglobin on days 0 and 7, and serum was isolated 14 days after the initiation of exposure. Serum samples were analyzed for IgG, IgG1 and IgG2a-specific antibody (Fig. 3) and for specific IgE antibody expression (Fig. 4). As reported previously, treatment with all doses of OVA induced vigorous IgG antibody responses in a dose-dependent manner (Dearman *et al.*, 2003; Herouet-Guicheney *et al.*, 2009) (Fig. 3). Bovine haemoglobin was considerably less immunogenic than OVA, such that treatment with 1% of this protein stimulated only low levels of IgG antibody in 4 out of 5 animals (Fig. 3) and doses of 0.5% and 0.25% failed to provoke any detectable antibody (data not shown). However, i.p. injection of 10% bovine haemoglobin stimulated specific IgG antibody at levels approaching those induced by exposure to 0.25% OVA. All doses of actinidin were moderately immunogenic and stimulated equivalent levels of IgG antibody production to those observed for 10% haemoglobin and 0.25% OVA. The distribution of IgG1 (upregulated with IgE antibody and often used as a surrogate marker for allergic responses) and IgG2a (reciprocal relationship with IgE) subclasses were examined for selected samples (Stevens *et al.*, 1988; Zakroff *et al.*, 1989). In spite of vigorous induction of IgG1 antibody responses, anti-OVA IgG2a responses were less marked, such that the ratio of IgG1 to IgG2a antibody

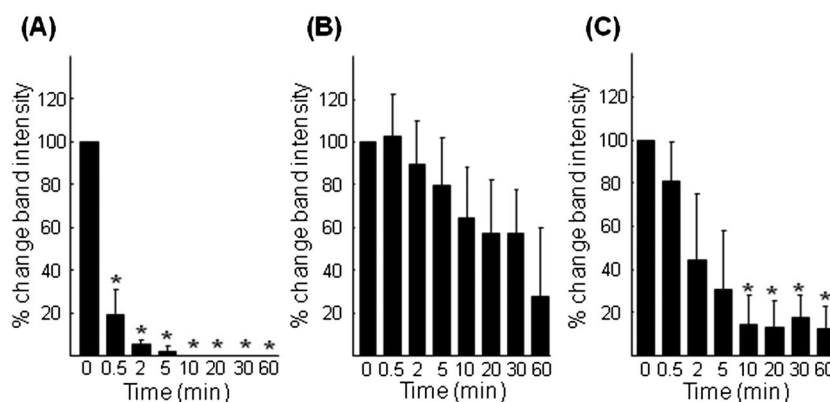


Figure 2. Pepsin digestion of haemoglobin and actinidin using simulated gastric fluid (SGF): densitometric analyses. Native haemoglobin (A), native actinidin (B) and reduced actinidin (C) were incubated at 37 °C with SGF for 0 to 60 min and samples analysed by SDS-PAGE gel electrophoresis as described in Fig. 1. Bands were quantified using a Li-cor gel scanner and Odyssey software. The integrated intensity values for each band were used to calculate the percentage change in intensity for each of the samples in comparison with the 0 min time point. The mean and SE percentage intensity change for three independent experiments is shown. One-way ANOVA and post-hoc Dunnett's test was conducted in comparison with the zero time point (* = $P < 0.01$).

responses was approximately 3.5:1 (Fig. 3). Interestingly, the subclass distribution of serum samples from actinidin-treated animals was less skewed, with a comparatively more vigorous IgG2a antibody response recorded such that the IgG1:IgG2a ratio was 1.6:1.

Exposure to OVA provoked marked IgE responses, with titres of 1 in 32, 1 in 8 and 1 in 4 recorded across the dose range, again similar to those observed previously (Dearman *et al.*, 2003; Herouet-Guicheney *et al.*, 2009). All five mice in the 1% OVA dose groups were also identified as IgE responders. However, neither dose of bovine haemoglobin stimulated detectable IgE antibody production, with a titre of zero recorded for pooled serum and with 0/5 IgE responders in both groups. In contrast, actinidin produced an allergenic response, with relatively high titre IgE antibody levels induced across the dose range (titres of 1 in 8 to 1 in 64 recorded). Furthermore, at the lowest sensitization dose of actinidin (0.25%), all individual serum samples tested positive for IgE, demonstrating that all mice were IgE responders.

Discussion

The stability of actinidin to digestion by pepsin and its ability to provoke IgG and IgE antibody responses after systemic exposure to BALB/c strain mice have been compared with a reference food allergen OVA, and with a control (non-sensitizing) protein bovine haemoglobin. Actinidin is a highly abundant protein in Green kiwifruit (*Actinidia deliciosa*), comprising up to 50% of soluble protein at harvest, and belongs to the family of thiol/sulphydryl proteases that includes the well-characterized allergens bromelain and papain (Nieuwenhuizen *et al.*, 2007). In many kiwifruit allergic populations (Austrian, Spanish, Dutch and Swiss, for example), it has been shown to be an important allergen (Bublin *et al.*, 2004, 2008, 2010; Palacin *et al.*, 2008; Pastorello *et al.*, 1998). IgE-binding activity to actinidin was associated with severe (anaphylactic) reactions in some of these patient populations (Palacin *et al.*, 2008). In other countries, however, notably for kiwi-allergic patients from the UK, actinidin was found not to be a major allergenic protein (Lucas *et al.*, 2007). The reasons for these geographical variations are unclear; it has been suggested that patterns of consumption may be

different (Lucas *et al.*, 2007), that different cultivars of kiwifruit contain different amounts of actinidin (Bublin *et al.*, 2004; Ciardiello *et al.*, 2009; Nieuwenhuizen *et al.*, 2007) and that the time of harvest impacts on the level of expression (Gavrovic-Jankulovic *et al.*, 2005). Up to 11 allergenic proteins have been identified to date in kiwifruit; however, a recent review concluded that actinidin was the most reliable allergenic marker for diagnostic purposes in most patient groups (Ballmer-Weber and Hoffmann-Sommergruber, 2011). Thus, in spite of some uncertainty about whether actinidin is always the major kiwifruit allergen, there is no doubt that it is an important food allergen and that any characterization strategies for allergens should be able to identify this protein as positive.

The pepsin digestibility assay revealed that the control protein bovine haemoglobin was very rapidly digested, which is consistent with its known lack of allergenicity and with published data in which this protein has been utilized as a model substrate for pepsin (Samloff and O'Dell, 1985). There is some controversy regarding the digestibility by pepsin of kiwifruit allergens, including actinidin. In addition, considerable variation in the molecular weight of the material has been observed. In the investigations described here, the major band for actinidin was recorded at 25 kDa; in other previous studies the major band has been reported variously as 25 kDa (Lucas *et al.*, 2007), 27 kDa (Bublin *et al.*, 2004) and 30 kDa (Pastorello *et al.*, 1998). This may be as a result of different preparation techniques or isolation of different isoforms and from different cultivars. In addition, in common with other proteolytic enzymes, actinidin is secreted in an inactive form as actinidin, which has a molecular weight of 39 kDa. It has been suggested that actinidin might only display allergenic potential after the pro-sequence is cleaved (Pastorello *et al.*, 1998). The material used herein is used commercially as a high-quality meat tenderizer, so it is produced in its proteolytically active form. In fact, the proteolytic activity of actinidin is believed to have played a major role in early difficulties with the identification of kiwi-allergic individuals employing conventional skin prick tests and the need, therefore, for fresh fruit prick-to-prick tests (Lucas *et al.*, 2003). There are several reports that demonstrate that actinidin is very rapidly digested by pepsin (within 0.5 min) (Lucas *et al.*, 2008; Fu *et al.*, 2002; Yagami *et al.*, 2000). Two of those studies utilized kiwifruit

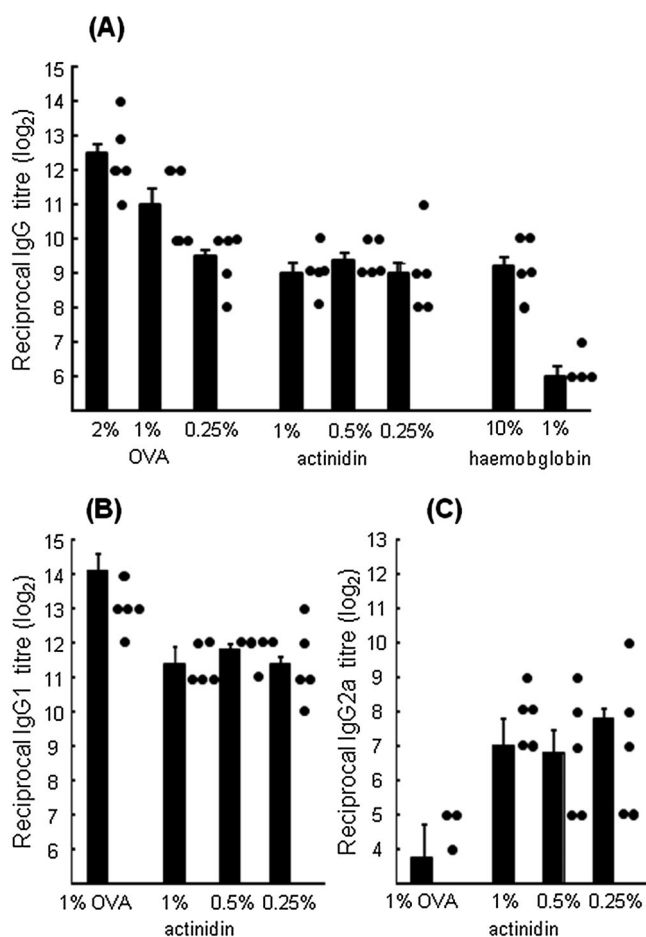


Figure 3. IgG subclass antibody responses induced by actinidin, haemoglobin and ovalbumin. Animals ($n=5$) received 250 μ l of 2%, 1% or 0.25% OVA, 1%, 0.5% or 0.25% actinidin or 10% or 1% haemoglobin in PBS by intraperitoneal (i.p.) injection on days 0 and 7. Seven days later animals were exsanguinated, and individual serum samples tested for the presence of specific IgG (A), IgG1 (B) or IgG2a (C) antibody by ELISA. Negative control serum samples (from naive animals) were tested concurrently and were routinely negative (data not shown). Data are expressed as individual responders ($\bullet$) and as mean (and SEM) IgG reciprocal titre ($\log_2$). There were no significant differences in responder frequencies (Fisher's exact test). IgG titre induced by 1% haemoglobin was significantly lower ($P < 0.01$) than all other groups; IgG titre induced by 2% OVA was significantly higher ($P < 0.01$) than all other groups (Student's t -test).

extracts, whereas in the third study (Fu *et al.*, 2002), the purified protein was examined. It has been reported that actinidin stability is very dependent upon the pH of the reaction, with resistance to pepsin digestion recorded when pH of 2.5 and above were used (Lucas *et al.*, 2008), rather than pH 1.2, the recommended pH for the assay for regulatory purposes (Thomas *et al.*, 2004). The pepsin–protein ratio will also play a role in the extent of digestion. In the study in which purified actinidin was digested rapidly by pepsin, a ratio of 13:1 was used (Fu *et al.*, 2002). In a subsequent study in which purified actinidin was found to be relatively stable to pepsin, SGF conditions of pH 2.0 and a pepsin–protein ratio of 1:20, said to be more physiologically relevant, were used (Bublin *et al.*, 2008). In the present study (which utilized standard conditions of pH 1.2 and 3:1 pepsin:protein), native actinidin was resistant to digestion with pepsin. It has been shown that actinidin exists as

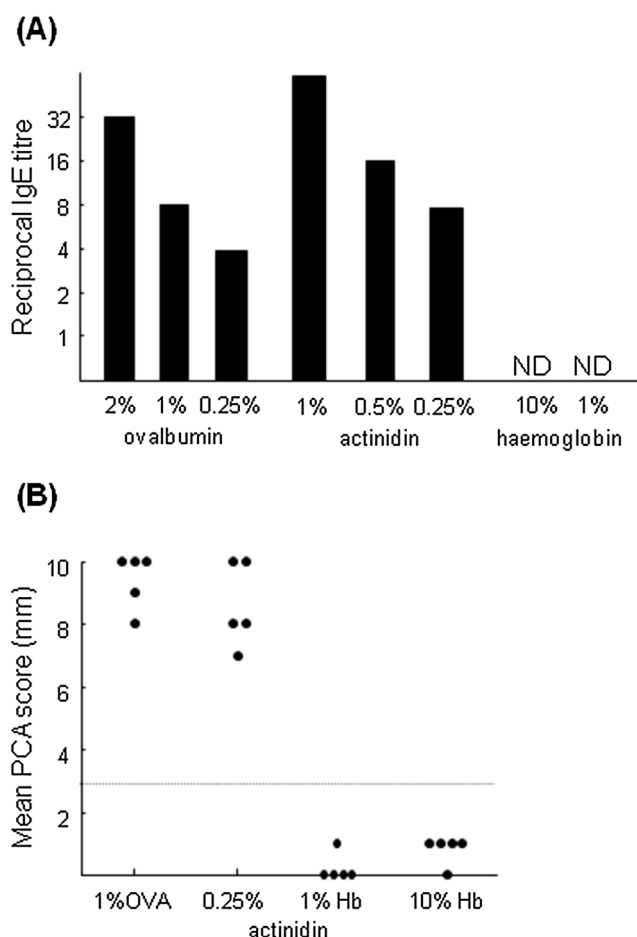


Figure 4. IgE antibody responses induced by actinidin, haemoglobin and ovalbumin. Animals ($n=5$) received 250 μ l of 2%, 1% or 0.25% OVA, 1%, 0.5% or 0.25% actinidin or 10% or 1% haemoglobin in phosphate-buffered saline (PBS) by intraperitoneal (i.p.) injection on days 0 and 7. Seven days later animals were exsanguinated, and individual and pooled serum samples tested for the presence of specific IgE antibody by homologous passive cutaneous anaphylaxis (PCA) assay. (A) Pooled serum samples were serially diluted to determine IgE titre on a treatment group basis. Data are displayed as mean and SE of the PCA reaction ($n=3$ to 4) for each dilution tested. ND=no detectable IgE with neat serum. OVA and actinidin-induced significantly higher IgE titres than did haemoglobin ($P < 0.01$) (Sign test). (B) Individual serum samples were tested (diluted 1 in 2) to determine the number of IgE responder animals; data are displayed as mean diameter of PCA reaction for each serum sample (tested in $n=2$ animals). Negative control serum samples (from naive animals) were tested concurrently. The horizontal line denotes the cut off for a positive PCA reaction; 3 mm. OVA and actinidin induced significantly higher responder frequencies than did haemoglobin ($P < 0.01$) (Fisher's exact test).

at least 10 different isoforms with the same molecular weight and cysteine protease activity but which vary in isoelectric point from acidic (pI 3.9) to basic (pI 9.3) (Nieuwenhuizen *et al.*, 2007). These isoforms may have differential allergenicity and it is also possible that they exhibit differential sensitivity to pepsin digestion.

Concerns have also been raised that the standard method of protein staining after electrophoretic separation (by Coomassie or other stains) may not be sufficiently sensitive. Several previous studies have demonstrated that immunoblotting of digested protein with either sera from patients or with

polyclonal antibodies raised in experimental animals detected fragments that were not stained by Coomassie (Guimaraes *et al.*, 2010; Lucas *et al.*, 2008). Regardless of the debate regarding the optimal conditions for analysis, it is of interest to consider the potential physiological relevance of the stability of proteins for the development of allergic responses. It has been suggested that the relationship simply reflects the fact that pepsin-resistant proteins will survive in the gut environment for a sufficient time to interact with the immune system and to generate an immune response. However, it has been shown that even those proteins that are digested very rapidly (within 1 min) by pepsin are still able to induce immune responses after oral exposure (Dearman *et al.*, 2002). This leads to the hypothesis that resistance to intracellular digestion, particularly within antigen-presenting cells (APC) of the immune system, may be more important. In order to generate an immune response, exogenous protein is broken down into peptides within endosomal and lysosomal compartments of APC and the peptides displayed on the cell surface. A complex series of reactions takes place within the cell, including unfolding (facilitated by low pH), proteolysis and reduction to break down disulphide bonds that appears to be critical for unfolding and efficient proteolysis during antigen processing (Li *et al.*, 2005; Maric *et al.*, 2001). In the current studies, the resistance of actinidin to digestion has also been examined after chemical reduction of disulphide bonds. Compared with the native material, the reduced material was significantly more labile to digestion with pepsin, with significant loss of intact protein observed after 10 min. It has been suggested that intact disulphide bonds are important for allergenicity, although in general the readout for immunogenicity has been binding of serum IgE, rather than the induction of IgE antibody responses (del Val *et al.*, 1999).

An alternative method for the assessment for protein allergenicity, and one that has the potential to identify novel allergens, is the measurement of induced IgE antibody responses *in vivo*; here assessed in BALB/c strain mice after systemic (intraperitoneal) exposure. In these studies, this route was used to avoid oral tolerance and down-regulation of immune and allergic responses that is common in rodent models (Dearman and Kimber, 2009). Intraperitoneal exposure, a route used frequently for experimental immunization to proteins, generally provokes vigorous immune responses. However, the method does not incorporate the use of adjuvants such as alum or cholera toxin, used frequently to maximize allergic responses (Lehrer and McClain, 2009). Although co-administration of adjuvant can enhance IgE antibody responses to proteins, and improve the sensitivity of such animal models, there is some concern that this approach will result in loss in selectivity, potentially causing false positive responses. It is important to be able to interpret a negative IgE result with confidence, thus, in the context of the experimental configuration described herein, the failure to generate an IgE antibody response is only interpreted as a lack of potential allergenicity under conditions where the protein induces a concordant and robust IgG antibody response. Thus, the low dose (1%) bovine haemoglobin that fails to stimulate either vigorous IgG or detectable IgE antibody is a potential false negative. However, at considerably higher doses (10%), IgG antibody responses that are equivalent to those provoked by either OVA or actinidin were recorded for bovine haemoglobin in the absence of detectable IgE, supporting a more substantive conclusion of no allergenic potential. That is, the animals have

been exposed to an amount of material sufficient to provoke an immune response, which is consistent with a foreign protein, but is not able to stimulate the quality of immune response necessary for the allergen associated induction of IgE antibody. Complete failure to generate an IgG antibody response may be as a result of a lack of immunogenicity in the mouse strain of choice, the preparation of the protein solution (protein structure, vehicle choice or a combination), or it may relate to prior dietary exposure to similar or cross-reactive proteins (Dearman and Kimber, 2009; Lehrer and McClain, 2009). In the case of bovine haemoglobin, given the degree of homology with murine haemoglobin, it is not surprising that it is relatively lacking in immunogenicity and that high doses are required for IgG responses.

In contrast, exposure to actinidin induced vigorous IgG and IgE antibody responses, even at relatively low doses (0.25%), consistent with allergenic potential. In addition to IgE antibody production, the subclass distribution of the IgG antibody response was investigated. Although IgE antibody is accepted as the major mediator of immediate hypersensitivity allergic reactions in humans, in mice a subclass of IgG (IgG1) can also sensitize mast cells and trigger anaphylaxis (Silva *et al.*, 2008). The IgG1 subclass is subject to similar immune regulation as is IgE and is therefore used as a surrogate marker for IgE antibody responses (Birmingham *et al.*, 2002; Robinson *et al.*, 1996). In contrast, IgG2a antibody production displays a reciprocal relationship with IgE and IgG1 antibody responses (Stevens *et al.*, 1988; Zakroff *et al.*, 1989). In the experiments reported herein, both allergens (OVA and actinidin) provoked vigorous IgG1 antibody responses, but also the expression of the IgG2a antibody, albeit at lower levels, which might be considered as a negative predictor of allergenicity. In many experimental models of allergenicity it is reported that proteins with known allergenic potential stimulate skewed responses with respect to IgE and IgG subclass distribution. For example, in a recent study with a panel of 29 food, pollen, latex, dander and mould allergens, immunization of BALB/c strain mice resulted in each case in high IgG1 levels and a complete absence of IgG2a (Roesler *et al.*, 2009). However, in that study the immunization protocol utilized the adjuvant alum, which is known to promote IgE and IgG1 antibody responses. Systemic (intraperitoneal) administration of allergens such as latex in the absence of alum has been shown to elicit a more mixed pattern of antibody responses, with both IgG1 and IgG2a subclasses, in addition to IgE (Kurup *et al.*, 1994). Furthermore, it is clear that only a fraction of IgG1 antibodies exhibit mast cell binding and anaphylactic properties, with this property being dependent on the glycosylation status of the molecule (Silva *et al.*, 2008). Therefore, although the distribution of IgG1 and IgG2a antibodies is of mechanistic interest, it is probably prudent to utilize IgE antibody as the primary marker of allergenicity and to examine total IgG antibody production as a marker of immunogenicity and exposure.

In conclusion, there is potential benefit in the development of more holistic approaches to be used in tandem with the assessment of pepsin stability in order to facilitate the safety assessment of proteins. The stability of proteins to pepsin is part of the current weight of evidence approach to protein allergenicity testing but there are questions regarding the most appropriate assay conditions. The absolute relationship between pepsin stability and *in vivo* response parameters has also been difficult to determine. The measurement of IgE-inducing

properties in BALB/c strain mice offers promise for a model that could be used in tandem with assessment of pepsin stability that can distinguish a number of allergens (including the important kiwifruit allergen actinidin) from non-allergens such as haemoglobin.

Acknowledgments

E.S. Foster's PhD studentship was supported fully by Syngenta Crop Protection.

References

- Aaronov D, Tasher D, Levine A, Somekh E, Serour F, Dalal I. 2008. Natural history of food allergy in infants and children in Israel. *Ann. Allergy Asthma Immunol.* **101**: 637–640. DOI: 10.1016/S1081-1206(10)60228-1.
- Astwood JD, Leach JN, Fuchs RL. 1996. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* **14**: 1269–1273.
- Ballmer-Weber BK, Hoffmann-Sommergruber K. 2011. Molecular diagnosis of fruit and vegetable allergy. *Curr. Opin. Allergy Clin. Immunol.* **11**: 229–235. DOI: 10.1097/ACI.0b013e3283464c74.
- Bindslev-Jensen C, Sten E, Earl LK, Crevel RW, Bindslev-Jensen U, Hansen TK, Stahl Skov P, Poulsen LK. 2003. Assessment of the potential allergenicity of ice structuring protein type III HPLC 12 using the FAO/WHO 2001 decision tree for novel foods. *Food Chem. Toxicol.* **41**: 81–87.
- Birmingham N, Thanosvorakul S, Gangur V. 2002. Relative immunogenicity of commonly allergenic foods versus rarely allergenic and nonallergenic foods in mice. *J. Food Prot.* **65**: 1988–1991.
- Bublin M, Mari A, Ebner C, Knulst A, Scheiner O, Hoffmann-Sommergruber K, Breiteneder H, Radauer C. 2004. IgE sensitization profiles toward green and gold kiwifruits differ among patients allergic to kiwifruit from 3 European countries. *J. Allergy Clin. Immunol.* **114**: 1169–1175.
- Bublin M, Pfister M, Radauer C, Oberhuber C, Bulley S, Dewitt AM, Lidholm J, Reese G, Vieths S, Breiteneder H, Hoffmann-Sommergruber K, Ballmer-Weber BK. 2010. Component-resolved diagnosis of kiwifruit allergy with purified natural and recombinant kiwifruit allergens. *J. Allergy Clin. Immunol.* **125**: 687–694. DOI:10.1016/j.jaci.
- Bublin M, Radauer C, Knulst A, Wagner S, Scheiner O, Mackie AR, Mills EN, Breiteneder H. 2008. Effects of gastrointestinal digestion and heating on the allergenicity of the kiwi allergens Act d 1, actinidin, and Act d 2, a thaumatin-like protein. *Mol. Nutr. Food Res.* **52**: 1130–1139. DOI: 10.1002/mnfr.200700167.
- Bruins ME, Janssen AE, Boom RM. 2001. Thermozyms and their applications: a review of recent literature and patents. *Appl. Biochem. Biotechnol.* **90**: 155–186.
- Ciardello MA, Giangrieco I, Tuppo L, Tamburrini M, Buccheri M, Palazzo P, Bernardi ML, Ferrara R, Mari A. 2009. Influence of the natural ripening stage, cold storage, and ethylene treatment on the protein and IgE-binding profiles of green and gold kiwi fruit extracts. *J. Agric. Food Chem.* **57**: 1565–1571. DOI: 10.1021/jf802966n.
- Dearman RJ, Caddick H, Stone S, Kenna JG, Basketter DA, Kimber I. 2002. Immunogenic properties of rapidly digested food proteins following gavage exposure of mice: a comparison of ovalbumin with a potato acid phosphatase preparation. *Food Chem. Toxicol.* **40**: 625–633.
- Dearman RJ, Kimber I. 2007. A mouse model for food allergy using intraperitoneal sensitization. *Methods* **41**: 91–98.
- Dearman RJ, Kimber I. 2009. Animal models of protein allergenicity: potential benefits, pitfalls and challenges. *Clin. Exp. Allergy* **39**: 458–468. DOI: 10.1111/j.1365-2222.2008.03194.x.
- Dearman RJ, Stone S, Caddick HT, Basketter DA, Kimber I. 2003. Evaluation of protein allergenic potential in mice: dose-response analyses. *Clin. Exp. Allergy* **33**: 1586–1594.
- del Val G, Yee BC, Lozano RM, Buchanan BB, Ermel RW, Lee YM, Frick OL. 1999. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. *J. Allergy Clin. Immunol.* **103**: 690–697.
- Derby CJ, Gowland MH, Hourihane JO. 2005. Sesame allergy in Britain: a questionnaire survey of members of the Anaphylaxis Campaign. *Pediatr. Allergy Immunol.* **16**: 171–175.
- Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. 2002. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* **196**: 1645–1651.
- Escudero C, Quirce S, Fernández-Nieto M, Miguel J, Cuesta J, Sastre J. 2003. Egg white proteins as inhalant allergens associated with baker's asthma. *Allergy* **58**: 616–620.
- Fine AJ. 1981. Hypersensitivity reaction to kiwi fruit (Chinese gooseberry, *Actinidia chinensis*). *J. Allergy Clin. Immunol.* **68**: 235–237.
- Fu TJ, Abbott UR, Hatzos C. 2002. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid—a comparative study. *J. Agric. Food Chem.* **50**: 7154–7160.
- Goodman RE, Tetteh AO. 2011. Suggested improvements for the allergenicity assessment of genetically modified plants used in foods. *Curr. Allergy Asthma Rep.* **11**: 317–324. DOI: 10.1007/s11882-011-0195-6.
- Gavrovic-Jankulovic M, Polovic N, Prusic S, Jankov RM, Atanaskovic-Markovic M, Vuckovic O, Cirkovic Velickovic T. 2005. Allergenic potency of kiwi fruit during fruit development. *Food Agric. Immunol.* **16**: 117–128.
- Guimaraes V, Drumare MF, Lereclus D, Gohar M, Lamourette P, Nevers MC, Vaisanen-Tunkelrott ML, Bernard H, Guillon B, Crémion C, Wal JM, Adel-Patient K. 2010. In vitro digestion of Cry1Ab proteins and analysis of the impact on their immunoreactivity. *J. Agric. Food Chem.* **58**: 3222–3231. DOI: 10.1021/jf903189j.
- Gupta RS, Springston EE, Warriar MR, Smith B, Kumar R, Pongracic J, Holl JL. 2011. The prevalence, severity, and distribution of childhood food allergy in the United States. *Pediatrics* **128**: e9–e17. DOI: 10.1542/peds.2011-0204.
- Herman RA, Woolhiser MM, Ladics GS, Korjagin VA, Schafer BW, Storer NP, Green SB, Kan L. 2007. Stability of a set of allergens and non-allergens in simulated gastric fluid. *Int. J. Food Sci. Nutr.* **58**: 125–141.
- Herouet-Guicheney C, Aldemir H, Bars R, de Barbeyrac D, Kennel P, Rouquié D, Stahl BU, Kimber I, Dearman RJ. 2009. Inter-laboratory comparisons of assessment of the allergenic potential of proteins in mice. *J. Appl. Toxicol.* **29**: 141–148. DOI:10.1002/jat.1391.
- Kurup VP, Kumar A, Choi H, Murali PS, Resnick A, Kelly KJ, Fink JN. 1994. Latex antigens induce IgE and eosinophils in mice. *Int. Arch. Allergy Immunol.* **103**: 370–377.
- Lack G, Chapman M, Kalsheker N, King V, Robinson C, Venables K, BSACI working party. 2002. Report on the potential allergenicity of genetically modified organisms and their products. *Clin. Exp. Allergy* **32**: 1131–1143.
- Ladics GS, Knippels LMJ, Penninks AH, Bannon GA, Goodman RE, Herouet-Guicheney C. 2010. Review of animal models designed to predict the potential allergenicity of novel proteins in genetically modified crops. *Regul. Toxicol. Pharmacol.* **56**: 212–224. DOI: 10.1016/j.yrtph.2009.09.018.
- Li P, Gregg JL, Wang N, Zhou D, O'Donnell P, Blum JS, Crotzer VL. 2005. Compartmentalization of class II antigen presentation: contribution of cytoplasmic and endosomal processing. *Immunol. Rev.* **207**: 206–217.
- Lehrer SB, McClain S. 2009. Utility of animal models for predicting human allergenicity. *Regul. Toxicol. Pharmacol.* **54**: S46–S51. DOI: 10.1016/j.yrtph.2009.01.001.
- Lucas JS, Cochrane SA, Warner JO, Hourihane JO. 2008. The effect of digestion and pH on the allergenicity of kiwifruit proteins. *Pediatr. Allergy Immunol.* **19**: 392–398.
- Lucas JS, Grimshaw KE, Collins K, Warner JO, Hourihane JO. 2004. Kiwi fruit is a significant allergen and is associated with differing patterns of reactivity in children and adults. *Clin. Exp. Allergy* **34**: 1115–1121.
- Lucas JS, Lewis SA, Hourihane JO. 2003. Kiwi fruit allergy: a review. *Pediatr. Allergy Immunol.* **14**: 420–428.
- Lucas JS, Nieuwenhuizen NJ, Atkinson RG, Macrae EA, Cochrane SA, Warner JO, Hourihane JO. 2007. Kiwifruit allergy: actinidin is not a major allergen in the United Kingdom. *Clin. Exp. Allergy* **37**: 1340–1348.
- Maric M, Arunachalam B, Phan UT, Dong C, Garrett WS, Cannon KS, Alfonso C, Karlsson L, Flavell RA, Cresswell P. 2001. Defective antigen processing in GILT-free mice. *Science* **294**: 1361–1365.
- Nieuwenhuizen NJ, Beuning LL, Sutherland PW, Sharma NN, Cooney JM, Bielecki LRF, Schröder R, MacRae EA, Atkinson RG. 2007. Identification and characterisation of acidic and novel basic forms of actinidin, the highly abundant cysteine protease from kiwifruit. *Funct. Plant Biol.* **34**: 946–961.

- Ormstad H, Groeng EC, Duffort O, Løvik M. 2003. The effect of endotoxin on the production of IgE, IgG1 and IgG2a antibodies against the cat allergen Fel d 1 in mice. *Toxicology* **188**: 309–318.
- Palacin A, Rodriguez J, Blanco C, Lopez-Torrejon G, Sánchez-Monge R, Varela J, Jiménez MA, Cumplido J, Carrillo T, Crespo JF, Salcedo G. 2008. Immunoglobulin E recognition patterns to purified Kiwifruit (*Actinidia deliciosa*) allergens in patients sensitized to Kiwi with different clinical symptoms. *Clin. Exp. Allergy* **38**: 1220–1228. DOI: 10.1111/j.1365-2222.2007.02927.x.
- Pastorello EA, Conti A, Pravettoni V, Farioli L, Rivolta F, Ansaloni R, Ispano M, Incorvaia C, Giuffrida MG, Ortolani C. 1998. Identification of actinidin as the major allergen of kiwi fruit. *J. Allergy Clin. Immunol.* **101**: 531–537.
- Prescott S, Allen KJ. 2011. Food allergy: riding the second wave of the allergy epidemic. *Pediatr. Allergy Immunol.* **22**: 155–160. DOI: 10.1111/j.1399-3038.2011.01145.x.
- Robinson MK, Babcock LS, Horn PA, Kawabata TT. 1996. Specific antibody responses to subtilisin Carlsberg (Alcalase) in mice: development of an intranasal exposure model. *Fundam. Appl. Toxicol.* **34**: 15–24.
- Roesler E, Weiss R, Weinberger EE, Fruehwirth A, Stoecklinger A, Mostböck S, Ferreira F, Thalhamer J, Scheiblhofer S. 2009. Immunize and disappear-safety-optimized mRNA vaccination with a panel of 29 allergens. *J. Allergy Clin. Immunol.* **124**: 1070–1077. DOI: 10.1016/j.jaci.2009.06.036.
- Samloff IM, O'Dell C. 1985. Inhibition of peptic activity by sucralfate. *Am. J. Med.* **79**: 15–18.
- Selgrade MK, Bowman CC, Ladics GS, Privalle L, Laessig SA. 2009. Safety assessment of biotechnology products for potential risk of food allergy: implications of new research. *Toxicol. Sci.* **110**: 31–39. DOI: 10.1093/toxsci/kfp075.
- Sharma P, Singh AK, Singh BP, Gaur SN, Arora N. 2011. Allergenicity assessment of osmotin, a pathogenesis-related protein, used for transgenic crops. *J. Agric. Food Chem.* **59**: 9990–9995. DOI: 10.1021/jf202265d.
- Sicherer SH, Sampson HA. 2009. Food allergy: recent advances in pathophysiology and treatment. *Annu. Rev. Med.* **60**: 261–277. DOI: 10.1146/annurev.med.60.042407.205711.
- Silva SR, Casabuono A, Jacysyn JF, Favoretto BC, Fernandes I, Macedo MS, Couto AS, Faquim-Mauro EL. 2008. Sialic acid residues are essential for the anaphylactic activity of murine IgG1 antibodies. *J. Immunol.* **181**: 8308–8314.
- Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, Vitetta ES. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* **334**: 255–258.
- Sun G, Palmer AF. 2008. Preparation of ultrapure bovine and human hemoglobin by anion exchange chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **867**: 1–7. DOI: 10.1016/j.jchromb.2008.02.014.
- Tay SS, Clark AT, Deighton J, King Y, Ewan PW. 2007. Patterns of immunoglobulin G responses to egg and peanut allergens are distinct: ovalbumin-specific immunoglobulin responses are ubiquitous, but peanut-specific immunoglobulin responses are up-regulated in peanut allergy. *Clin. Exp. Allergy* **37**: 1512–1518.
- Thomas K, Aalbers M, Bannon GA, Bartels M, Dearman RJ, Esdaile DJ, Fu TJ, Glatt CM, Hadfield N, Hatzos C, Hefle SL, Heylings JR, Goodman RE, Henry B, Herouet C, Holsapple M, Ladics GS, Landry TD, MacIntosh SC, Rice EA, Privalle LS, Steiner HY, Teshima R, Van Ree R, Woolhiser M, Zawodny J. 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regul. Toxicol. Pharmacol.* **39**: 87–98.
- Untersmayr E, Diesner SC, Oostingh GJ, Selzle K, Pfaller T, Schultz C, Zhang Y, Krishnamurthy D, Starkl P, Knittelfelder R, Förster-Waldl E, Pollak A, Scheiner O, Pöschl U, Jensen-Jarolim E, Duschl A. 2010. Nitration of the egg-allergen ovalbumin enhances protein allergenicity but reduces the risk for oral sensitization in a murine model of food allergy. *PLoS One* **5**: e14210. DOI: 10.1371/journal.pone.0014210.
- Yagami T, Haishima Y, Nakamura A, Osuna H, Ikezawa Z. 2000. Digestibility of allergens extracted from natural rubber latex and vegetable foods. *J. Allergy Clin. Immunol.* **106**: 752–762.
- Zakroff SG, Beck L, Platzer EG, Spiegelberg HL. 1989. The IgE and IgG subclass responses of mice to four helminth parasites. *Cell. Immunol.* **119**: 193–201.