

The effect of digestion and pH on the allergenicity of kiwifruit proteins

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It is suggested that patients with oral allergy syndrome (OAS) respond to pepsin-sensitive allergens, and systemic reactors identify pepsin-resistant allergens. We sought to assess the digestibility of kiwifruit proteins in simulated gastric fluid (SGF), and to compare the immunogenicity of the digests in patients with isolated oral and systemic reactions to kiwifruit. In addition, the effect of pH on digestibility of kiwifruit proteins was investigated. The *in vitro* resistance of kiwifruit proteins to digestion was determined using SGF. G-immunoglobulin (IgE) binding to digested proteins was investigated by Western blotting using sera from children and adults (aged 5–72 yr) with systemic reactions and patients with isolated oral symptoms. To determine whether pH conditions influence digestion of kiwifruit extracts, digestion at pHs 1.5–7 were compared by SDS-PAGE. Patients with systemic reactions showed IgE binding to digestion-resistant allergens, but patients with oral symptoms reacted only to digestion-labile allergens. An increase in pH from 1.5 to 2.5 significantly reduced pepsin breakdown of kiwifruit allergens. Immunoreactive digested protein fragments were detectable by immunoblot but not Coomassie stain. This study confirms a difference in the lability of food allergens recognized by patients with systemic reactions and those with OAS. Pepsin digestion of kiwifruit proteins was impaired by hypoacidic conditions suggesting that patients with hypoacidic gastric conditions are at increased risk of systemic absorption of allergens. The data indicate that commonly used methods for predicting allergenicity of novel proteins using Coomassie stains may be flawed.

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Kiwifruit is now one of the most common causes of food allergy (1, 2). Although patients often present with mild oral allergy syndrome (OAS), severe systemic reactions are not uncommon, particularly in children (3).

Foods contain a wide variety of proteins, yet only a few are allergens. The reason why some proteins are highly allergenic and others are not remains poorly understood, but certain chemico-physical properties appear to be associated with allergenicity. Most allergens are said to have a molecular weight of 10–70 kDa and are typically stable to changes in heat and pH, and to digestion. They generally have an acid isoelectric

point (pI) and are soluble for absorption across the gastrointestinal tract. However, many non-allergenic proteins also show these properties. Moreover, many allergens do not have these properties, e.g., profilins are not stable to digestion and lipid transfer proteins do not have an acid pI.

Several studies have suggested that food allergens are resistant to pepsin digestion, whereas non-allergenic proteins are more likely to be rapidly and completely digested (4, 5). However, a number of studies have shown some food allergens to be rapidly digested (6–8). Furthermore, analysis of the avocado allergen (Prs a 1),

shows it to be extensively digested, but the resulting peptides remain reactive *in vivo* and *in vitro* (9). It is generally believed that patients with OAS react to labile allergens, whilst those with systemic reactions react to pepsin-resistant proteins.

With the introduction of novel and genetically modified foods, it has become necessary to devise methods to identify potential allergens and various assessment strategies have been developed. The FAO/WHO decision pathway (10, 11) for pre-marketing assessment of the allergenicity of GM foods focuses on the source of the gene, the sequence homology of the newly introduced protein to known allergens, the immunochemical binding of the newly introduced protein with IgE from the serum of individuals with known allergies to the transferred genetic material, and the physicochemical properties of the newly introduced protein including digestion. Thus, evaluation of protein digestibility using pepsin is currently a factor in predicting the allergenic potential of a novel GM protein. More recent guidelines for the risk assessment of GM foods in Europe recognize that there is no absolute correlation between pepsin digestion and allergenicity but suggest that rapid and extensive degradation may be helpful in determining potential allergenicity (12).

A study by Yagami showed that all allergens in kiwifruit were readily digested by simulated gastric fluid (SGF) when assessed by means of SDS-PAGE and Western blotting (8) using sera from patients with latex allergy, some of whom had fruit allergy. Other studies have confirmed that kiwi proteins are heat-labile (13, 14). Until now, no study has used sera from patients recruited on the basis of having kiwifruit allergy to assess the effects of digestion on the allergenicity of kiwifruit, and no study has directly compared IgE reactivity of patients with systemic and OAS symptoms to any digested food.

Recent studies have highlighted that allergen digestibility is influenced by the pH of gastric juices (15), a hypoacidic environment potentially increasing the absorption of undigested proteins and the risk of sensitization (16). We also examined the effect of pH in SGF on digestion of kiwifruit proteins.

The aim of this study was to assess the digestibility of kiwifruit proteins to SGF, and to assess the immunogenicity of the digested products by Western blotting. In particular, it was designed to compare the digestibility of allergens recognized by patients with systemic and those with isolated oral allergy.

Methods

The Southampton and South West Hants Joint Research Ethics Committee approved the study (LREC number 045/00 and 055/01).

Subjects and clinical investigations

Patients were part of a larger study examining the clinical characteristics of kiwifruit allergy (3), and included adults and children (age range 5–72 yr). All patients had a history strongly supportive of IgE mediated allergy to kiwifruit allergy. All patients had IgE binding to kiwifruit proteins confirmed by Western blotting. FEIA-CAP was not used in this study to determine specific IgE to kiwifruit as we have previously found it to have poor sensitivity in patients with positive food challenges (3), and to correlate poorly with IgE binding on Western blots. Patients were excluded from the study if they had a negative prick-to-prick skin test using fresh fruit (3). Classification of patients into two groups, OAS or systemic reactors, was on the basis of self reported symptoms by structured questionnaire. About 12 patients were assigned to the OAS group on the basis that they had had at least three allergic reactions to kiwifruit during which they had pruritus, urticaria or swelling confined to the oral mucosa. They were excluded from this group if they had ever had generalized or systemic reactions to kiwifruit. About 12 patients were assigned to the systemic reactors group on the basis of ever having had a previous allergic reaction to kiwifruit involving the respiratory or cardiovascular systems, or if they had had generalized urticaria or angioedema. A pool of sera from five non-atopic volunteers was used as a negative control.

Preparation of kiwifruit extracts

Extracts of kiwifruit protein were made from fresh *Actinidia deliciosa*, Hayward variety, using 'ready-to-eat' fruit purchased from a local supermarket. Fruit was peeled and pureed and some of the seeds were crushed. The pulp was homogenized 1:2 w/v in PBS (pH 7.4), and stored on ice whilst vortexing every 15 min for 2 h. The subsequent homogenate was centrifuged (3500 rpm, 30 min, 4°C), and the supernatant dialysed over 48 h at 4°C against water. The specimen was frozen at –80°C and then lyophilized and the resulting protein powder was frozen at –80°C until required.

Simulated gastric fluid

A ratio of 10 U of pepsin activity/ μ g of test protein was used, based on an evaluation of the average activity of pepsin recommended in the US Pharmacopoeia (2000) and used in previous studies (17). Pepsin A (Sigma, Gillingham, UK) was purchased as lyophilized powder with 2235 U activity/mg solid. 3 M HCL was added to 30 ml of water until the pH reached 1.5. About 61 mg of NaCl was then added to 30 ml of HCL solution (35 mM NaCl). About 28 mg of pepsin A was added to 3.5 ml of solution. The resulting SGF was heated to 37°C in a water bath.

Digestion of extract

About 34 mg of lyophilized kiwifruit extract protein was re-suspended in 1 ml cold (4°C) water and 0.08 ml (2.72 mg protein) was added to 1.56 ml of warmed SGF, vortexed and immediately placed in a 37°C water bath. Samples of 60 μ l were removed at 0.5, 2, 5, 10, 20, 30 and 60 min after initiation of the incubation. Each 60 μ l was immediately quenched by addition of 70 μ l sodium bicarbonate solution (pH 10), 50 μ l lithium dodecylsulphate sample buffer (Invitrogen, Paisley, UK) and 20 μ l reducing agent (Invitrogen). They were then heated to 70°C for 10 min and stored at -20°C. The zero time point protein digestion samples were prepared by quenching the pepsin in the sodium bicarbonate/sample buffer solution before adding the protein extract.

Kiwifruit digestion under different pH conditions

To determine whether pH conditions influence digestion of kiwifruit extract and its immunogenicity, solutions were made as for SGF (35 mM NaCl, 10 U pepsin activity/ μ g test protein), but with pH of 2, 2.5, 3, 5 and 7. The pH was adjusted using 3M HCl.

Controls

Control samples for protein stability (kiwifruit extract in water at 37°C, no SGF), pH stability (kiwifruit extract in SGF without pepsin) and pepsin auto-digestion (pepsin without kiwifruit protein) were treated in exactly the same way.

β -Lactoglobulin (BLG), which is resistant to digestion, was used as a control allergen. A 34 mg/ml solution was made using lyophilized BLG (Sigma), and was treated identically to the kiwifruit extract solution.

SDS-PAGE and Western blots of digested kiwifruit

For SDS-PAGE, 10 μ g kiwifruit protein extract from each time point was subjected to SDS-PAGE under reducing conditions as previously described (18). For BLG, a single protein, 5 μ g was used. Gels were stained with EZblue (Sigma).

For Western blotting, 10 μ g of the digested extracts were separated by SDS-PAGE under reducing conditions and then transferred onto PVDF membranes (0.2 μ m pore; Invitrogen) at 30 V for 1 h using XCell II blotting apparatus (Invitrogen). Membranes were blocked in 3% bovine serum albumin (BSA) in TBS with 0.1% Tween-20 for 1 h at room temperature. IgE binding to kiwifruit proteins was determined by incubating the separated extracts with the two pools of sera. A pool of sera from five non-atopic subjects was used as a control. Sera were diluted 1 in 10, in a diluent of 0.3% BSA in TBS with 0.1% Tween-20, and the strips were incubated at room temperature over-night. Membranes were then incubated in 1:500 biotin-labelled goat anti-human IgE (Vector BA 3040) followed by 1:120,000 ALP-linked extravidin (Sigma) each for 45 min. Following each incubation, membranes were washed twice with TBS containing 0.05% Tween-20 (TBS-T) and twice in TBS alone. IgE binding sites were identified by developing with BCIP/NBT for 15 min.

Results

SDS-PAGE of digested proteins

Visualisation of the SDS gel showed rapid and complete digestion of the kiwifruit proteins by SGF at pH 1.5 (Fig. 1). Original full-length proteins were undetectable 0.5 min after the start of digestion but a digestion fragment, or possible protein aggregate (approx. 70 kDa), was observed up to 2 min. In comparison, the control protein BLG was resistant to digestion and remained visually unchanged after 60 min of digestion (Fig. 1). Kiwifruit extract incubated at 37°C in ultra-pure water showed no significant degradation over 1 h and kiwifruit extract incubated with SGF without pepsin (i.e. pH 1.5, 35 mM NaCl) also showed no significant loss of protein bands over 1 h (data not shown). This suggests that protein breakdown was predominantly due to pepsin.

The pH of the SGF had a significant effect on pepsin activity. Rapid digestion of proteins occurred at pH $\leq$ 2, but by 10 min digestion of extract by SGF with a pH of 2.5 remained

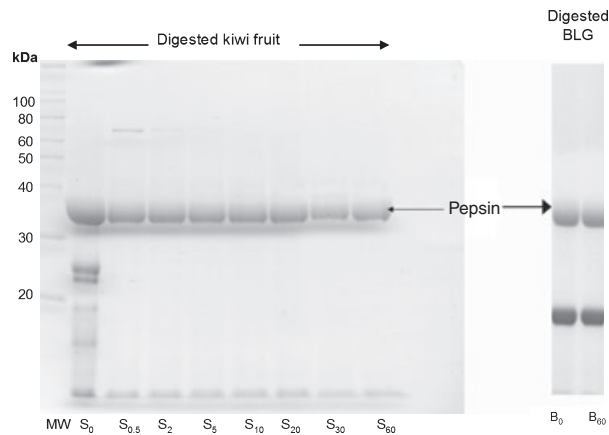


Fig. 1. Kiwi extract digested in SGF (pH 1.5) at different time points 0.5–60 min (S_{0.5}, S₆₀), stained with EZblue (Sigma). S₀ shows kiwi extract added to inactivated pepsin. About 10 µg of extract per lane were run on 12% NuPAGE gels under reduced conditions. B-lactoglobulin in inactivated pepsin (B₀) and after 60 min digestion in SGF pH 1.5 (B₆₀). MW = molecular weight markers (Magic Mark; Invitrogen).

minimal, and even after an hour protein bands were clearly visible in extracts treated with SGF with a pH ≥ 3 (Fig. 2).

Western blotting

Despite numerous optimization studies using a variety of secondary antibodies and blocking conditions, it was not possible to eliminate non-specific binding to kiwifruit proteins with molecular weights of approx. 21 kDa (data not shown). These bands were present when the protein strips were incubated with either controls of no sera or controls of sera from non-atopic patients. Corresponding bands in kiwifruit allergic patients were therefore not considered significant.

Western blotting using pooled sera from patients with symptoms of OAS to kiwifruit showed IgE binding to proteins in untreated kiwifruit extract with molecular weights of 30, 38, 40, 42, 60 and 80 kDa (Fig. 3, lane K). Unfortunately, the molecular weight of pepsin (39 kDa) is similar to some of the allergens in kiwifruit, and IgE binding to allergens of 38, 40 and 42 kDa was ‘blocked’ by this protein as demonstrated in lane S₀ where the extract was added to inactivated pepsin so that digestion has not occurred. After 0.5 min in SGF, the allergens of 30 and 80 kDa had disappeared, and by 10 min it appeared that all allergens had been digested. The extracts treated with water, instead

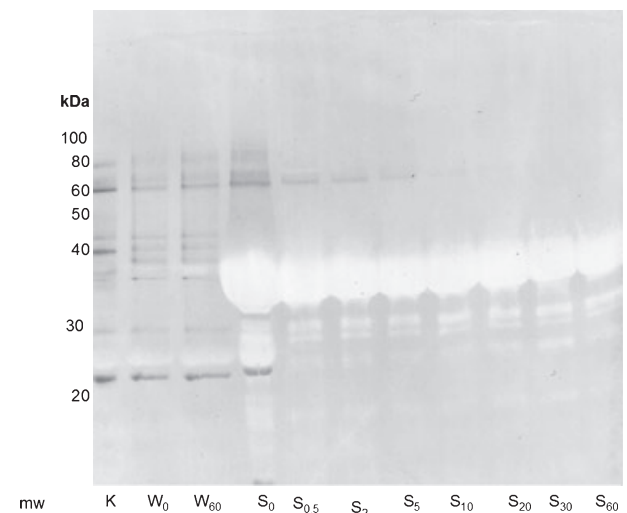


Fig. 3. Western blot using sera from patients with symptoms of OAS. Kiwi fruit extract was digested using SGF with pH 1.5 at times ranging from 0.5 min (S_{0.5}) to 1 h (S₆₀). Lane S₀ has kiwi extract added to inactivated pepsin. Lane K: kiwi extract with no incubation in SGF or control fluid. Lanes W₀ and W₆₀: the extract was incubated with water instead of SGF as a control.

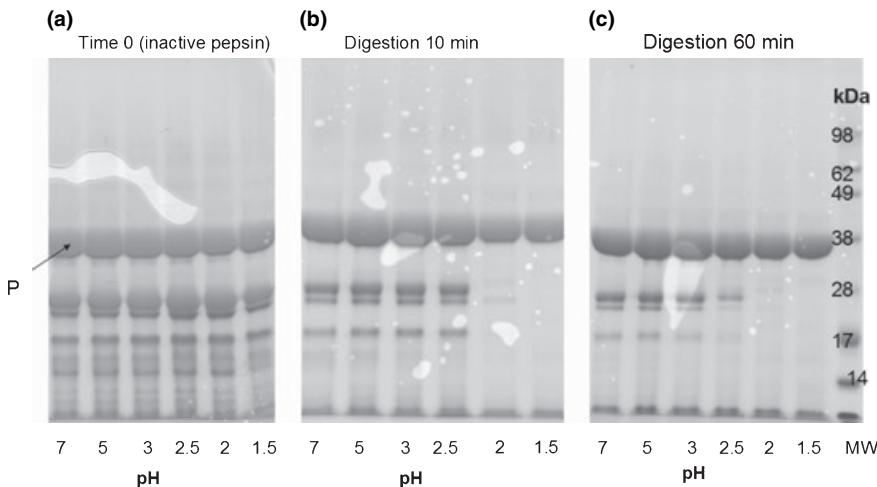


Fig. 2. SDS-PAGE of kiwi extract treated with SGF with pHs 1.5, 2, 2.5, 3, 5 and 7 stained with EZblue (Sigma). (a) Extracts added to inactivated pepsin, (b) proteins after 10 min in SGF and (c) extracts after an hour in SGF. P = pepsin. MW = molecular weight markers (SeeBlue Plus2; Invitrogen).

of SGF, demonstrated no difference in immunogenicity after 1 h (W_{60}) in comparison to time zero (W_0) confirming that the changes seen over time in the digested extracts were due to effects of SGF.

Western blotting using pooled sera from patients with systemic symptoms to kiwifruit showed IgE binding to proteins in untreated kiwifruit extract with molecular weights of 25, 28, 30, 38, 40, 42, 60 and 80 kDa (Fig. 4). Again the pepsin caused a physical barrier to IgE binding to allergens with molecular weights 38–42 kDa. As with the sera from patients with OAS, after 0.5 min the allergens with molecular weights of 25, 28 and 80 kDa has been digested, and binding to a band at 62 kDa persisted for 10 min.

However, in marked contrast to the blotting results using sera from patients with OAS, binding to the 30 kDa allergen continued, and was still evident after 1 h digestion. Also, there was evidence of new allergenic protein digests after 10 min digestion, with new bands of molecular weights of 22 and 25 kDa. These new protein digests remained present after 1 h of digestion. A band also appeared at 70 kDa after 0.5 min digestion, that then reduced in intensity after longer periods of digestion. This binding could be to the 70 kDa band noted during SDS-PAGE of digested proteins (Fig. 1). Therefore there were significant differences between IgE binding to digested proteins by systemic reactors and patients with OAS, with systemic reactors not just recognising digestion stable epitopes, but

also binding to epitopes that were not bound prior to digestion.

Discussion

Resistance to digestion is generally regarded as a characteristic of food allergens (19, 20), and pepsin stability is currently a factor that is considered in deciding the probability that a novel protein is allergenic (10). However, the stability of most allergens has not yet been determined, and some studies have questioned the association between allergenicity and digestive stability (6–8).

Astwood et al. (19) investigated the stability of common allergenic foods including peanut, soy, egg and milk relative to non-allergens. They reported that all non-allergenic proteins were rapidly digested, lasting 15 s in SGF, in comparison to allergenic foods which lasted 8–60 min. Some subsequent studies have supported their findings (21), but with some quantitative differences in the times of stability, presumably due to variation in methodology. However, other studies have failed to find an association between digestive stability and allergenicity (6, 8). At least some of these discrepancies are due to methodological issues (22), for example standardization of assays and no agreed time frame for stability.

If digestibility is to be considered part of the safety assessment of novel proteins, digestion assay protocols clearly need to be standardized. A multi-centre study from nine laboratories used a common protocol for evaluating the *in vitro* digestibility of common allergens and found it to be reproducible, yielding consistent results when performed using the same proteins at different laboratories (17). Their protocol was followed for this study. A limitation of the method is the 'blocking' of proteins by pepsin (39 kDa) on SDS-PAGE and Western blots. This was particularly relevant because the major allergen of the study population had a molecular weight of 38 kDa.

Most kiwifruit proteins were rapidly digested, with only one faint protein band visible with Coomassie staining after 0.5 min digestion. This supports the findings of Yagami et al. who showed that all allergens in kiwifruit were readily digested by SGF when assessed by means of SDS-PAGE (8).

Until now no study has directly compared IgE reactivity of patients with systemic and OAS symptoms to digested food. Despite no protein bands visible on staining, the pool of sera from our patients with systemic reactions demonstrated IgE binding by Western blotting to

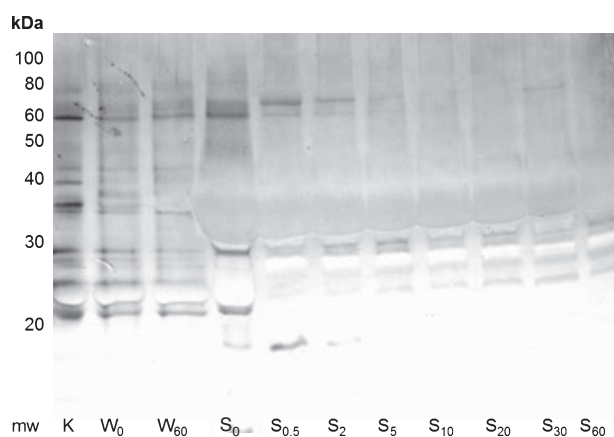


Fig. 4. Western blot using sera from patients with systemic symptoms on eating kiwi fruit. Kiwi fruit extract was digested using SGF with pH 1.5 at times ranging from 0.5 min ($S_{0.5}$) to 1 h (S_{60}). Lane S_0 has kiwi extract added to inactivated pepsin. Lane K: kiwi extract with no treatment. Lanes W_0 and W_{60} : the extract was incubated with water instead of SGF as a control.

proteins persisting after 1 h of simulated digestion. Interestingly, new IgE binding sites appeared after 20 min suggesting that digestion may even increase the IgE reactivity. These findings contrasted with those using sera from the pool of patients with OAS, who failed to show IgE binding to extract after it had undergone simulated gastric digestion for 10 min. Likewise Yagami's patients (8) with latex allergy, some of whom had fruit allergy, failed to show IgE binding to kiwifruit after several minutes of digestion. A limitation of this study was the need to use pooled sera due to limited stored volumes. The findings described may therefore be due to antibodies from only some individuals or even a single patient. Future studies need to be designed to investigate these findings in individuals with systemic and localized reactions.

It is generally believed that 'complete' food allergens are stable to gastric digestion, and absorption of the intact allergens allows sensitization, and on subsequent ingestions, systemic reactions to the food. Sensitization to pollens and latex is likely to occur, other than by ingestion, with fruit allergy occurring as a cross-reaction to homologous proteins, and symptoms to the digestion-labile fruit proteins restricted to the oral mucosa (OAS). The most recent guidelines on evaluation of novel proteins in GM plants recognize that the correlation between protein stability and allergenicity is not absolute (12). Although it has long been hypothesized that the digestibility of the responsible allergens determines whether a person develops systemic symptoms or OAS, this is the first study to confirm a difference in the lability of allergens recognized by patients with systemic reactions and those with OAS. These unique findings, if confirmed in different populations and to different food types, may provide the basis for the first test to predict which patients will proceed to have severe reactions.

Pepsin digestion of kiwifruit proteins was affected by hypoacidic conditions. Reducing the acidity of the SGF from pH 1.5 to 2.5 (i.e. 10-fold reduction in hydrogen ion concentration) strongly reduced pepsin digestion, presumably increasing the sensitization capacity of the protein. Similar findings were found by a group looking at parvalbumin from fish (15). They went on to compare IgE responses to labile food allergens in gastroenterology patients prior to, and 3 months after starting H₂-blockers (16), and reported a significant increase in food sensitization. Their findings are perhaps surprising because many patients receive antacid treatment, and there have not been reports of these

patients developing new food allergies. However, our data pertaining to protein digestion supports their *in vivo* findings and if confirmed will not only be relevant for patients receiving antacids, but will have implications for other groups with gastric hypoacidity. Newborns have a gastric pH approx. four and perhaps this hypoacidic state is responsible for the increased sensitization observed in infants who are weaned early.

Limited sera prevented testing individual patients on the digested and undigested kiwi protein extracts and this work needs to be done. The results of this study would be further strengthened by assessing skin test reactivity to digested and non-digested allergens. These findings could then have broad implications on the diagnosis of kiwi allergic patients as the IgE reactivity profile could be used to diagnose patients with a higher risk for systemic reactions. Such work was outside the scope of this study.

In summary, this study highlights several important findings. Lability of all kiwifruit proteins to pepsin digestion was suggested by SDS-PAGE and Coomassie staining. However, although this is the method advocated to assess lability of novel proteins by the WHO (10), it is clear that simply using an approach of pepsin digestion followed by SDS-PAGE and Coomassie staining to assess digestibility in relation to allergenicity may be flawed. As illustrated by the findings here, staining with Coomassie (EZBlue is reported to detect 10 ng protein) was not sufficiently sensitive and IgE binding occurred to allergens not visible on the stained gels. It is therefore extremely important to address the stability of allergens in relation to IgE reactivity. However, for novel foods suitable sera may not be available. In these cases, staining with silver nitrate may provide visible evidence of protein bands.

It has previously been hypothesized that clinical severity of symptoms is due to lability of allergens, but this is the first published work to confirm the association by comparing IgE binding to digested food using sera from patients with OAS and with systemic symptoms. The pooled IgE from patients with systemic symptoms recognized epitopes that were digestion resistant, and interestingly, IgE binding sites not present in undigested kiwifruit were recognized by systemic reactors following fruit digestion. This is important because it highlights that digestion does not just result in loss of allergenicity by breakdown to smaller fragments. The process is dynamic and it is possible that as larger proteins are broken down, the peptides can then aggregate or dissociate. There is therefore the potential for forma-

tion or unmasking of new allergenic protein digest products as evidenced by these results.

Acknowledgments

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