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REVIEW

Kiwifruit as a food allergen source

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Abstract: Since its first appearance on the market, kiwifruit has become very popular in the human diet due to its pleasant taste, low caloric value and high content of vitamin C. However, kiwifruit allergy has become a frequent cause of type I hypersensitivity in the western society. The molecular basis for kiwifruit allergy has been ascribed to up-to-now 11 identified IgE reactive molecules. They are proteins and glycoproteins with a molecular mass between 10 and 50 kDa. The major kiwifruit allergen is a cysteine protease denoted as Act d 1, which represents 50 % of the soluble protein extract. Due to differences in the abundance of the protein components and biological activity, the quality of kiwifruit extracts intended for allergy diagnosis can vary in content and amount of IgE reactive molecules. In addition, the quality of allergen extracts for allergy diagnosis depends on the fruit ripening stage and storage conditions. In terms of clinical reactivity, it has become evident that kiwifruit allergy is not a homogeneous disorder. Different patterns of IgE reactivity accompany several clinical subgroups that have been identified in different geographical regions. In the last decade, enormous progress has been made in the isolation and characterization of kiwifruit allergens. This paper presents an overview of the structural features of kiwifruit allergens.

Keywords: allergy; kiwifruit; food allergens; IgE reactivity.

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1. INTRODUCTION

Since its first appearance on the fruit market, kiwifruit has become very popular in the human diet due to its pleasant taste, low caloric value and high content of vitamin C; and numerous investigations on health-promoting properties of this fruit have been reported.[1,2] Kiwifruit has also found extensive application in the food industry as a meat tenderizer,[3] milk coagulant[4] and fruit ingredient in jams and jellies, syrups, confectionery, *etc*.[5]

However, simultaneously, kiwifruit is a good example of the possible consequences encountered by the introduction of a novel item into the human diet. In 30 years, from the first report of allergic reaction in 1981,[6] kiwifruit has become one of the top ten sources of food allergy, as shown in recent studies from Finland, Sweden and France.[7]

Kiwifruit was formally described for the first time in 1847 and given the Latin name *Actinidia chinensis.* The plant is native to the Yangtze valley of southeast China and has been a favorite of the local population for centuries.[8] The transformation of a small, hard, and wild Chinese berry into the fleshier, tastier kiwifruit began in 1904 when a school teacher from New Zealand arrived back from a short visit to China with seeds of what was then called Chinese or Ichang gooseberry, and from these a local nurseryman produced two plants from which almost all today's cultivars outside of China descend. Kiwifruit benefited from the warm, humid climate and volcanic soil of New Zealand's north island and soon became popular in the area, in the beginning valued more as a decorative vine with snowy white flowers than for its fruit. Over the next three decades, gardeners developed superior kiwifruit vines through careful selection, pruning, and grafting. During World War II, American soldiers stationed in New Zealand developed a liking for the taste of this fruit and in 1962, when New Zealand began with the export of this fruit to the USA, as part of a marketing campaign, they renamed it from Chinese gooseberry to kiwifruit, after the national bird of New Zealand.[9,10]

Kiwifruit belongs to the large genus of *Actinidia* and was first known under the botanical name *A. chinensis.* However, this species was discovered to be

polymorphic comprising several varieties (var. *chinensis*, var. *hispida* and var. *setosa*). In 1986, it was concluded that enough differences between the varieties exist to reclassify *A. chinensis* var. *chinensis* and *A. chinensis* var. *hispida* into two different species. Kiwifruit that is commercially grown and readily available worldwide is now known under the botanical name *A. deliciosa* (A. Chev) C.F. Liang & A.R. Ferguson var. *deliciosa*.[11] Until 1999, the world kiwifruit production was mostly based on the green-fleshed cultivar *Actinidia deliciosa* cv Hayward, when the yellow-fleshed cultivar *A. chinensis* cv Hort16A became available on the international market under the commercial name Zespri Gold. These two varieties not only differ in taste, but also in their allergenicity.

2. CLINICAL MANIFESTATIONS OF KIWIFRUIT ALLERGY

Since it was first described in 1981,[6] kiwifruit allergy has been observed with increasing frequency in western populations.[12] Symptoms of kiwifruit allergy vary from mild symptoms localized to the oral mucosa to severe systemic reactions, particularly in young children.[12] Kiwifruit allergy can be the result of cross-reactivity with pollen and latex, the most frequent association being with birch and grass pollinosis;[13] however, monosensitizations have also been reported. Symptoms severity and sensitization patterns of kiwifruit allergy are geographically dependent, with different allergen sensitization patterns seen across Europe. More severe symptoms to kiwifruit allergy in allergic patients were recorded in the birch-free Mediterranean area than in central Europe.[14] Aleman *et al*. provided evidence that kiwifruit allergy is not a homogeneous disorder because several clinical subgroups can be established.[15]

Food allergy is a consequence of primary sensitization of the immune system to food allergens or from primary sensitization to inhalant allergens (pollens or latexes in allergies to plant foods) in atopic persons. It has been postulated that direct sensitization by food allergens *via* the oral route is only possible when the allergens possess resistance to proteolysis in the digestive tract. This feature is regarded as decisive for the potential of food allergens to induce severe systemic reactions mediated by immunoglobulin E antibodies.

Structural similarities of proteins from different sources provide an explanation for a number of clinically observed cross-reactivity cases.[13] Due to cross-reactivity between plant and pollen allergens, patients with allergy to pollens often report oral allergy syndrome (OAS) after ingestion of certain fruits, nuts and vegetables. Pollen–food cross-reactive IgE antibodies are usually implicated in mild symptoms of allergy, such as OAS. The major birch pollen allergen Bet v 1 and the pollen profilins, the major source of cross-reactive IgE binding epitopes, are sensitive to pepsin digestion, and therefore the clinical symptoms of allergy are restricted to the oral cavity.

3. MOLECULAR BASIS OF KIWIFRUIT ALLERGY

The molecular basis of kiwifruit allergy is attributed to eleven International Union of Immunological Societies (IUIS) nominated kiwifruit allergens (www.allergen.org) (Table I).

TABLE I. Kiwifruit allergens

Allergen	Biochemical name	*MW* / kDa (SDS–PAGE)
Act d 1	Cysteine protease (actinidin)	30
Act d 2	Thaumatin-like protein	24
Act d 3	–	40
Act d 4	Phytocystatin	11
Act d 5	Kiwellin	26
Act d 6	Pectin methylesterase inhibitor	18
Act d 7	Pectin methylesterase	50
Act d 8	Pathogenesis-related protein PR-10	17
Act d 9	Profilin	14
Act d 10	Lipid transfer protein 1	10
Act d 11	Major latex protein/ripening-related protein family	17

3.1. Act d 1

Actinidin (EC 3.4.22.14) is a cysteine protease from the papain superfamily and is abundant in kiwifruit.[16] Beginning from the 1970s, this enzyme has been studied in detail and its 3-dimensional structure, amino acid and nucleotide sequences are known.[17–19] The mature form of actinidin is comprised of a single polypeptide chain organized into two domains with 3 disulphide bridges and has a molecular weight of 23.8 kDa and a p*I* value of 3.5.[17,20,21] Nieuwenhuizen *et al.* identified more than 10 different mRNA molecules encoding actinidin isoforms with predicted p*I* values ranging from acidic (3.9) to basic (9.3).[22] Actinidin has a wide pH activity range (4–10) and wide substrate specificity, preferentially hydrolyzing the amide and ester bonds at the carboxyl side of lysine residues.[23,24]

Actinidin represents more than 50 % of total soluble protein content in the green-fleshed kiwifruit cultivar (*A. deliciosa* cv. Hayward), but this protein appears to be at a very low level in Hort16A, the most important commercial cultivar of gold kiwifruit (*A. chinensis*).[16,22] Immunolocalization showed that actinidin was present inside the vacuole of the small cells of the outer pericarp of mature *A. deliciosa* fruit at harvest.[22] Several other cysteine proteases, which are actinidin homologues from the papain family, are also accumulated at high levels in specific tissues or cell types, including papain from papaya, bromelain from pineapple and ficin from fig.

The physiological role of these enzymes is unknown, but it has been proposed that they function as storage proteins or are part of a defense mechanism against insects, plant diseases and other forms of stress.[25,26]

Actinidin (Act d 1) is a kiwifruit allergen and is considered a marker of monosensitization to this allergen food source, since no cross-reactivity with birch or grass pollen has been observed.[15,20,27,28] In most studies, actinidin was identified as a major allergen in kiwifruit allergic patients.[7,25,27–29] An exception to this was a study performed in 2007 by Lucas *et al*. on kiwifruit allergic patients in the United Kingdom in which none of the 30 patients showed IgE binding to purified actinidin in Western blot.[30] Actinidin retains allergenicity after thermal treatment, a significant feature in terms of the possible development of allergic reactions after the consumption of food products containing processed kiwifruit.[21]

Although some studies indicate that actinidin is susceptible to degradation upon passage through the gastrointestinal tract,[31] others showed that actinidin retains its enzymatic activity under conditions of gastric and intestinal digestion and aids in the digestion of food proteins.[32,33] In a 2012 study by Čavić *et al.*, the proteolytical activity of actinidin, which leads to changes in the morphology and adhesion of intestinal epithelial cells, was proposed as a possible route for oral sensitization to this allergen.[34]

3.2. Act d 2

The second IgE binding molecule isolated from *A. deliciosa* was a thaumatin-like protein (TLP) and it was denominated Act d 2.[13] Act d 2, as well as other TL proteins, belongs to the pathogenesis related (PR) 5 family of proteins.[35] It is a protein of 225 amino acids, with the first 24 representing a signal peptide that designates this protein to the apoplast. It has basic p*I* values of 9.4 and 9.5.[13,14] A potential *N*-glycosylation site is located on $N^{189}FS$ and glycosylation was suggested to exist based on its ability to bind to concanavalin A lectin.[13] Kiwi TLP, similar to other members of the PR 5 family, possesses antifungal activity against *Botrytis cynerea*, *Mycosphaerella arachidicola*[36] and *Saccharomyces carlsbergensis.*[13] The anomalous migration of kiwifruit TLP in SDS–PAGE is due to an unusually high number of S–S bridges, a peculiarity common for TLPs.[13] Namely, it has 8 disulphide bridges between cysteines Cys^{33}–Cys^{224}, Cys^{74}–Cys^{84}, Cys^{89}–Cys^{95}, Cys^{140}–Cys^{213}, Cys^{146}–Cys^{196}, Cys^{154}–Cys^{164}, Cys^{168}–Cys^{177} and Cys^{178}–Cys^{183}.

There are opposing studies concerning TLP stability under simulated gastrointestinal conditions.[13,31] The first obtained results suggested that purified TLP was digested in simulated gastric fluid (SGF) in only 1 min, while TLP in crude extract resisted digestion for 8 min.[13] However, later studies suggested that the stability of TLP during simulated gastrointestinal digestion largely depended on the enzyme:substrate ratio employed in the experimental setting, as well as whether reducing or non-reducing conditions were employed for SDS–PAGE analysis of the reaction mixture. Under non-reducing conditions, 25 % of the allergen re-

mained intact following *in vitro* gastric digestion and during duodenal digestion residual intact Act d 2 was still present when analyzed by SDS–PAGE. Under reducing conditions, the allergen promptly disappeared.[31] The observed differences stem from the different digestion protocols used in the experiments, *i.e.*, different amounts of pepsin used for the simulated *in vitro* digestion. However, it can be assumed, as suggested by a later study[31] and by similar results obtained for milk[37] and wheat[38] allergens, that disulfide bonds are important in the resistance of this and other allergens to digestion with proteases commonly en-countered in the gastrointestinal tract.

Thermal stability experiments suggest that Act d 2 aggregates following heating at pH 7, but not at pH 2.[31] These results show that cross-linking of Act d 2 molecules at pH 7 by intermolecular disulfide bonds was induced by heating. The cleavage of disulfide bonds at neutral pH and high temperature was previously observed for thaumatin,[39] but also in unrelated proteins including ovalbumin and transferrin.[40] The most probable mechanism of this reaction is β-elimination, in which a base-catalyzed subtraction of a β-proton from a cysteine results in cleavage of disulfide bonds. This creates intermolecular disulfide linkages and induces aggregation. In contrast, thermal denaturation at acidic conditions (such as those found in juice) reversibly unfolded the protein.[31]

Members of the TLP family have a role as allergens in a wide panel of plant foods and in several pollens, although there is little experimental evidence of plant foods and/or pollen cross-reactivity. Gavrović-Jankuović *et al.*[13] showed positive skin prick tests (SPT) in 80 % of the tested polysensitized patients (4 out of 5) and IgE reactivity in Western blot (7 out of 7 in crude extract). In a study by Palacin *et al.*,[14] *in vitro* (specific IgE detected in 64 % and 88 % of individual sera by ELISA and immunodetection assays, respectively) and *in vivo* (52 % of positive SPT responses) reactivity pointed to Act d 2 as the second major allergen in the analyzed kiwi-sensitized population of Spain. Immunoblotting experiments by Bublin *et al.*, showed that in extracts prepared from kiwifruit jam and whey, Act d 2 was present and its allergenic activity was not decreased by technological treatments.[31]

3.3. Act d 3

Act d 3 is a strongly glycosylated 40 kDa protein present in two genetic variants, and is homologous to hypothetical hydrolases from castor bean and other plant species.[14,41] The high sequence identity with the putative *Ricinus communis* protease suggests a functional role for Act d 3 in kiwifruits that could explain the presence in preparations of the purified allergen of possible self-degradation products that retain IgE-binding potency.[14]

Act d 3.02 seems to be a minor component of kiwifruit extracts, yet, in contrast, it represents a major kiwi allergen based on its high specific IgE prevalence

(62 %) in sera from kiwi-sensitized patients. The potential clinical relevance of Act d 3 was further supported by the statistical correlation between IgE levels to this allergen and anaphylactic symptoms. All these data are in agreement with those observed previously for Act d 3.01, both *in vitro* (66 % of sera with specific IgE) and *in vivo* (13 out of 15 patients with positive SPT responses).[41] Moreover, Act d 3 could probably correspond to the 38-kDa protein described by Lucas *et al*. as the major kiwifruit allergen in the United Kingdom.[30] Thirty six percent of the 22 patients with combined kiwifruit/pollen or kiwifruit/pollen/latex allergy were sensitized to Act d 3, as reported by Bublin *et al*.[29] Moreover, these authors claimed that IgE binding to highly cross-reactive allergens (rAct d 8, rAct d 9) or Act d 3 was not a clinically specific marker for kiwifruit allergy in the presence of pollen or pollen/latex sensitization.[29]

The complex glycans (cross-reactive carbohydrate determinants) attached to Act d 3 can be a source of cross-reactivity between kiwifruit and other plant foods and pollens, the actual clinical relevance of which remains to be explored.[14]

3.4. Act d 4

Act d 4 is a 11 kDa allergen first reported as an IgE binding component of kiwifruit extract.[28] It belongs to the family of cysteine proteinase inhibitors (CPI) named phytocystatins.[42] There are 3 isoforms of phytocystatins present in kiwifruit, with isoform 1 being the most abundant.[42] Act d 4 was identified and isolated as isoform 1.[43]

Act d 4 is a type I plant cystatin synthesized as a pre-protein of 116 amino acids, with the first 26 amino acids representing a signal sequence that is cleaved off from the mature protein.[42,43] Immuno-tissue print results indicated that CPI is most abundant in the outer layer of the pericarp, near the peel and the innermost part of the pulp – sites where it could act as a natural barrier against pathogens entering the fruit.[44] It is a glycoprotein with a p*I* of 6.9 which binds Con A lectin, mannose-specific banana lectin and fucose-specific *Aleuria aurantia* lectin.[43] The molecular masses of the mature protein were determined by MALDI to be 10902.5 Da and 11055.2 Da.[43] These different masses observed could probably be attributed to the presence of different glycosylation isoforms present in kiwifruit.

Act d 4, similar to other type I plant cystatins, showed antifungal activity against two phytopathogenic fungi (*Alternaria radicina* and *B. cinerea*), by inhibiting fungal spore germination. *In vivo*, Act d 4 was able to prevent artificial infection of apple and carrot with spore suspensions of *B. cinerea* and *A. radicina*, respectively. It also exerted activity on both intracellular and fermentation fluid proteinases.[44] Act d 4 influenced the growth of phytopathogenic bacteria *Agrobacterium tumefaciens* (76.2 % growth inhibition using 15 μM CPI), *Burkholderia cepacia* (75.6 % growth inhibition) and, to a lesser extent, *Erwinia ca-*

rotovora (44.4 % growth inhibition) by inhibiting proteinases that are excreted by these bacteria.[45]

Act d 4 was first identified as an IgE binding component of kiwifruit extract.[28] However, a positive skin prick reactivity with Act d 4 was induced in three kiwifruit allergic patients, as well as the upregulation of CD63 and CD203c molecules in the basophile activation assay. IgE reactivity was detected in dot blot analysis and subsequent negative Western blot analysis using sera from six kiwifruit patients, which suggested the presence of conformational IgE epitopes on the Act d 4 molecule. As an activator of effector cells in type I hypersensitivity Act d 4 is a functional allergen contributing to the clinical symptoms of kiwifruit allergy.[43]

3.5. Act d 5

The fifth allergen detected in kiwifruit extract was named kiwellin and was designated as Act d 5.[46] Kiwellin was first identified in green kiwifruit and described as an allergen and one of the major protein components of this fruit.[46] It was also identified in the gold kiwifruit species, where it appears as the most abundant protein component.[47] Anomalous behavior of Act d 5 was observed in SDS-PAGE (traveling as a 20 kDa band in non-reducing and a 28 kDa band in reducing conditions), which, as observed for TLP, is a consequence of disulphide bridges, as it contains 14 cysteine residues. It contains 189 amino acid residues and sequence heterogeneity was found at position 61, where His replaced Tyr in approximately 40 % of the protein molecules.

Kiwellin is cleaved into the following four peptides: kissper, KiTH1, KiTH2 and KiTH 3.[47] Kissper is a 39-residue peptide isolated in good yield from the edible part of kiwifruit; its amino acid sequence showed 100 % identity with the first 39 residues of the *N*-terminal region of kiwellin.[48] Kissper is derived from the processing of the precursor kiwellin through the cleavage of the peptide bond between Thr39 and Thr40. The capacity of kissper to permeabilize synthetic membranes was tested, and while kissper showed anion selectivity,[48] it was at concentration values generally lower than those reported for several pore-forming peptides, such as defensins, thionins, cecropins, cryptidin, duramycin, *etc*.[49,50] The high amount of kissper found in ripe kiwi fruit and its strong resistance to proteolysis suggest that it could very likely affect the gastrointestinal physiology.[48]

KiTH was detected on SDS–PAGE as a 20-kDa band. Elucidation and analysis of the primary structure of purified KiTH revealed 100 % amino acid sequence identity with the C-terminal region (residues 40–189) of kiwellin. KiTH and kissper were isolated from green kiwifruit in approximately stoichiometric amounts, which suggested that both were produced following a proteolytic cleavage of kiwellin.[47] KiTH and kissper were not detected in gold kiwifruit extract, but it cannot be excluded that they were present in very low, undetectable

amounts.[47] The observation that their presence in green kiwifruit was correlated with a high amount of actinidin suggested a possible involvement of this protease in their generation. Two KiTH forms found in the green kiwifruit extracts derived from *in vivo* cleavage between Thr^{39} and Thr^{40} (site 1) and between His^{41} and Ser^{42} (site 2) of kiwellin, were also identified as products of *in vitro* digestion by actinidin. A third form of KiTH, showing two additional residues at the N terminus, was obtained after *in vitro* enzymatic cleavage of kiwellin, suggesting a significant effects of the environmental conditions on the specificity of the proteolytic action. The experimental data demonstrated that KiTH and kissper are produced following *in vitro* proteolytic processing by actinidin and that environmental conditions may affect the ratio of the digestion products.[47]

Serological tests and Western blot analysis showed that kiwellin is specifically recognized by IgE of patients allergic to kiwifruit.[46] Similar to kiwellin from green kiwifruit,[46] the homologous protein from gold kiwifruit displays IgE-binding capacity. Both proteins were detected by the same sera, and even the levels of the signal on Western blot were comparable, thus suggesting conservation of the IgE-binding epitopes. KiTH was also detected as an IgE binding molecule.[47] The obtained results suggested that (i) kiwellin may have a hidden IgE-binding epitope that becomes available in KiTH, following the removal of kissper, and (ii) kissper might be an IgE-binding epitope by itself.[47]

On testing a population of subjects allergic to kiwifruit using the standard protocol for SPT, eight out of 29 (28 %) had a positive reaction to Act d 5. The observation that some subjects had a positive reaction either at neutral or acidic pH values suggests that this allergen, depending on the experimental conditions, may expose different epitopes. CD measurements under different experimental conditions indicated that the three-dimensional structure of Act d 5 is modulated by the solvent pH and polarity. Therefore, it may be hypothesized that, depending on the environments encountered, this allergen may undergo *in vivo* conformational changes and expose different epitopes, inducing the synthesis/interaction of different specific IgEs.[51]

3.6. Act d 6

A protein acting as a powerful inhibitor of plant pectin methylesterase (PMEI) was isolated from kiwifruit and denoted as Act d 6.[52] This protein is comprised of 152 amino-acid residues, accounting for a molecular mass of 16277 Da, with a predominant alpha-helix conformation in the secondary structure.[52] The protein has five cysteine residues but neither tryptophan nor methionine.[52] Analysis of fragments obtained after digestion of the protein alkylated without previous reduction identified two disulfide bridges connecting cysteines Cys^{9}–Cys^{18}, and Cys^{74}–Cys^{114}, while Cys^{140} bears a free thiol group. A database search indicated a similarity between PMEI and plant invertase inhibitors. In

particular, the four Cys residues, which in PMEI are involved in the disulfide bridges, are conserved. A comparison of the sequence of these inhibitors confirms the existence of a novel class of proteins with significant sequence conservation, comprising plant proteins acting as inhibitors of sugar metabolism enzymes, and probably involved in various steps of plant development.[52]

Considering that PMEI interacts with pectin methylesterase (PME), which is localized in the cell wall, it is probable that a signal sequence is required to direct the protein toward its final cellular localization. In fact, preliminary experiments of tissue immunological staining indicate that Act d 6 is concentrated in a layer close to the cell membrane.[52]

The relative expression levels of the PMEI genes in kiwifruit, analyzed by competitive PCR, increased with progression of fruit maturation. Given that the PME activity also showed its highest level at the fully ripened stage of maturation, the increase in PMEI expression may not indicate direct inhibitory effects on the PME activity and fruit maturation process.[53]

3.7. Act d 7

Act d 7 belongs to the family of pectin methylesterases (PME), a class of proteins involved in pectin metabolism during different physiological and pathological processes, such as fruit ripening[54] and response to pathogen attack.[55] PME was purified from a salt extract of kiwifruit cell wall by a combination of ion exchange and affinity chromatography (on pectin methylesterase inhibitor PMEI).[56] PME consists of two forms (PME_1 and PME_2). Both isoforms have a neutral p*I* of 7.3.[56] The molecular weight of purified kiwi PME, determined by both gel filtration and SDS–PAGE, was 50 kDa.[56] Act d 7 is a monomeric protein.[56] Kiwi PME was reported to be glycosylated with a molecular mass of 57 kDa,[57] however in this case, glycosylation may approximately account for 30 % of the total molecular mass of the protein.[58] The two isoforms of PME differ in their degree of glycosylation as shown by different retention times on concanavalin A–sepharose with respect to a glucose gradient.[57]

Kiwi PME showed two activity optima at pH 6.5 and 8.0–8.5, both in the presence and in the absence of 100 mM NaCl. The enzyme has a pH and salt dependent activity. It shows lowered activity at the lower pH optimum in the absence of salt, which may be due to the adverse effect of specific charged groups on the enzyme/substrate interaction.[58] The two isoforms also differ in thermostability, with PME_1 being more stable than PME_2.[57]

Binding experiments, performed by surface plasmon resonance, showed that PMEI strongly interacts with immobilized kiwi PME, as indicated by the extremely low dissociation rates observed at pH values ranging from 3.5 to 8.0.[58] The observation that only extreme pH conditions can dissociate the complex kiwi PME–PMEI may address the hypothesis that, *in vivo*, kiwi PME is irreversibly

inactivated by PMEI when it has no further physiological function at the end of the ripening process.[58]

In Western blot analysis, 13 % of patients had a positive IgE directed to purified allergen. Patients positive to Act d 7 were also positive to Api g 5, Ana c 2 and Hev b 4, which are known as allergens containing cross-reactive carbohydrate determinants (CCDs).[59] This can be explained by the high cross reactivity of CCDs from different allergen sources.[7]

3.8. Act d 8

Act d 8 is a homologue to the major birch pollen allergen Bet v 1.[60] Act d 8 (from *A. deliciosa*) and Act c 8 (from *A. chinensis*) are encoded by open reading frames (ORFs) of 471 and 474 nucleotides, corresponding to 157 and 158 amino acid residues, respectively.[61] There are thirteen ORF sequences corresponding to Act d 8 isoforms detected in green kiwi extract, while six were detected in gold kiwi extract.[61] The amino acid sequence identity between Act d 8 and Act c 8 was 70 % and to Bet v 1 (CAA54696) 53 and 54 %, respectively.[61] The predicted molecular masses of Act d 8 and Act c 8 are 16922 Da and 17387 Da, and their calculated p*I* values are 5.36 and 5.82, respectively.[61]

Transcripts for Act c 8 and Act d 8 are considerably less abundant, with Act c 8 appearing to be more highly expressed than Act d 8 in both green and gold kiwifruit.[61]

The secondary structures of recombinantly produced Act d 8 and Act c 8 were determined by CD spectroscopy and the spectra were similar to CD spectra obtained with rBet v 1.0101.[61]

IgE binding of purified rAct d 8 and rAct c 8 was confirmed in both ELISA and immunoblot experiments on the sera from eight kiwifruit/birch pollen allergic patients. Both purified proteins were able to bind IgE from patient sera in both ELISA and immunoblot. The cross-reactivity of rBet v 1.0101, rAct d 8, and rAct c 8 was assayed in an IgE ELISA inhibition assay. Pretreatment of the sera from individual kiwifruit/birch pollen allergic patients with rAct d 8 or rAct c 8 as inhibitor resulted in reduced IgE binding to rBet v 1.0101, while IgE binding to rAct d 8 and rAct c 8 was completely inhibited by pre-incubation with rBet v 1.0101.[61]

The results of the localization studies show that a Bet v 1-related protein was recognized in the peripheral pulp by a polyclonal anti-Bet v 1 antibody in green and gold kiwifruit.[61]

Allergic symptoms elicited by a member of the Bet v 1 family are usually confined to the oral mucosa or angioedema of the lips. Such mild symptoms have been described in Bet v 1-mediated fruit allergy to apple[62] and cherry.[63] These findings could be explained by heat liability and low resistance to digestion of the Bet v 1 homologous proteins.

Birch pollen-related food allergy is highly prevalent and often perennial. Recent study has shown that high food allergen-specific IgG_4/IgE ratios seem associated with food tolerance, potentially because specific IgG_4 blocks IgE binding to food allergens. Thus, the presence of food allergen-specific IgG_4 antibodies is no diagnostic marker for birch pollen-related food allergy.[64]

3.9. Act d 9

Act d 9 or profilin is an actin-binding protein involved in the dynamic turnover and restructuring of the actin cytoskeleton.[65] It is found in all eukaryotic organisms in most cells. Profilin is important for spatially and temporally controlled growth of actin microfilaments.[65]

Profilin binds sequences rich in the amino acid proline in diverse proteins. While most of profilin in a cell is bound to actin, profilins have over 50 different binding partners. Many of these are related to actin regulation, but profilin also seems to be involved in activities in the nucleus, such as mRNA splicing.[66] Profilin binds some variants of membrane phospholipids. The function of this interaction is the sequestration of profilin in an "inactive" form, from where it can be released by action of the enzyme phospholipase C. IgE reactive profilins are present in birch,[67] grass[68] and other pollen.[69–71]

A primary structure comparison of Act d 9 and profilins from other allergen sources revealed amino acid sequence identity with Cap a 2 from bell pepper (93 %), Hev b 8 from latex (90 %) and Bet v 2 from birch pollen (75 %).

rAct d 9 demonstrated IgE binding reactivity *in vitro* in 36 % patients with combined kiwifruit/pollen or kiwifruit/pollen/latex allergy.[29]

3.10. Act d 10

Act d 10 belongs to the family of plant lipid transfer proteins or LTPs.[72] Plant LTPs are widely distributed, structurally related, small proteins involved in defense mechanisms. Although their lipid binding ability has been well reported, the biological function of LTPs is still largely unknown. The plant LTP family includes two subfamilies according to their molecular masses: the 9-kDa LTP1 and the 7-kDa LTP2. Although LTP1 and LTP2 share a common compact fold consisting of four α-helices stabilized by four disulfide bridges, the pairing partners of cysteines are not completely conserved between the two subfamilies, that also display a low overall sequence similarity (about 30 % identity).[73] To date, 63 LTPs have been characterized as allergens, 46 of them expressed in edible parts of plants, almost all of them belonging to the LTP1 protein subfamily, and just two to the LTP2 subfamily (www.allergome.org).

LTP was detected and purified in both gold and green kiwifruit as Act c 10 and Act d 10, respectively. Both proteins are present in seed extract, but not in pulp extract.[72] Direct protein sequencing of the purified protein allowed the

identification of two different isoforms of Act d 10. Possible additional isoforms, suggested by elution profiles obtained during purification, were not identified probably because of the low yield.[72]

MALDI–TOF mass spectrometry of purified nAct d 10 provided two mass values, 9.464 and 9.484 kDa, which are in good agreement with the values deduced from the amino acid sequence of the two isoforms having alanine (9.458 kDa) or threonine (9.488 kDa) as N-terminal residues, respectively.[72]

Analysis by SDS–PAGE and RP-HPLC of LTP samples subjected to digestion in SGF showed that nAct c 10 and nAct d 10, similarly to nPru p 3, are resistant to gastric digestion. Moreover, similar to the results reported by Cavatorta *et al.*,[74] Pru p 3 was partially digested by trypsin, whereas nAct d 10 and nAct c 10 appeared to be resistant.[72]

A homology search in Uniprot protein database realized using the BLAST algorithm (www.expasy.org) showed the sequence identity between Act d 10 and other already known allergenic LTPs to be not very high, ranging between 55 % with Ara h 9 (isoform Ara h 9.0201) and 35 % with Par j 2. The identities among the full-length amino acid sequence of the six allergenic LTPs: Act d 10, Ara h 9, Art v 3, Cor a 8, Mor n 3 and Pru p 3 are in the range from 42 to 70 %. The sequence identity values between Act d 10 and other allergenic LTPs, such as Api g 2 (celery stalk), Cit s 3 (orange), Fra a 3 (strawberry), Lac s 1 (lettuce), Len c 3 (lentil), Lyc e 3 (tomato), Mal d 3 (apple), Ory s 14 (rice), Pla or 3 (plane tree pollen), Pru du 3 (almond), Pyr c 3 (pear), Sin a 3 (mustard), Tri a 14 (wheat), Vit v 1 (grape), Zea m 14 (maize) are found in the narrow range of 40–55 %.[72]

3.11. Act d 11

Act d 11 is a 17-kDa protein found in variable amounts in extracts of green kiwifruit. This is a ripening-related protein, the amount of which is influenced by natural ripening and post harvesting treatments, including exposure to the plant hormone ethylene.[75] Act d 11 displays the highest sequence identity with members of the major latex protein/ripening-related protein (MLP/RRP) family, which belongs to the Bet v 1 superfamily.[76] A lower sequence identity is shared with members of the PR-10 protein family, including Bet v 1.[75]

Several antigenic regions of the surface of Bet v 1 and of co-recognized allergens were described following studies based on the mapping of conserved residues,[77,78] phage-displayed allergen mimotope technology[79] and X-ray crystallography.[80] Most of the amino-acid residues, which belong to B-cell or T-cell epitopes in Bet v 1 or in Bet v 1-related allergens,[77,81] are conserved in Act d 11 and cluster mainly in the regions comprising the p-loop motif and the protein C-terminal domain, where the local sequence identity is significantly high.[75]

The residue Glu[45] was reported to be critically important in Bet v 1 for IgG and IgE binding.[81] Like several MLP/RRPs, Act d 11 shares E45 with Bet v 1 and with most of the homologous allergens. The capacity of Act d 11 to inhibit, at least partially, IgE binding to Bet v 1 and to homologues, such as Cor a 1, Dau c 1 and Mal d 1, suggests epitope-sharing regions higher than that inferable from the low overall sequence identity.[75]

Act d 11 showed IgE reactivity in SPT, double blind placebo-controlled food challenge and immunoblot. Act d 11 was able to inhibit partially Bet v 1, Mal d 1 and Cor a 1 IgE binding. Soluble Bet v 1 achieved 100 % IgE inhibition of almost all Act d 11 positive sera.[75]

4. CROSS-REACTIVITY BETWEEN KIWIFRUIT AND LATEX AND/OR POLLEN ALLERGENS

Cross-reactivity (CR) occurs when an adaptive immune response to a particular antigen causes reactivity to other antigens that are structurally related to the inducer.[82] The World Health Organization guidelines for the prediction of allergenicity specify that a protein can be considered to cross-react with an allergen if they share at least 35 % sequence similarity in a fragment of 80 amino acids or complete identity with a peptide of 6–8 amino acids from an allergen. Latex-fruit syndrome is the association of latex allergy and allergy to plant foods, which affects up to 50 % of latex-allergic patients.[83] The foods most frequently involved are banana (28 %), avocado (28 %), chestnut (24 %) and kiwi (20 %). With these foods, clinical symptoms are often severe, as is the case with other foods less frequently related to latex (fig, papaya and tomato). Allergy to latex usually precedes food allergy, although this is not always the case. Frequently, the spectrum of food allergies increases with time.[83]

Patients allergic to pollen from birch and other Fagales show symptoms of allergy to plant foods. Pollinosis precedes the symptoms induced by the foods. These tend to be slight, characteristically OAS, and occur following ingestion of the raw food. The main culprit allergen, which is involved in more than 90 % of patients with allergy to plant foods associated with allergy to birch pollen, is Bet v 1,[84] a PR-10, which gives rise to cross-reactivity with its homologues in these foods. In kiwifruit, the two Bet v 1 homologues identified so far in green and gold specimens are Act d 8 and Act c 8,[61] but also Act d 11.[75]

Profilins are structural proteins that are both ubiquitous and very well conserved during evolution. They are considered incomplete allergens, capable of inducing sensitization by inhalation, but not by ingestion, due to their lability against peptic digestion. Thus, whilst in the north of Europe, it is associated with allergy to birch pollen,[85] while in Spain, it is more frequent and associated mainly with pollinoses due to grasses.[86] The clinical manifestation of this food allergy is OAS induced by the raw food. Several foods could be involved, given

that many allergenic profilins have been described in plant foods that are eaten raw. Profilin present in green kiwifruit extract was designated Act d 9.[29]

In the study of Gavrović-Jankulović *et al*., a molecular basis of IgE cross-reactivity between meadow fescue pollen and kiwifruit has been found between Fes p 4, a 36-kDa meadow fescue allergen and a 24-kDa kiwifruit protein.[13]

5. DIAGNOSIS OF KIWIFRUIT ALLERGY

At present, the diagnosis of kiwifruit allergy is unsatisfactory. *In vitro* and *in vivo* tests based on commercially available kiwifruit extracts frequently fail to detect specific IgE, resulting in a low sensitivity.[29] Diagnosis performed by skin prick testing with kiwifruit extract has a sensitivity of only 40 to 50 %, while the measurement of food specific serum IgE has a sensitivity of 17 to 60 %.[87] Due to the poor correlation between a suggestive case history and a skin prick test (SPT) performed with commercial kiwifruit protein extracts, improved *in vivo* diagnosis of kiwifruit allergy necessitates the use of fresh fruit, such as in the prick–prick technique. However, although prick-to-prick tests with fresh kiwifruit have a higher sensitivity (83 to 100 %), they also have a low specificity (31 %) and are difficult to standardize.[12,15] The low diagnostic sensitivity of *in vitro* and *in vivo* tests is related to the low level or absence of kiwifruit proteins from commercially available kiwifruit extracts. Preparation of higher quality extracts that would give reproducible results is limited by the natural variability of the plant source material.[29] It was also shown that the natural ripening stage, cold storage, and ethylene treatment influence the protein composition and IgE-binding profiles of both green and gold kiwi fruit extracts.[88,89]

Two recent studies showed that the use of individual kiwifruit allergens increased the diagnostic sensitivity compared with use of commercial extracts. In a study by Bublin *et al*., the authors evaluated the use of individual allergens for component-resolved *in vitro* diagnosis of kiwifruit allergy. The study was performed on thirty patients with a positive double-blind placebo-controlled food challenge (DBCFC) to kiwifruit. Specific IgE to 7 individual allergens and allergen extracts was measured by ImmunoCAP. The use of individual allergens raised the diagnosis sensitivity from the 17 % obtained with commercial extract to 77 %, but the diagnostic specificity was lowered from 100 to 30 %. Using only kiwi allergens Act d 1, Act d 2, Act d 4, and Act d 5 gave a diagnostic sensitivity of 40 %, whereas diagnostic specificity remained high (90 %).[29] The performance of a component-based allergen micro-array for the diagnosis of kiwifruit allergy was evaluated by Bublin *et al*. The specific IgE and IgG4 levels to a panel of nine kiwifruit allergens where measured in sera of 237 individuals with kiwifruit allergy. The panel of kiwifruit allergens showed a diagnostic sensitivity of 66 %, a specificity of 56 % and a positive predictive value of 73 %.[7]

Molecular basis of kiwifruit allergy has been extensively investigated in the last decade and huge progress has been made in the isolation and characterization of kiwifruit allergens. However, kiwifruit allergen extracts are still employed in clinical settings for *in vivo* allergy diagnosis by the skin prick test. In order to improve performance of allergy diagnosis, it seems that the component-resolved concept with a selected panel of natural and/or recombinant kiwifruit allergens will replace allergen extracts in future allergy testing.

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И З В О Д

ПЛОД КИВИЈА КАО ИЗВОР АЛЕРГЕНА ХРАНЕ

МИЛИЦА ПОПОВИЋ, МИЛИЦА ГРОЗДАНОВИЋ и МАРИЈА ГАВРОВИЋ-ЈАНКУЛОВИЋ

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Од првог појављивања на тржишту плод кивија је постао изузетно популаран састојак хумане исхране услед пријатног укуса, ниске калоријске вредности и високог садржаја витамина С. Међутим, алергија на киви је постала учестали узрок преосетљивости типа I у западном друштву. До сада је откривено 11 IgE везујућих молекула који чине молекулску основу алергије на киви. То су протеини и гликопротеини молекулских маса између 10 и 50 kDa. Главни алерген кивија је цистеин-протеаза означена као Act d 1, која сачињава 50 % растворних протеина плода кивија. Услед разлике у заступљености протеинских компоненти и биолошкој активности, квалитет протеинских екстраката кивија који се употребљавају у дијагностификовању алергије може варирати у садржају и количини IgE реактивних молекула. Такође, квалитет алергених екстраката зависи од степена зрелости воћа приликом брања, као и од услова складиштења воћа након брања. По питању клиничке реактивности постало је очигледно да алергија на плод кивија не представља хомогени поремећај. Различити обрасци IgE реактивности уочени су код неколицине клиничких подгрупа које су идентификоване у различитим географским регијама. Током последње деценије начињен је велики напредак у изоловању и карактеризацији IgE везујућих протеина кивија. У оквиру овог рада даћемо преглед структурних особина алергених протеина кивија.

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