

Allergy to kiwi in patients with baker's asthma: identification of potential cross-reactive allergens

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Background: Baker's asthma is a frequent IgE-mediated occupational disorder mainly provoked by inhalation of cereal flour. Allergy to kiwifruit has been increasingly reported in the past few years. No association between both allergic disorders has been described so far.

Methods: Twenty patients with occupational asthma caused by wheat flour inhalation were studied. Kiwi allergens Act d 1 and Act d 2 were purified by cation-exchange chromatography. Wheat, rye, and kiwi extracts, purified kiwi allergens, and model plant glycoproteins were analyzed by IgE immunodetection, enzyme-linked immunosorbent assay (ELISA), and inhibition ELISAs.

Results: Kiwifruit ingestion elicited oral allergy syndrome in 7 of the 20 patients (35%) with baker's asthma. Positive specific IgE and skin prick test responses to this fruit were found in all these kiwi allergic patients, and IgE to Act d 1 and Act d 2 was detected in 57% and 43%, respectively, of the corresponding sera. Actinidin Act d 1 and bromelain (harboring cross-reactive carbohydrate determinants) reached above 50% inhibition of the IgE binding to wheat and/or kiwi extracts.

Conclusions: A potential association between respiratory allergy to cereal flour and allergy to kiwifruit has been disclosed. Cross-reactive carbohydrate determinants and thiol-proteases homologous to Act d 1 are responsible for wheat-kiwi cross-reactivity in some patients.

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INTRODUCTION

Baker's asthma, which is one of the leading causes of occupational asthma, is mainly provoked by an IgE-mediated allergic response to inhalation of cereal flour or baking additives.^{1,2} A high prevalence of this disease (approximately 10%) has been reported among bakery workers, flour millers, and cereal flour processors.^{1,2} A wide array of allergens has been associated with the baker's asthma condition, including fungal α -amylase and other additives,³ but the primary ones are salt-soluble proteins derived from wheat and the secondary ones are from rye and barley flour.⁴ Members of several cereal salt-soluble protein families, particularly α -amylase inhibitors, peroxidases, thioredoxins, and nonspecific lipid transfer proteins,^{5–8} have been identified as potential relevant allergens in wheat flour. Interestingly, most of them seem to be also involved in wheat IgE-mediated food allergy.⁹

On the other hand, allergy to fruits and vegetables is among the most frequent food IgE-mediated disease in the adult European population.¹⁰ In this context, allergy to kiwi-

fruit (*Actinidia deliciosa*) appears to be on the rise in the past few years, and it is mainly associated with pollinosis, mild and local symptoms (oral allergy syndrome), and, in some patients, latex fruit syndrome.^{11–13} Two main kiwifruit allergens have been identified and sufficiently tested in different series of patients with allergy to kiwi: Act d 1, the 30-kDa thiol-protease actinidin,¹⁴ and Act d 2, a 24-kDa thaumatin-like protein, which is probably glycosylated.^{15,16} Act d 1 is now considered a major kiwifruit allergen, whereas the sensitization prevalence to Act d 2 is still uncertain.^{14,18} Three additional kiwifruit allergens have been recently described: Act d 3 (a 40-kDa glycoprotein),¹⁹ Act d 4 (a 11-kDa cystatin), and Act d 5 (a 28-kDa kiwellin),²⁰ which seem to be relevant in kiwi allergy but whose actual importance in sensitization to this fruit remains to be established.

Apart from the associations of kiwi and pollen (grass, birch) allergy¹¹ and kiwi-latex fruit syndrome,¹³ no link between the baker's asthma condition and kiwifruit allergy has yet been detected. Such a link is herein reported, as well as the identification of thiol-proteases and cross-reactive carbohydrate determinants (CCDs)²¹ as potential inducers of wheat-kiwi cross-reactivity.

METHODS

Patients and Sera

Twenty consecutive patients with baker's asthma were recruited from the Servicio de Alergia, Fundación Jiménez Díaz, Madrid, Spain. The criteria for selection were (1) a convincing clinical history of occupational asthma provoked by inhalation of wheat flour in the workplace (the baker's

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asthma condition was further confirmed by specific inhalation challenge tests in all recruited patients, using methods previously described²²); (2) a positive skin prick test (SPT) response to a homemade wheat flour extract; and (3) a specific IgE level to wheat flour greater than 0.35 kU/L (mean, 16.3 kU/L; range, 0.51 to >100 kU/L), as determined using the Phadia CAP System FEIA (Phadia, Uppsala, Sweden). Demographic and clinical data of the studied patients are summarized in Table 1. Seven patients (patients 1 to 7 in Table 1) also showed allergy to kiwifruit, as indicated by a consistent clinical history of repeated episodes of oral allergy syndrome (itching and/or swelling of the oral mucosa and lips) on ingestion of this fruit. All these kiwi allergic patients showed specific IgE and a positive SPT response to kiwifruit. The remaining patients (patients 8 to 20) ate kiwi on a regular basis without any ill effect. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Fundación Jiménez Díaz (Madrid).

Four pools of sera were used for immunodetection assays: (1) 20 serum samples from all recruited patients, (2) 7 serum samples from patients with baker's asthma and allergy to kiwifruit, (3) 13 serum samples from selected patients non-sensitized to kiwifruit, and (4) a negative control pool from 3 bakers nonallergic to wheat (negative specific IgE and SPT response). Individual serum samples from study patients and control bakers were used in specific IgE determination and inhibition ELISAs.

Preparation of Extracts From Cereal Flours and Kiwifruit
Flours from bread wheat (*Triticum aestivum* cv Chinese Spring) and rye (*Secale cereale* cv INIA C/171M) were defatted with cold acetone (2 × 1:10 [wt/vol], 2 hours, 4°C), dried, and then extracted with 0.5M sodium chloride (2 × 1:5 [wt/vol], 1 hour, 4°C). The salt extract (albumin plus globulin protein fraction) was centrifuged (12,100g, 30 minutes, 4°C), and the supernatant dialyzed (cutoff point, 3.5 kDa) against water and freeze-dried. This salt extract comprised the most relevant allergens associated with baker's asthma.^{4,5}

Fruits of green kiwi (*A. deliciosa* cv Hayward) were peeled, cut into short pieces, and freeze-dried. The dry pulps were defatted with cold acetone (2 × 1:5 [wt/vol], 1 hour, 4°C) and then with ethanol and ether (1:3 [vol/vol]; 3 × 1:5 [wt/vol], 1 hour, 4°C). After drying, the defatted pulps were extracted with phosphate-buffered saline (PBS) buffer (0.1M sodium phosphate, pH 7.0, 0.15M sodium chloride; 1 × 1:5 [wt/vol], 1 hour, 4°C). After centrifugation (12,100g, 30 minutes, 4°C), the supernatant was treated with (NH₄)₂SO₄ (80% saturation) and then centrifuged under the same conditions. The precipitate was dissolved in PBS buffer, dialyzed (cutoff point, 3.5 kDa) against water, and freeze-dried. The main kiwifruit allergens (Act d 1, Act d 2, and Act d 3) have been previously shown to be extracted by this protocol.¹⁶ Protein concentration was quantified according to the method of Bradford.²³

Table 1. Demographic and Clinical Data of the Study Patients

Patient No.	Age, y/ sex	Specific IgE (CAP), kU/L		Specific IgE to kiwi (ELISA), OD units	Skin prick test, mm			Symptoms with kiwi	Sensitization to other noncereal plant foods
		Wheat	Rye		Wheat	Rye	Kiwi		
1	35/M	74.2	20.7	1.7	7	8	12	OAS	K, W
2	29/M	18.0	7.9	1.2	6	3	5	OAS	K, M
3	27/M	7.8	7.4	0.4	5	4	8	OAS	K, M, W, C, S
4	22/M	3.0	2	0.6	3	5	6	OAS/CU	K
5	26/M	>100	>100	0.4	7	4	10	OAS	K, M, A, B, C
6	21/F	19.0	17.9	2.0	4	3	4	OAS	K, M, A, P, Ma
7	30/M	2.4	1.7	0.4	4	... ^a	6	OAS	K, M, Pe
8	36/M	1.6	1.2	0.4	7	6	...	No	Pn
9	39/M	25.8	15.6	...	5	7	...	No	No
10	35/M	24.3	39.3	...	8	4	ND	No	No
11	28/M	2.5	3.8	1.2	4	4	3	No	No
12	60/M	3.4	4.0	0.5	6	3	...	No	No
13	54/M	2.4	0.6	...	3	5	9	No	No
14	27/M	5.0	5.0	...	7	5	...	No	No
15	42/F	3.7	2.7	...	6	5	...	No	No
16	54/M	1.6	1.1	...	6	7	6	No	No
17	26/M	74.6	58.4	...	11	...	...	No	No
18	43/M	13.8	26.9	...	12	3	...	No	No
19	34/F	2.0	1.0	...	4	3	...	No	No
20	41/F	1.7	0.7	...	3	3	...	No	No

Abbreviations: A, avocado; B, banana; C, chestnut; CU, contact urticaria; ELISA, enzyme-linked immunosorbent assay; K, kiwi; M, melon; Ma, mango; ND, not determined; OAS, oral allergy syndrome; P, pineapple; Pe, peach; Pn, peanut; S, sunflower seed; W, walnut.

^a Ellipses indicate that the OD (optical density) value was less than 0.32 units or the skin prick test response was less than 3 mm.

Isolation and Identification of Act d 1 and Act d 2 Allergens

The kiwifruit extract was fractionated by cation-exchange chromatography on a Mono S HR 5/5 column (Amersham Bioscience, Uppsala, Sweden), equilibrated with 20-mmol/L formic acid (pH 4.0), and eluted with a linear gradient of sodium chloride in the same buffer (0–0.5M in 140 minutes; 0.5 mL/min). Fractions were dialyzed (cutoff point, 3.5 kDa) against water, freeze-dried, and redissolved in water, and their protein content was quantified by the commercial bicinchoninic acid test (Pierce, Cheshire, England). The fractions containing Act d 1 or Act d 2 were identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) fractionation and IgE immunodetection with a serum pool from patients with baker's asthma with allergy to kiwi (Table 1 and Fig 1). Purity of the isolated allergens was confirmed by the presence of a single electrophoretic band of the expected apparent molecular size and no heterogeneity in the determined N-terminal amino acid sequence (30 kDa and LPSYV for Act d 1 and 20 kDa and ATFNI for Act d 2). Both sequences perfectly matched with those previously reported for kiwi actinidin¹⁴ and thaumatin-like protein,¹⁵ respectively.

Immunodetection

Extracts from wheat, rye, and kiwifruit (15 µg of protein), purified kiwi allergens (3 µg), and commercial plant glycoproteins (3 µg of horseradish peroxidase and bromelain; Sigma, St Louis, Missouri) were separated by SDS-PAGE on Bio-Rad Miniprotein System gels (15% polyacrylamide), according to Laemmli,²⁴ and electrotransferred (transfer buffer: 50-mmol/L Tris, 50-mmol/L boric acid, pH 8.3) to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts). After blocking with 5% bovine serum albumin (BSA) (wt/vol) in PBS buffer, replica membranes were incubated overnight with each pool of sera from patients with baker's asthma or control bakers described herein (patients and sera, 1:5 dilution), washed, and then treated with mouse anti-human IgE monoclonal antibody HE-2 ascitic fluid (1:3,000 dilution, 1 hour).²⁵ After washing, a rabbit anti-mouse IgG peroxidase-conjugated antibody (1:5,000 dilution, 1 hour; Dako A/S, Glostrup, Denmark) was added, and detec-

tion of IgE-binding components was achieved by enhanced chemiluminescence, following the manufacturer's instructions (Amersham Biosciences, Little Chalfont, England).

Alternatively, replica blocked membranes were analyzed to detect potential CCDs, following a method previously reported.²⁶ Briefly, replica blocked membranes were immunodetected with an anticomplex asparagine-linked glycans rabbit serum (1:40,000 dilution; provided by Dr M. J. Chrispeels, University of California, San Diego, California), then treated with anti-rabbit IgG peroxidase-conjugated antibody (1:20,000 dilution; Sigma), and finally revealed by enhanced chemiluminescence.

Specific IgE Determination and Inhibition ELISAs

Specific IgE determination to wheat and rye flour and kiwi extracts (15 µg/mL), as well as to purified Act d 1, Act d 2 (3 µg/mL), and commercial bromelain (15 µg/mL), was performed by a method previously described,²⁷ testing the 3 different pools of sera (1:6 dilution) from patients with baker's asthma. BSA (3 µg/mL) and the serum pool from bakers nonallergic to wheat were used as negative controls. Specific IgE levels were considered positive when greater than 0.32 OD (optical density) units (the highest value of mean + 3 × SD of the OD determined for the different negative controls).

Inhibition ELISAs were performed by the same method, using 15 µg/mL of wheat or kiwifruit extract as solid phase, the same extracts and purified Act d 1, Act d 2 (3 µg/mL), and bromelain (15 µg/mL) as inhibitors, and different individual sera (1:6 dilution) from the selected patients. Each serum was preincubated with the corresponding inhibitor during 3 hours at 25°C. All tests were performed in duplicate.

Statistical Analysis

Comparisons between groups (patients with baker's asthma with or without associated kiwi allergy) were performed using the Mann-Whitney *U* test involving nonparametric data. The prevalences of sensitization to individual allergens were compared by the χ^2 test with Yates correction. Correlation between variables was analyzed by the Spearman ρ . $P < .05$ was considered statistically significant. All tests were

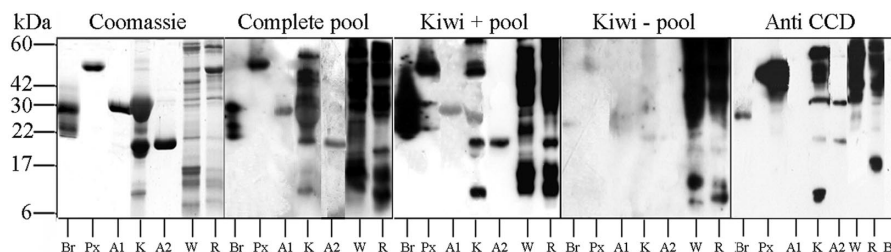


Figure 1. Protein staining (Coomassie) and immunodetection with IgE of a serum pool from all ($n = 20$) selected patients with baker's asthma (complete pool), those with ($n = 7$; kiwi + pool) or without ($n = 13$; kiwi - pool) associated kiwi allergy, or IgG of a rabbit polyclonal serum to plant complex glycans (anti-CCD) of the following samples separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis: plant glycoproteins bromelain (Br) and horseradish peroxidase (Px), purified kiwi allergens Act d 1 (A1) and Act d 2 (A2), and extracts from wheat (W) and rye (R) flour and kiwifruit (K). Bovine serum albumin (Bs) was used as negative control for anti-CCD.

performed using SPSS statistical software version 12 for Windows (SPSS Inc, Chicago, Illinois).

RESULTS

Seven of 20 recruited patients with baker's asthma (35%) reported local symptoms (oral allergy syndrome) on kiwifruit ingestion (Table 1), and for this reason they avoided eating this fruit. All these kiwi allergic patients showed specific IgE and positive SPT response to this fruit, whereas such test results were positive in only 4 (IgE) and 3 (SPT) of the 13 patients without clinical symptoms to kiwi. Interestingly, most of the patients with kiwifruit hypersensitivity were also sensitized to other noncereal plant food, particularly to melon (Table 1).

The differences summarized herein between both subgroups (with or without kiwi allergy) of patients with baker's asthma were reflected in the immunoblots obtained using the corresponding pool of sera from each subgroup (Fig 1). IgE from the serum samples of patients with kiwi allergy recognized a wide array of bands from approximately 6 kDa to above 60 kDa in the kiwifruit, wheat, and rye flour extracts. Besides, IgE bound to purified Act d 1 and Act d 2 and strongly to model plant glycoproteins harboring CCDs, such as horseradish peroxidase and bromelain. This IgE recognition profile was similar to that revealed when testing the serum pool from all selected patients with baker's asthma (Fig 1, complete pool). In contrast, the pool of sera from the subgroup of bakers not sensitized to kiwifruit showed a strong reaction with widespread molecular size components of the cereal extracts but a weak, or even null, recognition of the extract and isolated allergens from kiwifruit and of plant glycoproteins (Fig 1, kiwi-pool). Plant complex glycans (CCDs) were detected by a specific rabbit antiserum (Fig 1, anti-CCDs), mainly in 30- to 60-kDa glycoproteins of the

wheat, rye, and kiwifruit extracts, the bromelain and peroxidase positive controls, and purified Act d 2 (2 bands of 20 and 30 kDa, suggesting different glycosylation degrees of the isolated allergen). No reaction was observed in the Act d 1 and BSA (negative control) samples, as expected.

ELISAs to quantify specific IgE levels to the different extracts and purified allergens indicated differences between the positive and negative kiwi allergic subgroups of selected patients (Fig 2). Percentages of patients with positive IgE levels, as well as mean IgE values, to wheat and rye extracts and bromelain were roughly similar in both subgroups. Conversely, the positive and negative kiwi allergic patients showed different degrees of positive response to the kiwi extract (71% vs 46%), Act d 1 (57% vs 15%), and Act d 2 (43% vs 8%). Statistical differences were detected for Act d 1 ($P = .05$) but not in other comparisons, probably by the limited number of samples analyzed. A strong correlation was found between the levels of specific IgE to wheat flour and kiwi ($r = .79$; $P < .001$).

Inhibition ELISAs testing isolated kiwi allergens and bromelain (CCDs) as inhibitors were performed with individual sera from the selected patients with baker's asthma (Fig 3). The results suggested a wide and different profile of sensitization among patients but uncovered a potential role of Act d 1 (and homologous thiol-proteases) and CCDs as allergens responsible for wheat-kiwi cross-reactivity. Inhibition values (more than 75%) observed for Act d 1 (serum sample 1) and bromelain (serum sample 6) of the IgE binding by both wheat and kiwi extracts illustrated the aforementioned issues. However, an extract-specific response to these allergens was detected in other sera (ie, serum samples 3, 4, and 5 for Act d 1 and serum samples 2 and 7 for bromelain). Kiwi Act d 2 did not seem to be a potent inhibitor against the wheat extract,

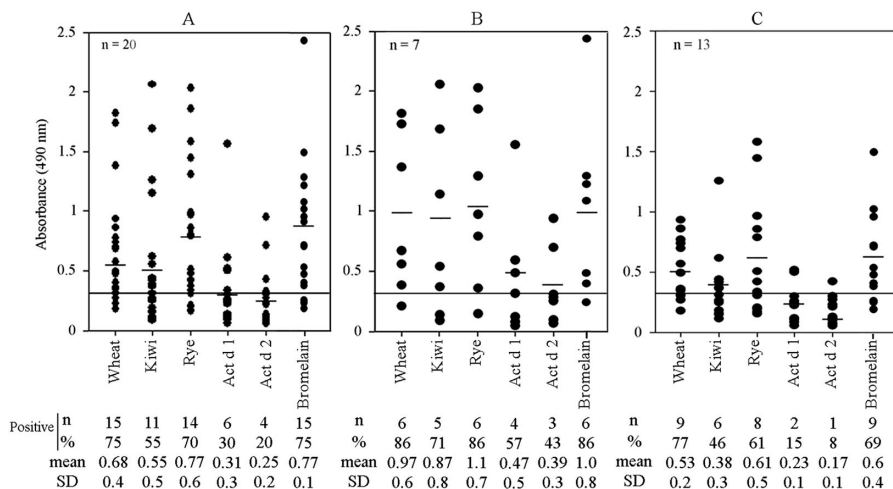


Figure 2. Specific IgE levels (OD units) to wheat, rye, and kiwi extracts and purified Act d 1, Act d 2, and bromelain of individual serum samples from 20 patients with baker's asthma. A, Whole set of individual sera; B and C, serum samples from the subgroup of patients with ($n = 7$) or without ($n = 13$) associated kiwi allergy. Values of 0.32 OD units or more (horizontal lines) are considered positive (see the "Methods" section). Mean values are marked (—) for each solid phase and serum pool.

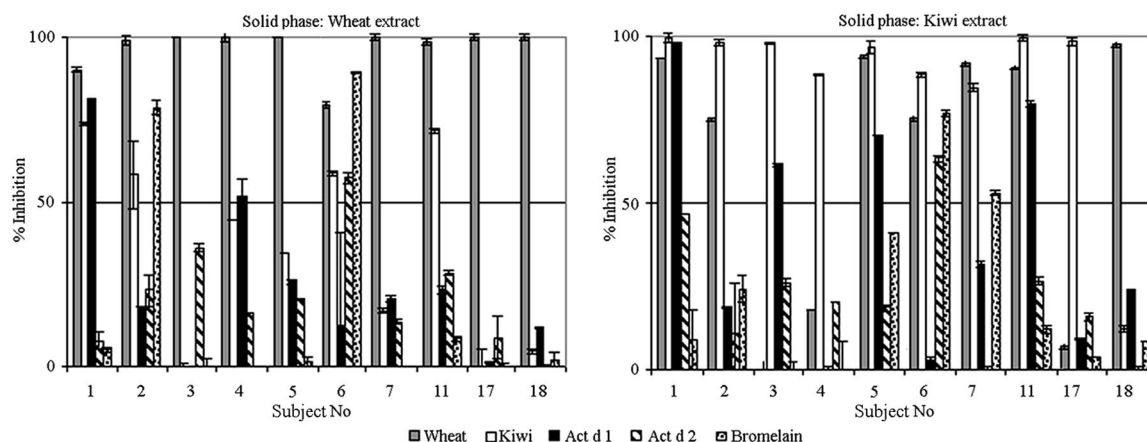


Figure 3. Inhibition enzyme-linked immunosorbent assays with the wheat or kiwi extract as solid phase and both extract and purified Act d 1, Act d 2, and bromelain as inhibitors. Patient numbers are as in Table 1.

except in the case of serum sample 6, which was also highly inhibited by CCDs (bromelain).

DISCUSSION

No association between allergy caused by inhalation of wheat flour (or other cereals) and allergy to kiwifruit ingestion has been previously reported. In contrast, a link between kiwi and latex allergy is well established as a variant of the latex fruit syndrome,^{11,13} although the allergens responsible for such cosensitization remain to be truly identified.^{16,28}

A recent study of patients with baker's asthma in Spain²² has allowed the identification of the potential wheat-kiwi cross-reactivity herein presented based on comparison of clinical and immunologic data between subgroups of patients with or without kiwi allergy. An unexpected proportion (35%) of the recruited patients with baker's asthma experienced oral allergy syndrome on kiwifruit ingestion and showed specific IgE and a positive SPT response to this fruit. In contrast, only 1 of 13 patients without clinical kiwi hypersensitivity displayed both positive IgE and SPT results. Furthermore, IgE of sera from the subgroup of patients with kiwi allergy exhibited a strong recognition of at least 5 components from 6 to 60 kDa of the kiwifruit extract, as well as of purified allergens Act d 1 and Act d 2 in immunodetection assays. Furthermore, a substantial number of these patients had specific IgE to the kiwi extract (71%) and to the purified

kiwi allergens (43% to 57%). Conversely, very weak immunodetection responses and a lower proportion (8% to 15% for the isolated allergens) of positive serum samples were found when testing IgE from the subgroup of patients without kiwi allergy.

The strong IgE-binding capacity of bromelain (and horseradish peroxidase) detected by both immunodetection and direct ELISAs together with its high inhibition values (>50%) against wheat and/or kiwi extract observed for 3 of 7 individual serum samples from the kiwi allergic patients point to CCDs (plant complex glycans) as being responsible for wheat-kiwi cross-reactivity. The same is true regarding Act d 1 (actinidin). A search of databanks looking for cereal proteins homologous to actinidin revealed the presence in wheat grain of α -tritricain, a member of a putative thiol-protease family, which shared 55% of amino acid sequence identity with Act d 1 (Fig 4). Actinidin Act d 1 has been defined as a major kiwifruit allergen,^{14,17,18} although recent data suggest a null recognition of the allergen by IgE of serum samples from kiwi allergic patients in the United Kingdom.²⁹ According to the results reported herein, Act d 1 is a major allergen in patients with both allergy to kiwifruit and asthma due to cereal flour inhalation; furthermore, cross-reactivity between this allergen and thiol-proteases present in cereal flours could account for the wheat-kiwi cosensitization found in such patients. Future studies comprising large num-

Alpha-Triticain	LPETVDWRKKGA VAAIKDQGGCGSCWAFSAIAA VEGINQIVT GD MIPLSE QELVDCDTSYN-EGCNGGLMD
Act d 1	LPSYVDWRSAGAV VDIKSQGECGGCWAFSAIA TV EGINKIVTGV LISLSE QELIDCGRTQNT RG CNGGYIT
Alpha-Triticain	YAF EF LI INNGGID SEED YPYKERDNRCDANKKN AKVVTID GYEDVPVNSEK SLQ KAVAN QPI SV AI EAGGR
Act d 1	DC FQ FI INNGG INTE ENYPYTAQDGE CN LDLQNEKY VTID TYENVPY NE WALQTAVTY Q PVS VA LDAA GD
Alpha-Triticain	AF QLYK SGIFT GT CGT ALDHGVA AVGY TENGKDY W LVRNS WGT W GED GY IR MERNIKASSG KCG IA VEP
Act d 1	AF KHYSS GIFT GPCGT AI DH AV TI VG Y TE GGIDY W LVKNS WDT W GE EGY MR ILRN VGG -AGTCGIAT MP
Alpha-Triticain	S YPTKTGENPPDSYNECPASTTCCCIY
Act d 1	S YPVKYN-NQNSKDGPGVVDGQRYSA

Figure 4. Alignment of amino acid sequences of wheat α -tritricain (accession No. Q0WXG8) and kiwi allergen Act d 1 (accession No. P00785). Identical residues are in bold letters (identity, 54.9%).

bers of both kiwi and wheat allergic patients are necessary to confirm the potential link of these 2 allergenic sources, as well as to further search for the role of CCDs and homologous thiol-proteases in wheat-kiwi cross-reactivity.

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