

Kiwellin, a Novel Protein from Kiwi Fruit. Purification, Biochemical Characterization and Identification as an Allergen*

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Kiwellin is a novel protein of 28 kDa isolated from kiwi (*Actinidia chinensis*) fruit. It is one of the three most abundant proteins present in the edible part of this fruit. Kiwellin has been purified by ion exchange chromatography. Its N-terminal amino acid sequence revealed high identity with that previously reported for a 28 kDa protein described as one of the most important kiwi allergens. This observation prompted us to fully characterize this protein. The complete primary structure, elucidated by direct sequencing, indicated that kiwellin is a cysteine-rich protein. Serological tests and Western Blotting analysis showed that kiwellin is specifically recognized by IgE of patients allergic to kiwi fruit.

KEY WORDS: Allergen; amino acid sequence; food protein; kiwellin; kiwi fruit.

1. INTRODUCTION

Plant-derived food is not only a source of important nutritional compounds, but also a source of bioactive molecules able to have effects on human health beyond nutritional aspects (Clydesdale, 1997; Roberfroid, 1999). The important role played by fruit and vegetables can be clearly stated, however a continuous increase of allergic reactions to their components is reported (Cuesta-Herranz *et al.*, 2000; Pastorello *et al.*, 2002). Plant allergens, being one of the most widespread allergenic substances, are hard to avoid. Therefore, their identification

and characterization is one of the keys for the diagnosis and treatment of allergic diseases (Dong-Dong and Shao-Heng, 2003). It appears that the relatively high amount of these proteins in plant-derived food play an important role in sensitizing genetically predisposed patients (atopic patients).

Kiwi fruit is an example of food reported to have significant effects on human health. In Chinese traditional medicine it was used for cancer prevention and therapy (Motohashi *et al.*, 2002). More recently, results obtained from *in vivo* and *in vitro* experiments associated kiwi fruit to various biological effects, such as antiviral (anti-HIV) activity (Gavrovic-Jankulovic *et al.*, 2002), protection against oxidative DNA damage (Collins *et al.*, 2003), anticancer activity (Collins *et al.*, 2003; Motohashi *et al.*, 2002), etc. On the other hand, literature describing negative effects of kiwi fruit consumption on subjects predisposed to allergy is also becoming increasingly larger (Lucas *et al.*, 2003).

Allergy to kiwi fruit was first described in 1981 (Fine, 1981), and has since been extensively

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⁵ Abbreviations: FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis.

described in the last twenty years. A wide range of symptoms, including localized oral allergy (OAS), laryngeal edema, urticaria, vomiting, cardiovascular collapse and life threatening anaphylaxis, have been reported (Lucas *et al.*, 2003). Kiwi fruit allergy has been frequently associated with allergy to other foods and to birch (Pastorello *et al.*, 1996; Voitenko *et al.*, 1997) and timothy grass (Pastorello *et al.*, 1996) pollen.

Identification of major allergens in kiwi fruit has so far resulted in conflicting and confusing results both in terms of number and relevance of allergens. In fact, different studies reported different dominant allergens, probably due to differences in both experimental procedures or study population used (Lucas *et al.*, 2003). Only some of them have been isolated and characterized. Actinidin (a 30 kDa protein), designated Act c 1, has been described as a major kiwi fruit allergen, binding IgE from more than 90% of patients with kiwi allergy (Pastorello *et al.*, 1998). Further, a 24 kDa protein, reported as a potential major kiwi allergen and designated Act c 2, has been isolated and biochemically characterized as a thaumatin-like protein, belonging to the family of PR-proteins (Gavrovic-Jankulovic *et al.*, 2002). A third allergen, only partially characterized, is a 43 kDa protein accounting only for 0.1% of total kiwi protein content (Moller *et al.*, 1997).

In the framework of a proteomic analysis of kiwi fruit, the aim of this study was to further analyze kiwi extracts in order to characterize new allergens. Kiwellin, a novel protein of 28 kDa accounting for approximately one third of kiwi total protein content, was isolated and fully characterized. We report here the purification and the complete amino acid sequence of this protein, and its immunological characterization as a new kiwi fruit allergen.

2. MATERIALS AND METHODS

2.1. Materials

Kiwi fruits were purchased from local markets. Trypsin was from Boehringer; bovine serum albumin, dithiothreitol, Tris, and 4-vinylpyridine from Sigma; DEAE-cellulose (type DE52) and SP-Sepharose from Whatman. HPLC⁵-grade acetonitrile was from Baker. Sequencer-grade reagents were from Applied Biosystems. All other reagents were of the highest commercially available quality.

2.2. Preparation of Kiwi Extracts

Kiwi fruits were homogenized in water. The pH of the homogenate was 3.5. After centrifugation, the supernatant, representing the soluble fraction (SF), was collected for further analysis. The pellet, containing the cell wall fraction, was resuspended in 0.5 M NaCl, adjusted to pH 8.3 by addition of NaOH, and homogenized again. After centrifugation, the supernatant (cell wall salt extract, CW) was collected. A total protein extract was obtained homogenizing kiwi fruit in 0.5 M NaCl, and collecting the supernatant obtained after centrifugation.

2.3. Protein Purification

The CW fraction, dialyzed against 10 mM Tris-HCl, pH 7.5, was loaded on a DE52 column equilibrated in the same buffer. The protein was eluted in the column flow-through, which was adjusted to pH 5.0 and loaded on a SP-Sepharose column, equilibrated in 10 mM sodium acetate, pH 5.0 (buffer A). Elution was carried out by increasing the concentration of buffer B (50 mM sodium acetate, pH 5.0, containing 0.5 M NaCl). The fractions containing the 28 kDa band (kiwellin) upon SDS-PAGE were pooled, dialyzed against 10 mM sodium acetate, pH 5.0, and purified by FPLC ion-exchange chromatography carried out on a Mono-S HR 10/10 column (Amersham-Pharmacia), equilibrated in the same buffer (buffer A). Flowrate was 3 ml/min. Elution was carried out increasing the concentration of buffer B, as above. The absorbance was recorded at 280 nm.

Protein samples were concentrated by ultrafiltration using Centriplus YM-3 filters (Amicon, Millipore). Protein concentrations were determined by the BIO-RAD Protein Assay (BIO-RAD), using calibration curves made with bovine serum albumin. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% gels.

2.4. Amino Acid Sequencing

Denaturation and alkylation of the sulfhydryl groups with 4-vinylpyridine was carried out as described (Camardella *et al.*, 1995). Tryptic digestions were carried out at an enzyme:protein ratio of 1:20 (w/w) on pyridylethylated samples (0.5 mg/ml) in 1% ammonium bicarbonate, at 37°C for two hours. Pepsin digestion was carried out at an enzyme:protein ratio of 1:70 (w/w) on

pyridylethylated samples (1 mg/ml) in 5% formic acid, at 37°C for two hours.

Reverse-phase HPLC of kiwi extracts and of tryptic peptides was performed on a Vydac C₈ column (0.21×25 cm), using a Beckman System Gold apparatus. Elution was accomplished by a linear gradient of eluant B (0.08% trifluoroacetic acid in acetonitrile) in eluant A (0.1% trifluoroacetic acid) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm. The separated fractions were manually collected and analyzed as needed.

Amino acid sequencing of intact protein or of peptides obtained by proteolytic digestion was performed with an Applied Biosystems Procise 492 automatic sequencer, equipped with on-line detection of phenylthiohydantoin amino acids. Protein sequence analyses were performed using softwares available on the ExPASy Proteomics Server (<http://www.expasy.org>).

2.5. Skin Prick Test

Skin prick test was performed on the volar face of the distal forearm using a commercially available kiwi extract (Stallergen). The diluent used to preserve the allergen extract and a solution of histamine 10 mg/ml were used as negative and positive control, respectively. The reaction was regarded as positive if the mean wheal diameter was > 3 mm.

2.6. Serological Tests

Biotinylation of kiwellin was carried out by the ECL Protein Biotinylation Module (Amersham Biosciences), according to the manufacturer's instructions. Capture Assay Radim Liquid Allergens (CARLA, Radim) tests (Olivieri *et al.*, 1993), for rapid specific IgE automated testing, were carried out on biotinylated samples according to the manufacturer's instructions. Sera of subjects with a history suggestive of allergy to kiwi fruit, and 1:500 dilutions of a stock 1 mg/ml solution of kiwellin were used. Levels of specific IgE higher than 0.5 kU/L (≥class 1) were considered positive.

2.7. Western Blotting Analysis

Western blotting was carried out after SDS-PAGE of 4 µg of pure kiwellin and 20 µg of total protein extract from kiwi fruit, followed by electroblotting onto polyvinylidene difluoride (PVDF) membranes. Blotted proteins were stained

with ponceau red and washed with water, then kept over night at 4°C in a blocking solution made of PBS containing 0.1% tween and 5% milk protein (Sigma). After further washing with the same solution, membranes were incubated with primary antibodies (1:5 dilutions of sera of allergic subjects, containing specific IgE) for 1 hr at room temperature. After removal of primary antibodies by washing with PBS containing 0.1% tween, membranes were incubated with the secondary antibody, i.e. a 1:1000 dilution of a goat anti-human IgE polyclonal antibody conjugated to horseradish peroxidase (Bethyl Laboratories, Inc.). Finally, immunoreacting allergen bands were revealed by incubation for 1 min with ECLTM Western Blotting Detection Reagents (Amersham Biosciences). Non-specific binding was tested using serum from a non-allergic patient. Non-specific binding of the anti-IgE antibody conjugate, measured in a similar blotting procedure omitting the incubation step with patient serum, appeared to be negligible.

3. RESULTS AND DISCUSSION

3.1. Kiwi Extract Analysis

The SF fraction, the CW extract and the total protein extract were analyzed by SDS-PAGE (Fig. 1). Three major bands were observed in the total protein extract, at 30, 28 and 24 kDa. The 30 kDa band was more abundant in the SF fraction, the 28 kDa band was more abundant in the CW fraction, and the 24 kDa band was present in approximately similar amounts in both fractions. After electroblotting onto PVDF, these bands were analyzed by N-terminal amino acid sequencing. The 30 and 24 kDa bands were identified as actinidin and thaumatin-like protein, respectively.

3.2. Identification of Kiwellin as a New Protein of Kiwi Fruit

The N-terminal sequence of the 28 kDa band (Fig. 1), which proceeded for 30 amino acid residues, showed no significant identity with sequences of proteins available in Data Banks and having known function. Nevertheless, it showed high identity with the cDNA-derived sequence of Grip 22, a "ripening-related" putative protein from grape (Davies and Robinson, 2000). Moreover, it showed very high identity with the N-terminal sequence of a 28 kDa

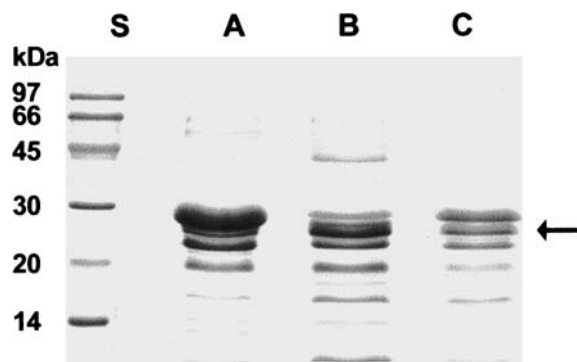


Fig. 1. 15% SDS-PAGE analysis of kiwi fruit extract. Soluble fraction (A, 20 µg), cell wall salt extract (B, 20 µg), and total protein extract (C, 10 µg). (S), low molecular weight standard proteins (Amersham Biosciences). Staining was carried out with Coomassie brilliant blue. The arrow indicates kiwelin.

protein described as one of the most important kiwi allergens (Pastorello *et al.*, 1998).

Following the purification procedure described in the Materials and Methods section, 4 mg of pure protein (Fig. 2), named kiwelin, were obtained from 100 g of peeled kiwi fruit.

3.3. Molecular Weight

The molecular weight of kiwelin, upon SDS-PAGE carried out under non-reducing conditions, was approximately 20 kDa (Fig. 2), different from that determined under reducing conditions (28 kDa). A similar behaviour was already described for other kiwi proteins (Gavrovic-Jankulovic *et al.*, 2002; Pastorello *et al.*, 1998). After reduction and pyridylethylation of cysteine residues, kiwelin showed a migration distance corresponding to a molecular mass slightly higher than 28 kDa, both under reducing and non-reducing conditions. This behaviour suggests that, as previously hypothesized for the thaumatin-like kiwi allergen (Gavrovic-Jankulovic *et al.*, 2002), the anomalous migration could be ascribed to the presence of disulfide bridges involving a high number of cysteine residues (see below).

3.4. Amino Acid Sequence Determination

After denaturation, reduction and alkylation of the sulfhydryl groups, kiwelin was digested with trypsin and with pepsin as described in Methods. The two hydrolysates were analyzed by reverse-phase HPLC (not shown). The chromatographic peaks were manually collected, vacuum-dried,

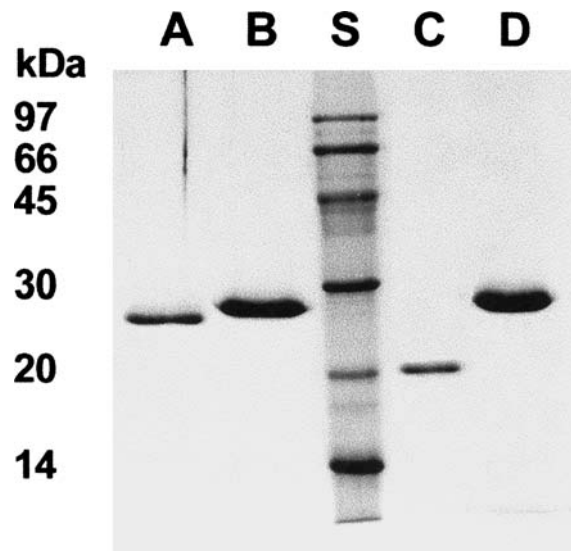


Fig. 2. 15% SDS-PAGE analysis of pure kiwelin under reducing (A, B) and non-reducing (C, D) conditions. Kiwelin (A, C), and pyridylethylated kiwelin (B, D). (S), low molecular weight standard proteins (Amersham Biosciences). Staining was carried out with Coomassie brilliant blue.

redissolved in 0.1% trifluoroacetic acid containing 20% acetonitrile, and sequenced. The complete sequence (Fig. 3) was established by alignment of the tryptic and peptic fragments with the sequence obtained from the N terminus of intact kiwelin and with the sequence of the above mentioned Grip 22 from grape (Davies and Robinson, 2000). Kiwelin contained 189 amino acid residues, and the sequence-derived molecular mass was 19,968 Da, in agreement with the data obtained upon SDS-PAGE carried out under non-reducing conditions (20 kDa). The protein contained 14 cysteine residues. A sequence heterogeneity was found at position 61, where His replaced Tyr in approximately 40% of the protein molecules.

3.5. Sequence Analysis

Homology search in Data Bank carried out by BLAST (Fig. 4) confirmed that the complete sequence of kiwelin has the highest identity (75%) with the cDNA-derived sequence of Grip 22 from grape (Davies and Robinson, 2000), whose mRNA is differentially expressed during fruit ripening and therefore defined “ripening-related”. High identity (ranging from 70% to 40%) was observed also with many genome-derived protein sequences from rice (*Oryza sativa*) of unknown function. The alignment

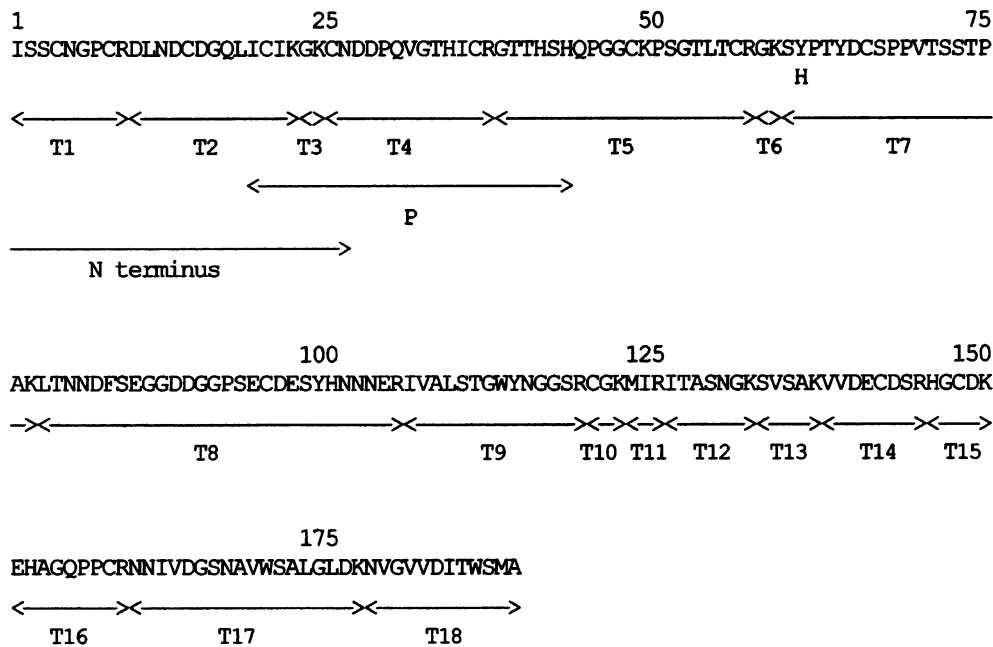


Fig. 3. Amino acid sequence of kiwelin. The tryptic peptides (T1–T18), a peptide obtained by hydrolysis with pepsin (P), and the sequence elucidated by automated Edman degradation from the N terminus are indicated below the sequence.

of kiwelin and Grip22 with the three most similar rice sequences (accession number Q7XVA8, Q6H5XO, Q9FWT5) showing 69, 68 and 55 percent identity, respectively, with the kiwi protein, is shown in Fig. 4. The similarities observed with other hypothetical plant (grape and rice) proteins

suggest that kiwelin is a member of a new class of proteins, whose biological role *in vivo* is worth to be investigated. A comparative analysis of the hypothetical tertiary structure of kiwelin, carried out using the 3D-PSSM programme (Kelley *et al.*, 2000), showed



Fig. 4. Alignment of the sequence of kiwelin with that of Grip22 from grape, and with those of three putative proteins from rice. Residues conserved in all sequences are grey-shaded.

Table 1. Skin Prick Test, Western Blotting, and Specific and Total IgE Levels of Patients Allergic (A, B) and Non-allergic (C) to Kiwi Fruit

Patient	Skin prick test	Western Blot	Specific IgE (kU/L)	Total IgE (kU/L)
A	+	+	1.2	250
B	-	+	0.9	230
C	-	-	0.1	25

fold similarities with different proteins such as, for example, Phl p 1, the 27 kDa major allergen from timothy grass (*Phleum pratense*) pollen (Ball *et al.*, 2005), a cysteine-rich glycoprotein representing one of the most important aeroallergens known to date. Similarities were also found with Barwin-like proteins, sharing a structural motif with various wound-induced and other pathogenesis-related proteins from potato, rubber trees and tobacco, and therefore suggested to be involved in widespread defense mechanisms in plants (Svensson *et al.*, 1992). The similarities observed are in line with the literature data indicating that in several cases plant defence proteins are also allergens (Hoffmann-Sommergruber, 2000).

3.6. Immunological Characterization

Two patients reporting significant symptoms upon ingestion of kiwi, and showing a positive (patient A) and a negative (patient B) skin prick test, were recruited for *in vitro* studies. The serum of a non-allergic individual (patient C) was used as a control. Sera of patients A and B contained specific IgE for kiwelling, as evaluated by performing the CARLA analysis with the biotinylated kiwi protein (Table 1). Western blotting analysis confirmed this result (Fig. 5). The total protein extract showed slightly different IgE-binding patterns with the individual sera. The components at approximately 28 (kiwelling) and 24 (thaumatin) kDa appeared as the most definite bands in both sera, whereas the bands at approximately 20 and 17 kDa showed a lower intensity in both sera. A component at approximately 14 kDa was detected only with the serum of patient B. Actinidin, showing a 30 kDa band on SDS-PAGE, was not recognized by the two sera.

Kiwi allergens at 14, 17, 20, 24 and 28 kDa had already been reported (Lucas *et al.*, 2003), although among them only the 24 kDa protein, identified as a thaumatin, was purified and characterized (Gavrovic-Jankulovic *et al.*, 2002). Kiwelling may correspond to the 28 kDa component

reported by Pastorello *et al.* (1998) as one of the most important kiwi allergens, since the N-terminal sequence reported is identical to that of kiwelling, except for two positions. In fact, we have identified in the reduced and alkylated kiwelling two Cys residues at position 4 and 8, instead of Arg and Ile, respectively. However, Pastorello *et al.* determined the sequence of the protein without previous alkylation treatment, therefore the Cys residues were not detectable under the experimental conditions of their analysis. Furthermore, the close similarity of the molecular weight may indicate identity of kiwelling with the 27 kDa component described by Gavrovic-Jankulovic *et al.* (2002), recognized by the sera of all the seven patients allergic to kiwi recruited for that study. Therefore, the results reported in this paper, together with the data available in the literature, indicate that kiwelling is an allergen of kiwi fruit. The recruitment of a higher number of patients allergic

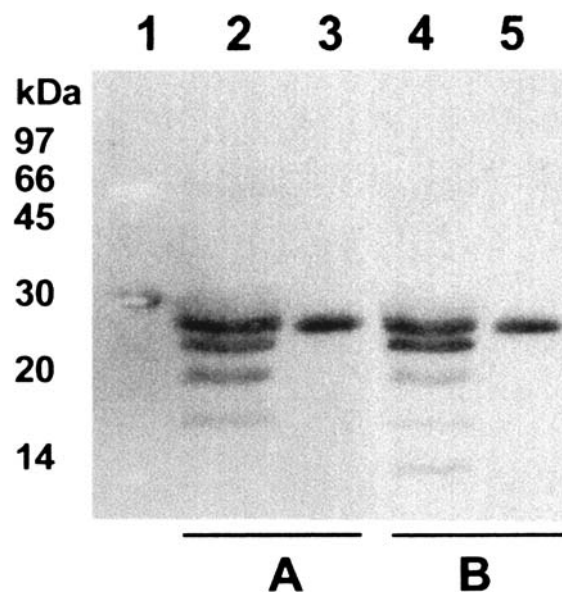


Fig. 5. Western blotting analysis of the binding between IgE of patients allergic to kiwi fruit and kiwelling. (A, B), patients with positive and negative skin prick test, respectively. Total kiwi protein extract (2, 4), and kiwelling (3, 5). (1), low molecular weight standard proteins (Amersham Biosciences).

to this fruit and the evaluation of their specific IgE produced against kiwellin will allow a better description of this allergen.

3.7. Conclusive Remarks

In most cases, protocols for the diagnosis of food allergy make use of total protein extracts. However, depending on the experimental procedure used and on the food characteristics (e.g., the ripening stage of a fruit), protein extracts may be variable in both the number and amount of the components. This heterogeneity may be at least one of the causes of some conflicting and confusing results reported in this field. Moreover, results obtained by using total protein extracts do not provide information about individual sensitivity towards single components of the investigated food, which would in turn result particularly useful in planning and monitoring desensitizing immunotherapies. Availability of purified and characterized allergens would help solving these problems, and also allow controlled and reproducible production of ipoallergenic derivatives (Bohle and Vieths, 2004).

In this framework, purification of kiwellin and its characterization as an important allergen of kiwi fruit add further insights into possible strategies for molecular diagnosis and therapy of kiwi allergy. Additional structural studies may shed light on the contribution of conformational epitopes in the recognition of specific IgE, and provide tools for studies on allergen-IgE interaction.

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REFERENCES

- Ball, T., Edstrom, W., Mauch, L., Schmitt, J., Leistler, B., Fiebig, H., Sperr, W. R., Hauswirth, A. W., Valent, P., Kraft, D., Almo, S. C., and Valenta, R. (2005). *FEBS J.* **272**: 217–227.
- Bohle, B., and Vieths, S. (2004). *Methods* **32**: 292–299.
- Camardella, L., Carratore, V., Ciardiello, M. A., Damonte, G., Benfatti, U., and De Flora, A. (1995). *Blood* **85**: 264–267.
- Clydesdale, F. M. (1997). *Nutr. Rev.* **55**: 413–422.
- Collins, A. R., Harrington, V., Drew, J., and Melvin, R. (2003). *Carcinogenesis* **24**: 511–515.
- Cuesta-Herranz, J., Lazaro, M., Figueredo, E., Igea, J. M., Umpierrez, A., and De-Las-Heras, M. (2000). *Clin. Exp. Allergy* **30**: 1411–1416.
- Davies, C., and Robinson, S. P. (2000). *Plant. Physiol.* **122**: 803–812.
- Dong-Dong, L. I., and Shao-Heng, H. E. (2003). *Acta Biochim. Biophys. Sinica* **35**: 789–792.
- Fine, A. J. (1981). *J. Allergy Clin. Immunol.* **68**: 235–237.
- Gavrovic-Jankulovic, M., Cirkovic, T., Vuckovic, O., Atanaskovic-Markovic, M., Petersen, A., Gojic, G., Burazer, L., and Jankov, R. M. (2002). *J. Allergy Clin. Immunol.* **110**: 805–810.
- Hoffmann-Sommergruber, K. (2000). *Int. Arch. Allergy Immunol.* **122**: 155–166.
- Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. E. (2000). *J. Mol. Biol.* **299**: 499–520.
- Lucas, J. S. A., Lewis, S. A., and Hourihane, J. O'B. (2003). *Pediatr. Allergy Immunol.* **14**: 420–428.
- Moller, M., Kayma, M., Steinhart, H., and Paschke, A. (1997). *Z. Lebensm. Unters. Forsch.* **205**: 364–369.
- Motohashi, N., Shirataki, Y., Kawase, M., Tani, S., Sakagami, H., Satoh, K., Kurihara, T., Nakashima, H., Mucsi, I., Varga, A., and Molnar, J. (2002). *J. Ethnopharmacol.* **81**: 357–364.
- Olivieri, V., Beccarini, I., Gallucci, G., Romano, T., and Santoro, F. (1993). *J. Immunol. Method.* **157**: 65–72.
- Pastorello, E. A., Conti, A., Pravettoni, V., Farioli, L., Rivolta, F., Ansaloni, R., Ispano, M., Incorvaia, C., Giuffrida, M. G., and Ortolani, C. (1998). *J. Allergy Clin. Immunol.* **101**: 531–537.
- Pastorello, E. A., Pravettoni, V., Calamari, A. M., Banfi, E., and Robino, A. M. (2002). *Allergy* **57**: 106–110.
- Pastorello, E. A., Pravettoni, V., Ispano, M., Farioli, L., Ansaloni, R., Rotondo, F., Incorvaia, C., Asman, I., Bengtsson, A., and Ortolani, C. (1996). *J. Allergy Clin. Immunol.* **98**: 601–610.
- Roberfroid, M. B. (1999). *J. Nutr.* **129**: 1398S–1401S.
- Svensson, B., Svendsen, I., Hojrup, P., Roepstorff, P., Ludvigsen, S., and Poulsen, F. M. (1992). *Biochemistry* **31**: 8767–8770.
- Voitenko, V., Poulsen, L. K., Nielsen, L., Norgaard, A., Bindslev-Jensen, C., and Skov, P. S. (1997). *Allergy* **52**: 136–143.