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# Influence of the Natural Ripening Stage, Cold Storage, and Ethylene Treatment on the Protein and IgE-Binding Profiles of Green and Gold Kiwi Fruit Extracts

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Kiwi fruit is an important source of food allergens, the number and relevance of which are still the object of investigation. Following a comparative analysis of the protein profiles in SDS-PAGE and IgE immunoblotting, a significant influence of conditions such as the ripening stage and the extraction method on the composition of green and gold kiwi fruit extracts was observed. Furthermore, the experimental data indicate that, mostly in the green species, a ripe fruit may have a different concentration of total proteins and a different amount of single components when ripeness is reached by different means of postharvest handling, such as ethylene exposure with or without previous cold storage. In summary, this study emphasizes the level of complexity associated with the preparation of extracts when a known and defined concentration of proteins/allergens is requested.

**KEYWORDS: Kiwi fruit; protein extracts; IgE binding; ripening; postharvest treatment**

## INTRODUCTION

Several literature reports describe kiwi fruit as an important source of food allergens. Ingestion of this fruit may elicit allergic reactions characterized by a wide range of symptoms, including local oral mucosa reactions, laryngeal edema, urticaria, vomiting, asthma, cardiovascular collapse, and life-threatening anaphylaxis (reviewed in ref *1*). Kiwi fruit allergy has been frequently associated with allergy to other foods including apple, avocado, banana, carrot, celery, hazelnut, and potato (*2*−*5*) and to pollens, such as those from birch, timothy grass, rye, and mugwort (*6*−*8*).

Following studies performed on protein extracts from green kiwi (*Actinidia deliciosa*) fruit (GrK), different authors have described different profiles of IgE-binding components. Results from Western blotting experiments provided evidence of IgE-binding bands of several molecular masses, including 13, 22, 30, and 67 kDa (*4*); 10−25 kDa (*7*); 12, 17, 24, and 28 kDa (*6, 9*); 15−94 kDa (*8*); 11, 22, 26, 27, and 32 kDa (*10*); 12, 24, 30, and 66 kDa (*11*); and 20 and 28 kDa (*12*).

Data concerning the number of allergenic proteins in kiwi fruit extracts, and their clinical relevance, are still fragmentary and sometimes conflicting, although several allergenic proteins have been officially named. Beyond variations related to the different studied cohorts and the experimental procedures, the fruit characteristics, depending on both ripening stages and storage treatment, might account for some of the observed mismatching results. Furthermore, in line with a recent paper (*12*), the presence in the GrK of a proteolytic activity due to the high amount of actinidin can provide a significant contribution affecting the profile of the IgE-binding components found in the protein extracts.

Several IgE-binding proteins have been identified in kiwi fruit (www.allergome.org), such as the above-mentioned actinidin (Act d 1), a thaumatin-like protein (TLP, Act d 2) (*13*), a 40-kDa protein (Act d 3) (*14*), kiwellin (Act d 5) (*15*), a phytocystatin and a chitinase (*10*), and several others. Actinidin and kiwellin (*12*) have also been found in the gold kiwi (*Actinidia chinensis*) fruit (GoK) (named Act c 1 and Act c 5, respectively). A biochemical characterization is available only for a few of these proteins, such as actinidin (*16*−*18*), TLP (*13, 19*), and kiwellin (*15, 20, 12*), but the characterization of their allergenicity is still very insufficient.

Although very often discrepancies in the results obtained in different laboratories are emphasized in the literature, a systematic study of the conditions that might affect the protein and

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allergen composition of kiwi fruit extracts has not been reported so far. To address this topic, a survey of the protein components in GrK and GoK fruits at different ripening stages and after postharvest handling has been performed using different extraction methods. Furthermore, the variation of the IgE-binding profiles in the protein extracts has been investigated using characterized sera from patients allergic to kiwi fruit.

## MATERIALS AND METHODS

**Kiwi Sample Collection.** Kiwi fruits, *A. deliciosa* cv. Hayward (green kiwi fruit, GrK) and *A. chinensis* (gold kiwi fruit, GoK), were from Italian plantations. Samples of GrK and GoK fruits were collected every 2 weeks, approximately, from July to November/December 2006. They were stored at −80 °C, after peeling and slicing. GrK and GoK samples were then collected at the ripening stage of the commercial harvest, with the following harvest indices: 6.2 and 9 °Brix for soluble solids content (SSC, determined by the MVM portable refractometer), 7 and 6 kg/0.5 $cm^2$ for flesh firmness (determined by the EFFEGI penetrometer equipped with a 8 mm tip), for GrK and GoK, respectively. They were divided into three aliquots. The first one was immediately frozen at −80 °C. The second one was stored at approximately 20 °C in a closed paper box, in presence of an equal amount (weight/weight) of apples as providers of ethylene until softening was achieved (12 days). This procedure did not allow the estimation of the exact ethylene quantity to which the fruits were exposed. Nevertheless, because kiwi fruits are extremely sensitive to ethylene, we can assume that it was largely exceeding the minimum amount able to induce fruit softening (5−10 ppb, see http://postharvest.ucdavis.edu/Produce/ProduceFacts/Fruit/kiwi.shtml for reference). The third aliquot was stored at 4 °C for approximately 2 months, followed by ethylene exposure until softening was achieved (1 week, approximately). Samples were also collected 40 and 30 days after the stage of commercial harvest of GrK and GoK fruits, respectively.

Samples of GrK fruits were also collected from July to December 2004. Their analysis (data not shown) produced results very similar to those obtained from the samples collected in the year 2006.

**Chemicals.** Bovine serum albumin (BSA) and Tris were from Sigma (Milan, Italy); acrylamide, bromochloroindolylphosphate (BCIP), nitroblue tetrazolium (NBT), and PVDF membrane were from Bio-Rad (Hercules, CA). Sequencer grade reagents were from Applied Biosystems (Foster City, CA). All other reagents were of the highest commercially available quality.

**Protein Extract Preparation.** For each trial three kiwi fruits were separately homogenized after the addition of distilled water at a 1:1 (w/w) ratio and analyzed. After centrifugation at 10400*g* for 30 min, the supernatant, representing the aqueous extract (AE), was collected for further analysis. The pellet, containing the cell wall fraction, was resuspended in 0.5 M NaCl and homogenized again. After centrifugation as above, the supernatant (salt extract of cell walls, SE) was collected. A total protein extract (TE) was obtained by homogenizing kiwi fruit after the addition of 1.0 M NaCl at a 1:1 (w/w) ratio and collecting the supernatant obtained after centrifugation.

Protein concentrations were determined by the Bio-Rad Protein Assay using calibration curves made with BSA.

**Protein Purification.** Actinidin, kiwellin, and KiTH were purified as previously described (*12*).

TLP was purified from kiwi fruit collected at the stage of commercial harvest. Kiwi fruits were homogenized in water. After centrifugation, the supernatant was discarded and the pellet, containing the cell wall fraction, was collected and homogenized again in the presence of 0.5 M NaCl, adjusted to pH 8.3. The extract, dialyzed against 10 mM Tris-HCl, pH 7.5, was loaded on a DE52 column equilibrated in the same buffer. Aliquots of the collected fractions were analyzed by reverse-phase HPLC on a Vydac $C_8$ column, eluted by a gradient of eluent B (0.08% TFA in acetonitrile) in eluent A (0.1% TFA). The protein was eluted in the column flow-through, which was adjusted to pH 5.0 and loaded on an 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 were analyzed by reverse-phase HPLC as reported above, and those containing TLP were pooled and dialyzed against 10 mM sodium acetate, pH 5.0, and further purified by FPLC ion-exchange chromatography carried out on a Mono-S HR 10/10 column (Amersham-Pharmacia, Milan, Italy), equilibrated in the same buffer (buffer A). The flow rate was 3 mL/min. Elution was carried out by increasing the concentration of buffer B, as above. The absorbance was recorded at 280 nm. The pure protein was finally obtained by elution from a gel filtration column Superdex 75 HR 10/30 (Amersham-Pharmacia) equilibrated in 50 mM Tris-HCl, pH 7.5, containing 0.25 M NaCl.

**Analysis of the Protein Components in SDS-PAGE and after Electroblotting.** The extracts were subjected to reducing 15% SDS-PAGE. Large volumes of protein extract solution were precipitated by adding 100% trichloroacetic acid (1:10, v/v). After 30 min on ice, the samples were centrifuged at 12000*g* for 10 min. The pellets were resuspended in the SDS-PAGE loading buffer and then loaded on the gel. Following electrophoresis, proteins were either stained with Coomassie Brilliant Blue or transferred onto PVDF membranes.

The analysis of the SDS-PAGE images and quantification of individual bands were carried out on dried gels. They were photographed and scanned on a Chemi Doc instrument (Bio-Rad), and the images were analyzed with the Quantity One software package (Bio-Rad). The relative intensity of the bands was expressed as percent of the sum of the intensity of all the examined bands within the sample lane.

For N-terminal amino acid sequence analysis, the transferred proteins were stained with Coomassie Brilliant Blue and the protein bands, after excision, were subjected to automated Edman degradation by an Applied Biosystems Procise 492 Automatic Sequencer (Applied Biosystems).

For immunoblotting experiments, the transferred proteins were stained with ponceau red.

**IgE Immunoblot.** Serum samples were obtained after informed written consent from kiwi-allergic subjects after the study protocol had been approved by the Institutional Ethical Committee (IDI-CE 192/2006). Serum pools were obtained by mixing equal volumes of individual sera from three subjects. Kiwi allergy was established on the basis of reliable reported clinical history, positive skin test with a commercial preparation or with the fresh fruit, and positive IgE detection on CAP system (Phadia, Uppsala, Sweden). IgE immunoblotting was carried out using the blocking solution and goat anti-human IgE contained in the Enzallergy Specific E kit (Bioallergy, Fiumicino, Italy). After washing with the blocking solution, membranes were incubated with the primary antibodies (1:4 dilutions of the kiwi allergic subject pooled sera) for 3 h. IgE−allergen complex was detected by incubation with secondary antibody, a 1:20 dilution of goat anti-human IgE conjugated to alkaline phosphatase for 18 h, followed by incubation with BCIP/NBT solution prepared according to the manufacturer. Nonspecific binding of the anti-IgE antibody conjugate was estimated in a similar blotting procedure, omitting the incubation step with patient pooled sera.

## RESULTS AND DISCUSSION

The aqueous extract (AE), the salt extract (SE), and the total protein extract (TE) were obtained as described under Materials and Methods.

**Influence of the Ripening Stage on the Protein Content of Kiwi Fruit TE.** Starting from the stage at which the fruit of each species reached its final size (approximately 110 days before the stage of commercial harvesting), the time course of the variation of the protein concentration was investigated. Rapidly increasing protein concentration values were observed in the time range from 110 ($t = -110$ days) to 40 ($t = -40$ days) days, approximately, before the stage of commercial harvesting ($t = 0$ days). Afterward, the protein concentration increased more slowly (**Figure 1**).

**Figures 2** and **3** show the SDS-PAGE analysis of the TE obtained from 10 mg of samples of GrK and GoK pulp. Variations in the relative amounts of the components were observed in the GrK (**Figure 2**) and, to a lesser extent, in the

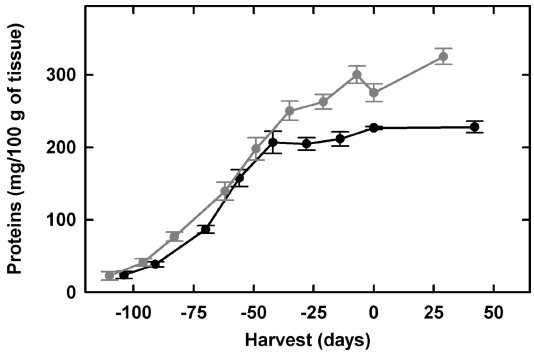


**Figure 1.** Variation of protein concentration, determined by the Bio-Rad Protein Assay, in the TE extracts as a function of the ripening stage of GrK (black) and GoK (gray). Bars indicate deviations from the mean values of three different measurements. The *X*-axis indicates the days of fruit harvest before (−) or after the stage of commercial harvest (time = 0).

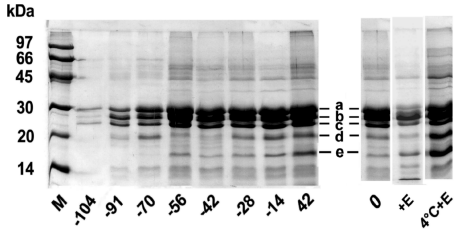


**Figure 2.** SDS-PAGE of the GrK total extracts obtained from 10 mg of fruit. Numbers below the lanes indicate the days before (−) and after the stage of commercial harvest (0). Extracts from fruits subjected to ethylene treatment with or without previous cold storage are indicated by 4 °C+E and +E, respectively. Actinidin, kiwellin, TLP, and the 20 and 17 kDa bands are indicated by a, b, c, d, and e, respectively. M, molecular weight markers.

GoK (**Figure 3**) extracts. Three proteins already appear as major components of the GrK extracts at the early harvesting stages, and their concentration increases during ripening. Electroblotting onto PVDF membrane and N-terminal amino acid sequencing of the GrK samples collected at two different stages ($t = -70$ days and $t = +42$ days) allowed the identification of these three components as the already known allergenic proteins actinidin (Act d 1), kiwellin (Act d 5), and TLP (Act d 2), showing 30, 28, and 24 kDa bands, respectively (**Figure 2**). In the GoK extracts there was only one major component, already described (*12*) as kiwellin (Act c 5) at every ripening stage (**Figure 3**), as confirmed by N-terminal amino acid sequencing of the electroblotted band from the two extracts of the GoK samples harvested at −83 and +29 days from the stage of commercial harvest.

In GrK, a 20 kDa band appeared, displaying the highest relative abundance at $t = -70$ days, decreasing at $t = -56$ days and again increasing during the final ripening stages. No sequence was detected for the band at $t = -70$ days. Attempts to determine the N-terminal amino acid sequence after deblocking reaction according to the method of ref *21* were unsuccessful, suggesting that the blocked N-terminal residue is not a serine or threonine. On the other hand, the band detected at the final ripening stages was identified by N-terminal amino acid sequencing as KiTH ( (*12*), see below). These results suggest the presence in GrK of two different protein components showing a 20 kDa band.

Recently, it has been reported that kiwellin may undergo in vivo proteolytic processing by actinidin. Kissper, a 4 kDa peptide (*20*), and the above-mentioned KiTH, a 16 kDa protein

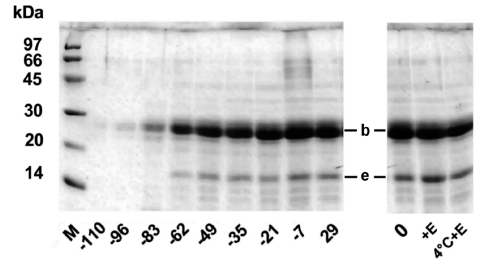


**Figure 3.** SDS-PAGE of the GoK total extracts obtained from 10 mg of fruit. Numbers below the lanes indicate the days before (−) and after the stage of commercial harvest (0). Extracts from fruits subjected to ethylene treatment with or without previous cold storage are indicated by 4 °C+E and +E, respectively. Kiwellin and the 17 kDa band are indicated by b and e, respectively. M, molecular weight markers.

migrating as a 20 kDa band in reducing SDS-PAGE (*12*), corresponding to the N-terminal and C-terminal regions of kiwellin, respectively, were identified as the products of the proteolytic cleavage. Actinidin, a thiol protease (*22*) present in a very high amount in GrK, is responsible for the processing of kiwellin (*12*). In GoK the presence of actinidin has also been reported (*23*), although in a much lower amount (*12*) than in GrK, and its protein band on SDS-PAGE is hidden by the high amount of kiwellin (**Figure 3**).

The data here reported indicate that the proteolytic processing of kiwellin is dependent on the ripening stage of the fruit. Nevertheless, it is worth noting that actinidin is already a major component of GrK during the early ripening stages, although a high concentration of KiTH has been observed only at the final ripening stages, suggesting that a regulatory control of the processing occurs. The presence in kiwi fruit of the thiol protease inhibitor phytocystatin has been reported (*24*), which, perhaps, may play a role in the regulation of the proteolytic activity of proteases such as actinidin.

A component showing a 17 kDa band was observed in both GrK and GoK extracts at $t = -56$ and $t = -62$ days, respectively, and its concentration increased during the ripening process. Experiments of IgE immunoblot showed that several individual sera of patients allergic to kiwi fruit recognized this protein (not shown), thus suggesting it as a potential new allergen. The N-terminal amino acid sequencing of the electroblotted band did not produce any sequence, suggesting a blocked N-terminus. Similar to the GrK 20 kDa band at $t = -70$ days (see above), deblocking reaction (*21*) was unsuccessful, suggesting that also in this case the blocked N-terminal residue is not a serine or threonine.

**Influence of Postharvest Handling on the Protein Profile of the Extracts.** The influence of postharvest handling on the protein content of the GrK and GoK extracts has been investigated by ethylene treatment with or without previous cold storage of samples of each species harvested at the ripening stage of commercial harvest.

The exposure of GrK and GoK fruits to the plant hormone ethylene produced a different effect on the protein concentration (**Figure 4**). In fact, the protein content of the GoK extracts was slightly increased as compared to the fruit without treatment, whereas a significant decrease of the protein concentration was measured in GrK. In contrast, several weeks of cold storage followed by ethylene exposure produced an opposite effect on the protein content of GrK, and an increase of the protein concentration was observed in the extract. The postharvest cold storage slightly affected the protein concentration of GoK, producing only a slight increase.

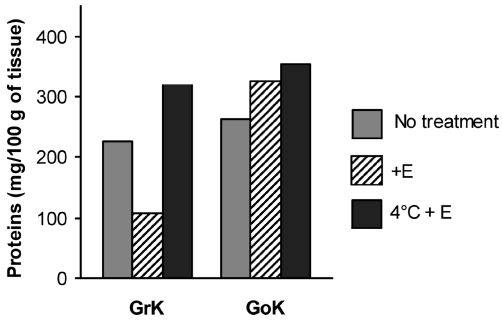


**Figure 4.** Protein content of extracts from fruits harvested at the stage of commercial harvest (no treatment) and subjected to ethylene treatment with (4 °C+E) or without (+E) cold storage. Protein concentration was determined by the Bio-Rad Protein Assay.

In line with literature reports on fruit subjected to postharvest treatments (*25*), the protein profiles obtained in SDS-PAGE show that the postharvest handling can affect the relative amount of some protein components (**Figures 2** and **3**). For instance, a GrK extract subjected to ethylene treatment after cold storage displays a relative abundance of protein components such as KiTH and the protein bands at 17 and 43 kDa higher than that observed in the extract of untreated fruit. The 43 kDa band was subjected to N-terminal amino acid sequencing of the electroblotted band. The sequence obtained (FTDGLIK) allowed its identification as Act d 3 (*14*). In contrast, the extract of ethylene-treated GrK without previous cold storage shows, at least apparently, only a slight variation in the relative amount of protein components. The postharvest handling of GoK apparently does not produce significant effects on the relative abundance of the major components.

The observation that the exposure of freshly collected GrK to ethylene produced a strong decrease of the total protein concentration suggests that a significant proteolytic degradation was induced. Because the ethylene treatment did not produce the same effect on GoK, a possible involvement of the high amount of the protease actinidin in GrK could be hypothesized. However, the SDS-PAGE profile of the extracts suggests that

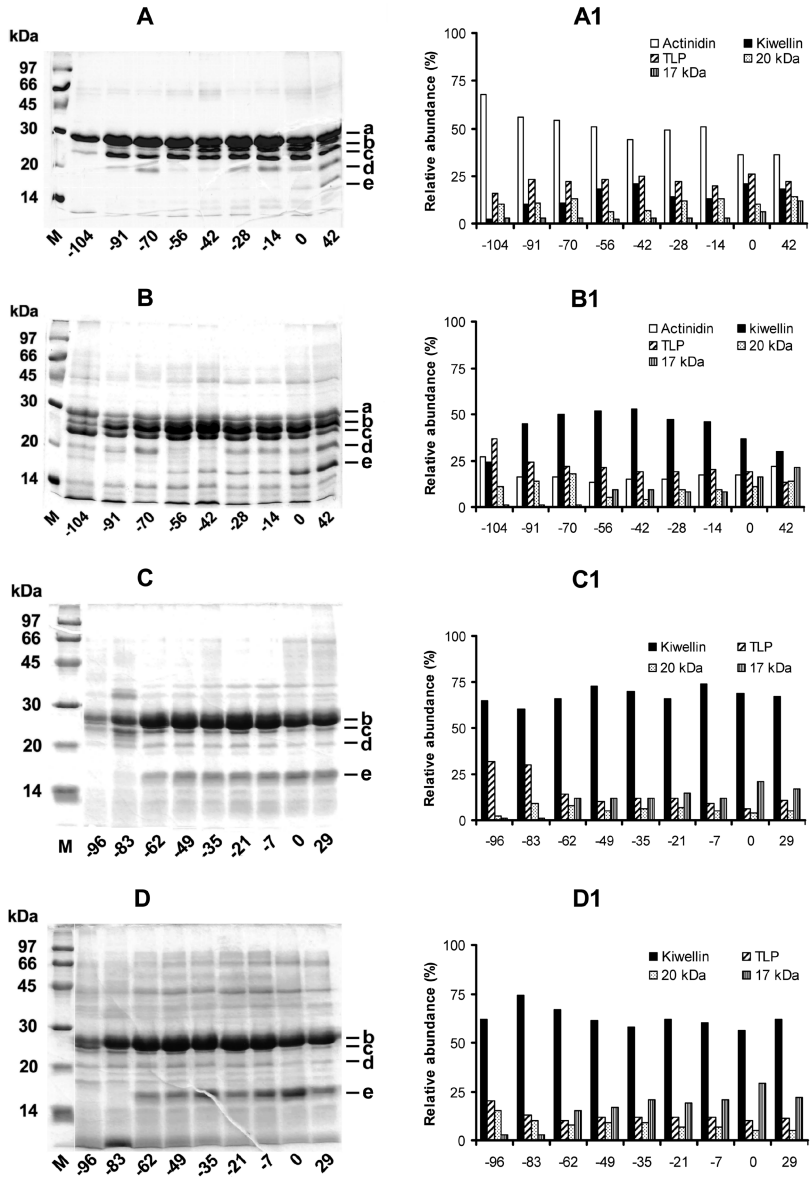


**Figure 5.** Protein profiles in SDS-PAGE (**A**−**D**) and relative abundance of the single components (**A1**−**D1**) of kiwi fruit extracts: (**A**, **A1**) AE fractions from GrK; (**B**, **B1**) SE fractions from GrK; (**C**, **C1**) AE fractions from GoK; (**D**, **D1**) SE fractions from GoK. Numbers below the lanes indicate the days before (−) and after the stage of commercial harvest (0). Actinidin, kiwellin, TLP, and the 20 and 17 kDa bands are indicated by a, b, c, d, and e, respectively. M, molecular weight markers.

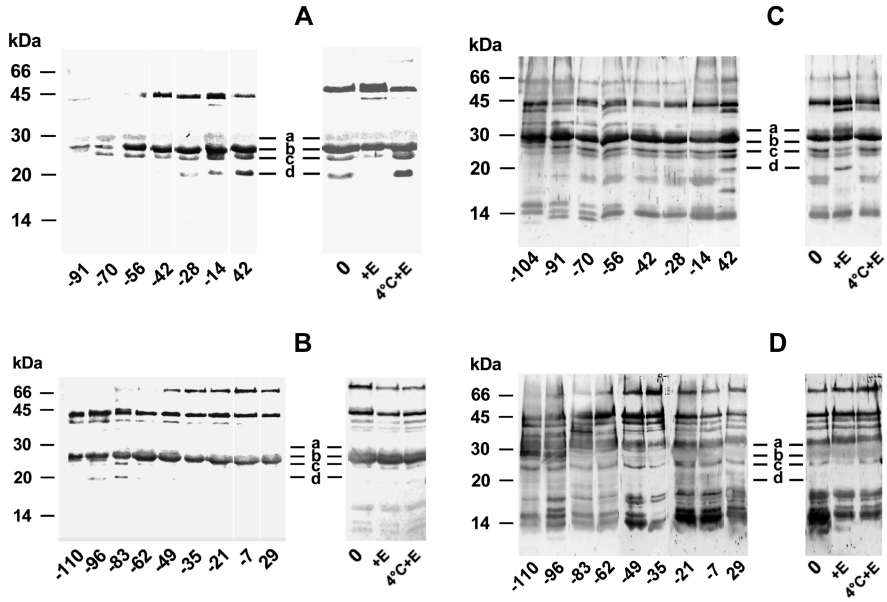


**Figure 6.** IgE-immunoblot analysis of GrK (**A**, **C**) and GoK (**B**, **D**): TE fractions probed with the serum pool 1 (**A**, **B**) and pool 2 (**C**, **D**). Numbers below the lanes indicate the days before (−) and after the stage of commercial harvest (0). Extracts from fruits subjected to ethylene treatment with or without previous cold storage are indicated by 4 °C+E and +E, respectively. Actinidin, kiwellin, TLP, and the 20 kDa band are indicated by a, b, c, and d, respectively.

a mechanism of generalized degradation acting on all of the protein components including actinidin, or a decrease in protein synthesis, was induced in the ethylene-treated GrK.

It is noteworthy that the separation of extracts by SDS-PAGE allows the analysis of the major components, whereas the proteins present in small amounts are normally overlooked. For instance, pectin methylesterase (PME), the allergenic protein Act d 7 (www.allergome.org), and pectin methylesterase inhibitor (PMEI), the allergenic protein Act d 6 (www.allergome.org), are two minor components of green kiwi fruit (*26−28*), the expression of which cannot be monitored by SDS-PAGE analysis of total extracts. Nevertheless, it is well-known (*26, 27*) that their amounts are strongly dependent on the ripening stage and postharvest treatments. In fact, PME is not detected in completely ripe fruit and is usually purified from unripe fruit harvested 1−2 weeks before the stage of commercial harvest. In contrast, PMEI is purified in good yields only from completely ripe fruit, following a postharvest cold storage and ethylene treatment.

**Influence of the Extraction Protocol on the Protein Pattern of the Extracts.** SDS-PAGE analysis of the AE and SE fractions of both GrK and GoK (**Figure 5**) was performed by loading samples of each protein extract and estimating the relative amount of the major components. Experimental trials showed that the addition of protease inhibitors to the extraction mixture did not affect the observed protein pattern (data not shown).

In GrK, the AE fraction (**Figure 5A**) shows a lower number of protein components than the fraction obtained by high salt extraction (**Figure 5B**). The relative abundance, expressed as a percentage (**Figure 5A1**,**B1**) of actinidin, kiwellin, TLP, KiTH, and the 17 kDa band, was analyzed. In GoK, the relative abundance of kiwellin, TLP, and the 20 and 17 kDa bands was analyzed (**Figure 5C1**,**D1**).

Actinidin is a major component of GrK and the most abundant protein of the AE fraction (**Figure 5A1**). TLP was observed in both fractions AE and SE, and its amount relative to the other components was lower in the GoK than in the GrK extracts. A 17 kDa component was only detected approximately 8 weeks before the stage of commercial harvesting of both GrK and GoK, and its amount increased during the ripening process.

Kiwellin is the most abundant component of the GoK extracts (**Figure 5C**,**C1**,**D**,**D1**). Its total amount increased during the ripening process. However, regardless of harvesting stage, postharvesting handling, and extraction protocol used, the relative abundance of kiwellin appeared to be almost constant, when compared to other components such as TLP and the 20 and 17 kDa proteins. In contrast, in GrK the amount of kiwellin showed a higher variation, and conditions such as the high salt extraction increased its concentration in the extracts maybe due to peculiar features of the pulp tissue different from those of GoK (**Figure 5A1**,**B1**). In fact, it was the most abundant component of the SE fraction, but it was detected in smaller amounts in the AE fraction of the final ripening stages. Perhaps, unlike actinidin and TLP, kiwellin has been overlooked for a long time due to its small amount in the AE fraction. In fact, the protocols chosen in many laboratories do not use a high ionic strength; thus, the extraction of cell wall proteins/allergens is not very efficient.

**Influence of the Ripening Stage and Postharvest Handling on the Profile of IgE-Binding Components.** For IgE-binding experiments, sera were preselected by testing them by immunoblotting using purified allergenic proteins (actinidin, kiwellin, KiTH, TLP). A pool (pool 1) was obtained by mixing three sera selected for the capacity to recognize kiwellin and KiTH and to not detect actinidin. This pool contained also IgE recognizing TLP. A second pool (pool 2) was obtained by mixing three sera of kiwi fruit-allergic patients selected for the absence of IgE specific for kiwellin. IgE recognizing actinidin and, to a lower extent, TLP had also been detected in sera of pool 2. These two serum pools were used in immunoblotting experiments to probe the TE extracts. Nonspecific binding of the anti-IgE antibody conjugate was estimated by omitting the incubation step with patient pooled sera. Only TLP showed a slight positive nonspecific signal, whereas actinidin, kiwellin, KiTH, and other unidentified components were not recognized by the anti-IgE antibody. As has been already reported (*10*), immunoblotting analysis displays differences between the GrK and GoK IgE-binding profiles. The pattern of IgE-binding components was affected by ripening and postharvest handling

of kiwi fruits, but it was also strongly dependent on the pool of sera used to probe the immunoblots.

IgE-binding profiles obtained with the serum pool 1 are shown in **Figure 6A**,**B**. Kiwellin was detected in all of the extracts of both species, whereas KiTH was observed in the green species during the final ripening stages, beginning from −28 days. In line with the results shown in **Figure 2**, KiTH appeared as a prominent IgE-binding band in GrK treated by cold storage, but it was almost undetectable in the fruit treated with ethylene without cold exposure. This observation suggested that the plant hormone may induce the degradation of KiTH produced during the final stages of fruit ripening on the plant and that the lower concentration of actinidin did not allow production of additional KiTH. In GoK, an IgE-binding band at a molecular mass similar to that of KiTH (20 kDa) was observed at the early stages of ripening. N-Terminal amino acid sequencing of the electroblotted band produced unclear results, suggesting the presence of low amounts of multiple sequences and the absence of KiTH.

Pool 1 clearly recognized TLP in the GrK extracts, but in the GoK species the very high amount of kiwellin hid, at least partially, the lower amount of TLP. IgE-binding components at 40−43 kDa were observed in both the GrK and GoK extracts. Probably, one of these IgE-binding bands corresponds to Act d 3, identified in the TE extracts of GrK by N-terminal amino acid sequencing. A not yet identified 66 kDa protein component, showing a significant IgE reactivity, was observed in most GoK ripening stages, whereas in GrK this was detected only in the fruit treated with ethylene after cold storage. Although it was a low-abundant component, the strong signal observed suggested that it might be an important allergen.

Immunoblotting with serum pool 2 of the GrK and GoK TE (**Figure 6C**,**D**) showed a higher number of IgE-binding components than immunoblots probed with serum pool 1. To reduce the bandwidth of the GrK actinidin and to increase the amount of the GoK actinidin, the concentration of which is very low, immunoblots were performed using a 3-fold higher amount of the GoK extract. Under these experimental conditions, a higher number of IgE-reactive bands were detected in the GoK extracts than in GrK, especially at molecular masses ranging from 24 to 66 kDa and below 17 kDa. In GrK, actinidin was clearly identified as an IgE-binding band at all of the ripening stages. In GoK, although some signals were observed at approximately 30 kDa, actinidin was not clearly detected. IgE-binding proteins at 40−43 kDa, already detected with serum pool 1 in both species, were observed also with serum pool 2. Moreover, the IgE-binding profiles obtained at different ripening stages showed high variability (**Figure 6**), especially in GoK and at molecular masses lower than 20 kDa. Several low-abundant components, overlooked in SDS-PAGE, displayed strong IgE-binding signals in immunoblots, and some of them were detected at only one or a few ripening stages. Control tests carried out with individual sera of control patients not allergic to kiwi (not shown) excluded that a significant nonspecific binding might have occurred.

Extracts of the GrK and GoK fruits displayed characteristic and distinct profiles of proteins and IgE-binding components. However, dependent on several conditions, variations of the protein content were observed. Results from this study underscore the level of complexity associated with the preparation of extracts when a known and defined concentration and relative abundance of single proteins and allergens is requested. In allergy diagnosis and immunotherapy the standardization of extract composition is of utmost importance. In this framework, an understanding of how some conditions affect the proteins/allergens pattern of extracts may provide very useful information, although the adoption of purified natural or recombinant single protein preparations replacing extracts is highly advised.

The influence of conditions such as ripening stages and extraction methods on the composition of extracts is especially important for the basic research and for the optimization of protein purification procedures. If the impact of kiwi fruit consumption on human health and the diagnostic and therapeutic aspects are considered, then attention has to be focused on the completely ripe and softened fruit, as it is normally eaten. Interestingly, the study here reported indicates that, especially in the green species, a ripe fruit may have a different concentration of total proteins and a different amount of single components when ripeness has been reached by different means of postharvest handling, such as ethylene exposure with or without cold storage. Therefore, we could hypothesize that the composition of kiwi fruit might be, at least partially, programmed and driven by acting on postharvest handling to obtain an ideal food with increased or decreased amount of proteins/allergens to address specific requests.

## ABBREVIATIONS USED

AE, aqueous protein extract; SE, salt protein extract; BCIP, bromochloroindolylphosphate; GrK, green kiwi fruit; GoK, gold kiwi fruit; NBT, nitroblue tetrazolium; TE, total protein extract; TFA, trifluoroacetic acid; TLP, thaumatin-like protein.

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