

Identification of actinidin as the major allergen of kiwi fruit

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Background: Allergic reactions to fruits and vegetables are among the most frequent food allergies in adults. Kiwi fruit (*Actinidia chinensis*) is commonly involved, causing local mucosal, systemic, or both types of symptoms by an IgE-mediated mechanism. In a previous study on 30 patients allergic to kiwi, we identified a major allergen of 30 kd against which all sera tested clearly reacted. Other allergens were detected at 12, 24, and 28 kd.

Objective: The aim of this study was to fully characterize the major kiwi fruit allergen of 30 kd.

Methods: Allergens were separated and purified by high-performance liquid chromatography with anion-exchange columns. The purity of the single proteins was checked by sodium dodecylsulfate-polyacrylamide gel electrophoresis, and their allergenicity was checked by immunoblotting with a pool of sera from patients allergic to kiwi. The allergens were characterized by isoelectrofocusing and amino acid sequencing, and periodic acid-Schiff stain was used to detect glycoproteins.

Results: Proteins of 30, 28, 24, and 17 kd were purified by high-performance liquid chromatography. IgE binding indicated the 30 kd protein, which showed an isoelectric point of 3.5, as the major allergen of kiwi. Determination of its partial amino acid sequence and comparison with the Swiss Protein Bank showed that this was actinidin, the main protein component of kiwi. The 24 and 28 kd proteins had the same N-terminal sequence, which did not correspond to any known protein. The 17 kd protein had a blocked N-terminal sequence.

Conclusions: These results demonstrate that the major allergen of kiwi fruit, Act c 1, is actinidin, a proteolytic enzyme belonging to the class of thiol-proteases. Two other allergens of 24 and 28 kd appear identical on amino acid sequencing. (*J Allergy Clin Immunol* 1998;101:531-7.)

Key words: Food allergy, *Actinidia chinensis*, actinidin

Thus far only a few food allergens have been characterized, compared with a large number of inhalant allergens. In the list of the acceptably characterized allergens recently published by the Subcommittee on Allergen Nomenclature

Abbreviations used

HPLC:	High-performance liquid chromatography
MW:	Molecular weight
PAS:	Periodic acid Schiff
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

of the International Union of Immunologic Societies,¹ it is surprising to see that the major allergens of only few foods have been accepted as definitely identified, sequenced, and functionally defined. However, some allergens from peanuts,² apples,³ and celery⁴ will probably soon be accepted because they have been completely characterized. The difference between these allergens and aeroallergens is mainly the difficulty of recruiting the large number of patients with IgE sensitization and clinically proved intolerance to a certain food needed to define the clinical importance of an allergen because of the lower prevalence of food allergy.

We recently studied the allergenic pattern of kiwi fruit (*Actinidia chinensis*) in a population of 30 patients allergic to this fruit as assessed by history, challenge tests, and IgE tests.⁵ Kiwi seems to be more and more involved in inducing allergic symptoms such as oral allergy syndrome, laryngeal edema, and even anaphylactic shock,⁶⁻⁸ possibly as a consequence of its growing consumption because of its easy cultivation, good taste, and high vitamin content. We reported that the major allergen was a protein with a molecular weight (MW) of about 30 kd, which was definitely the most important allergen of kiwi because all patients' sera reacted against it. Other allergens with MWs of 28, 24, and 12 kd appeared important either because of the large number of patients reacting to them or for their part in the clinical cross-reactivity of kiwi allergy with pollinosis to grass, birch, or both.

The aim of this study was the full characterization of kiwi allergens, particularly the 30 kd major allergen.

METHODS

Kiwi-sensitive patients

A pool of sera from 30 patients with allergic symptoms to kiwi who were positive for kiwi-specific IgE antibodies was used to check the in vitro IgE-binding capacity of the fractions of kiwi

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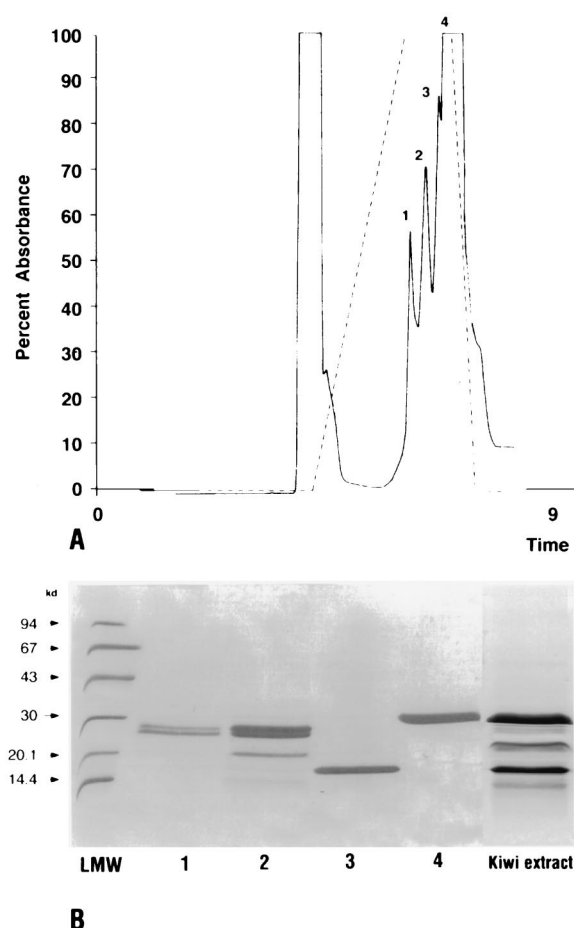


FIG. 1. Anion-exchange chromatogram of crude kiwi extract fractionated over HPLC RESOURCE Q column (A) and SDS-PAGE analysis of fractions from anion-exchange column of concentrated kiwi extract (B). Gel was stained with Coomassie Brilliant Blue. In SDS-PAGE analysis fractions were loaded as follows: 42 μ g for fraction 1, 166 μ g for fraction 2, 154 μ g for fraction 3, and 160 μ g for fraction 4.

purified from the crude extract. The patients' clinical characteristics, the results of oral provocation tests with kiwi, and the levels of kiwi-specific IgE for each patient have been reported fully elsewhere.⁵ The sera of seven of these 30 patients were used to check the IgE immunoblotting of kiwi in reducing and nonreducing conditions.

Extract

The pulp of fresh fruit was homogenized (1:2 wt/vol) in 0.1 mol/L potassium phosphate buffer (pH 7). The homogenate was centrifuged at 40,000 g at 4°C for 30 minutes, and the supernatant was then dialyzed for 48 hours at 4°C against 0.1 mol/L phosphate buffer by using Visking dialysis tubing (Serva, Heidelberg, Germany) with a MW cut-off of 8000 d. At the end of this period, the extract was centrifuged again at 10,000 g for 5 minutes to remove any precipitate and stored at -20°C. The protein content of the kiwi extract, determined by using Folin reagent according to the method of Lowry et al.,⁹ was 2.2 mg/ml. After preliminary experiments, the extract was concentrated by centrifuging at 3000 g at 4°C for 3 hours with a Centriprep 3 (Amicon Inc., Beverly, Mass.). A protein concen-

tration of 11 mg/ml in a final volume of 1 ml was obtained from a starting volume of 15 ml.

We also prepared an extract by using a thiol protease inhibitor, N-(N-[L-3-trans-carboxyoxirane-2-carbonyl]-L-leucyl)-agmatine (E64) (Boehringer Mannheim GmbH, Mannheim, Germany). Two micrograms per milliliter of the inhibitor was dissolved in the buffer used for homogenizing the fruit, according to the protocol suggested for papain and other thiol proteases.¹⁰

High-performance liquid chromatography (HPLC)

The crude extract was purified by analytic anion-exchange chromatography with an HPLC system (LKB BROMMA) by using the RESOURCE Q column (1 ml) (Pharmacia-Biotech, Uppsala, Sweden). The mobile phase was 20 mmol/L Tris HCl (pH 7.5). Absorbance was monitored at 280 nm with a flow rate of 1 ml/min. Two hundred microliters of the kiwi extract with a protein concentration of 11 mg/ml were loaded onto the column, applying a linear NaCl gradient from 0 to 0.5 mol/L. The fractions collected from HPLC were stored at -20°C after measuring their protein content by the method of Warburg and Christian.¹¹

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was then carried out to determine the purity of each fraction and the corresponding MW. The fractions were subsequently submitted to an IgE-immunoblotting procedure by using the pooled sera from the allergic patients to verify the IgE-binding capacity.

Electrophoresis and immunoblotting

Electrophoresis was done with a discontinuous buffer system in a sodium dodecylsulfate-polyacrylamide gradient gel with a 6% stacking gel and a 7.5% to 20% separation gradient gel at 6 mA for 16 hours in a BIORAD Protein Ixi vertical electrophoresis Slab Cell (Biorad Laboratories, Richmond, Calif.), essentially according to the method of Neville.¹²

Samples of the crude kiwi extract and purified fractions were diluted 1:2 in sample buffer containing Tris (hydroxymethyl-amino methane) at pH 6.1, 2% sodium dodecylsulfate, 3% β -mercaptoethanol, 5% glycerol, and 0.001% bromophenol blue. The diluted samples and low MW markers (Pharmacia Biotech) were denatured by heating at 100°C for 5 minutes. They then underwent centrifugation at 10,000 g for 5 minutes. As reported earlier,⁵ an aliquot of each fraction was stained with a solution of Coomassie Brilliant Blue R-250 (Pharmacia) for 1 hour to establish purity and MW. The sample containing the 30 kD protein was also stained with silver (Pharmacia) as a further control.

SDS-PAGE experiments were done with the purified fractions of 30, 24, and 28 kD after incubation for 1, 4, and 24 hours at room temperature to see whether these changes caused any splitting of the protein, hence the appearance of the 17 kD fraction from the 30 kD band and a change in the ratio between the 24 to 28 kD bands.

To assess the enzymatic activity of any of the components, SDS-PAGE was repeated on the extract prepared with the thiol protease inhibitor E64.

For IgE-immunoblotting, each purified fraction and the crude kiwi extract (reduced and not reduced) were electroblotted onto nitrocellulose paper (pore size, 0.45 μ m; Amersham, Bucks, U.K.) by using a Trans-Blot Cell obtained from Biorad at 045 Å, 100 V for 4 hours at 4°C. A strip of the nitrocellulose blot was placed in blocking solution (phosphate buffered saline [pH 7.4] with 0.5% Tween 20) for 30 minutes at room temper-

ature and then incubated overnight with the pooled serum diluted 1:4 in blocking solution. The paper was washed three times in normal saline with 0.5% Tween 20 for 10 minutes with rocking and then incubated for 6 hours with ^{125} I-labeled anti-human IgE antiserum (Pharmacia) diluted 1:4 in blocking solution followed by washing and exposure on x-ray film for 5 days.

Immunoblotting after SDS-PAGE of either the crude extract or the fractions from ion-exchange HPLC was also done on hydrophobic membranes (ProBlot, Applied Biosystem) to determine the sequence of the separate components directly. SDS-PAGE and IgE-immunoblotting experiments were repeated in nonreducing conditions (without 2-mercaptoethanol) to see whether SDS-PAGE altered the electrophoretic mobility of the 30 kd protein.

To show the specific IgE-binding capacity of the purified 30 kd protein, immunoblots were done with sera from allergic patients without kiwi-specific IgE that contained low (five patients) and high (three patients) total IgE and sera from four nonallergic individuals. One immunoblot was done without addition of serum (i.e., with buffer control).

Isoelectrofocusing

The 30 kd fraction was focused by an LKB 2117 MULTIPHOR II system (LKB Bromma) on a polyacrylamide gel plate (30% acrylamide, 0.8% bisacrylamide), with a pH gradient of 3.5 to 10. Five microliters of sample were applied at an electric current of 1000 V at constant voltage for 3 hours by using a 2197 LKB Power Supply (LKB). The gel was fixed and stained with Coomassie Brilliant Blue R-250 (Pharmacia) according to the manufacturer's directions.

Amino acid sequence

Protein and peptide sequence analysis was done on an Applied Biosystems 470A gas-phase sequencer (Applied Biosystems, Foster City, Calif.) equipped with a 120A phenylthiohydantoin-amino acid derivative analyzer. All chemicals were from Applied Biosystems.

Periodic acid-Schiff (PAS) stain

PAS staining was done to detect glycosylation of proteins. The minigels were blotted onto ProBlot membrane (Applied Biosystems), as described by Towbin and Gordon,¹³ at 10° C and 120 mA constant current for 60 minutes, except that methanol and sodium dodecylsulfate were omitted in the transfer buffer. Two identical membranes were blotted at the same time, one stained with Coomassie R-250 and the other used for PAS staining for glycoproteins. For PAS staining, the membrane was fixed in 12% trichloroacetic acid and immediately tested with a 7% acetic acid solution containing 3% KIO₄ and maintained at 4° C for 30 minutes. The membrane was then immersed in 0.1 mol/L HCl containing 1% potassium pyrosulfite until the amber color disappeared. The reducing solution was discarded and replaced with Schiff's reagent, and the membrane was kept in the dark in this reagent at 4° C overnight. Glycoproteins appeared as purple bands and became increasingly evident after several washings with distilled water. Milk whey proteins were used as controls.

Tryptic digestion and separation of the peptides

For sequence analysis, the protein was modified by reduction in 6 mol/L guanidinium chloride, 0.001 mol/L ethylenediamine tetraacetic acid, and 0.1 mol/L Tris-HCl (pH 8.3). Dithiothreitol (0.002 mol/L) was added, and the reduction proceeded

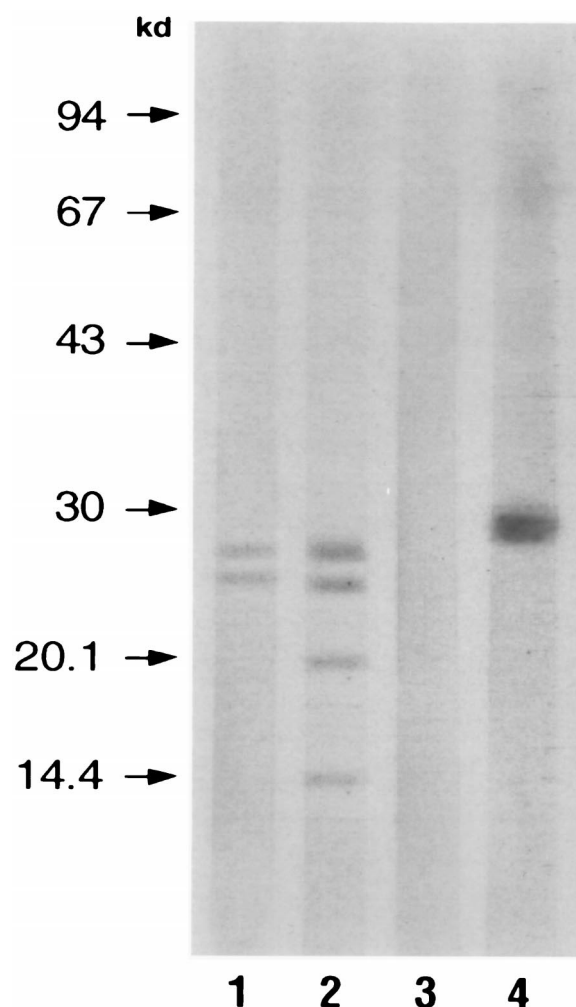


FIG. 2. IgE binding of four peaks (24 to 28, 17, and 30 kd) from anion-exchange column of concentrated kiwi extract by immunoblotting. Lane 3 corresponds to 17 kd fraction. Protein loaded for each fraction (per centimeter of gel) was as follows: lane 1, 16.8 μ g; lane 2, 66.6 μ g; lane 3, 94.2 μ g; and lane 4, 64.2 μ g.

under nitrogen, in the dark, at 37° C for 1 hour. S-Carboxamidomethylation was performed with 0.1 mol/L iodoacetamide in the same conditions as for reduction of the protein. The reduced, carboxamidomethylated protein was digested with 1% trypsin (TPCK) (Worthington Biochemical Corp., Freehold, N.J.) in 0.2 mol/L N-ethylmorpholine acetate (pH 8.3) at 37° C for 1 hour. The reaction was stopped by freezing at -40° C. The peptides obtained were purified by reverse-phase-HPLC (Varian LC 5000, Palo Alto, Calif.) on a C₁₈ Vydac column in 0.1% trifluoroacetic acid and eluted with a linear gradient of 0% to 70% acetonitrile in 0.1% trifluoroacetic acid for 60 minutes at a flow rate of 0.7 ml/min.

RESULTS

HPLC and SDS-PAGE/immunoblotting

Pilot experiments were conducted with the Resource Q (1 ml) anion-exchange column connected to the HPLC system to establish the optimal buffer system and salt gradient (linear from 0 to 0.5 mol/L of NaCl). This

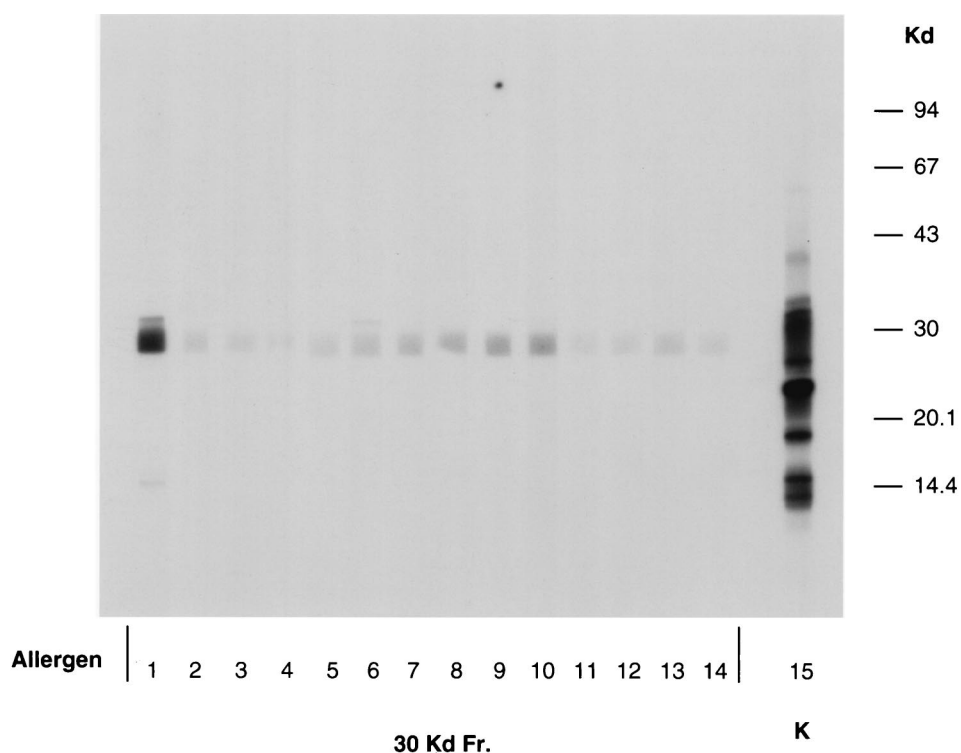


FIG. 3. IgE binding of 30 kD component obtained with pooled sera from patients allergic to kiwi (*line 1*), buffer control (*line 2*), sera from atopic patients with no kiwi-specific IgE with low total IgE (*lines 3 to 7*) and with high total IgE (*lines 8 to 10*), and sera from nonatopic subjects (*lines 11 to 14*). *Line 15* shows IgE immunoblotting with the whole kiwi extract. For each lane, 32 μ g of 30 kD kiwi protein was loaded. *Fr.*, Fraction.

procedure, conducted on a concentrated kiwi extract of 11 mg/ml (the unconcentrated extract had too low a protein concentration), led to the separation of four peaks (Fig. 1, *A*), which, analyzed in SDS-PAGE, showed good purification. Preliminary separation of the kiwi extract by gel filtration on a Superdex 75 HR 10/30 column did not significantly improve the purification.

The four fractions from the anion-exchange column, analyzed by SDS-PAGE, are depicted in Fig. 1, *B*. Two proteins with molecular weights of 24 and 28 kD were detected in the first and second fractions (protein concentrations, 0.28 mg/ml and 1.11 mg/ml, respectively), one protein of 17 kD was found in the third fraction (protein concentration, 1.03 mg/ml), and one protein of 30 kD was found in the fourth fraction (protein concentration, 1.07 mg/ml). Because peak 4 was very close to peak 3, we collected the small volume corresponding to the highest part of the peak, so as to obtain a sufficiently pure 30 kD protein. The 17 and 30 kD proteins appeared adequately purified and thus suitable for further analysis.

SDS-PAGE of purified fractions of 30 and 24/28 kD kept at room temperature for different periods did not show the appearance of any new band from the fraction containing the 30 kD allergen or any change in the ratio between the 24 and 28 kD bands. The good purification of the 30 kD protein was demonstrated by SDS-PAGE

staining with Coomassie Brilliant Blue R-250 and silver stain because only this band was stained (data not shown).

SDS-PAGE of the kiwi extract treated with the specific protease inhibitor E64 showed a large increase in the 30 kD band but not in the 24 to 28 kD band (data not shown).

Fig. 2 reports the IgE immunoblotting of the four peaks, showing the IgE-binding capacity of the bands at 30, 24, and 28 kD. The band at 17 kD had no IgE binding, as already observed.⁵

Electrophoresis and immunoblotting of kiwi extract with the pooled serum under reducing and nonreducing conditions showed that the allergens at 30, 24, and 28 kD had a lower MW when the nonreduced extract was used. The resolution of the proteins differed in the two conditions, the result being better with the reduced extract.

Fig. 3 shows the IgE binding of the 30 kD component with sera from allergic patients with no kiwi-specific IgE (with low and high total IgE), nonallergic patients, and the buffer control. Very weak aspecific binding was detected only with sera with high total IgE.

PAS staining

The results of PAS staining for glycosylation on the 30 kD protein and on a positive control exclude any glycosylation of this protein from kiwi extract (Fig. 4).

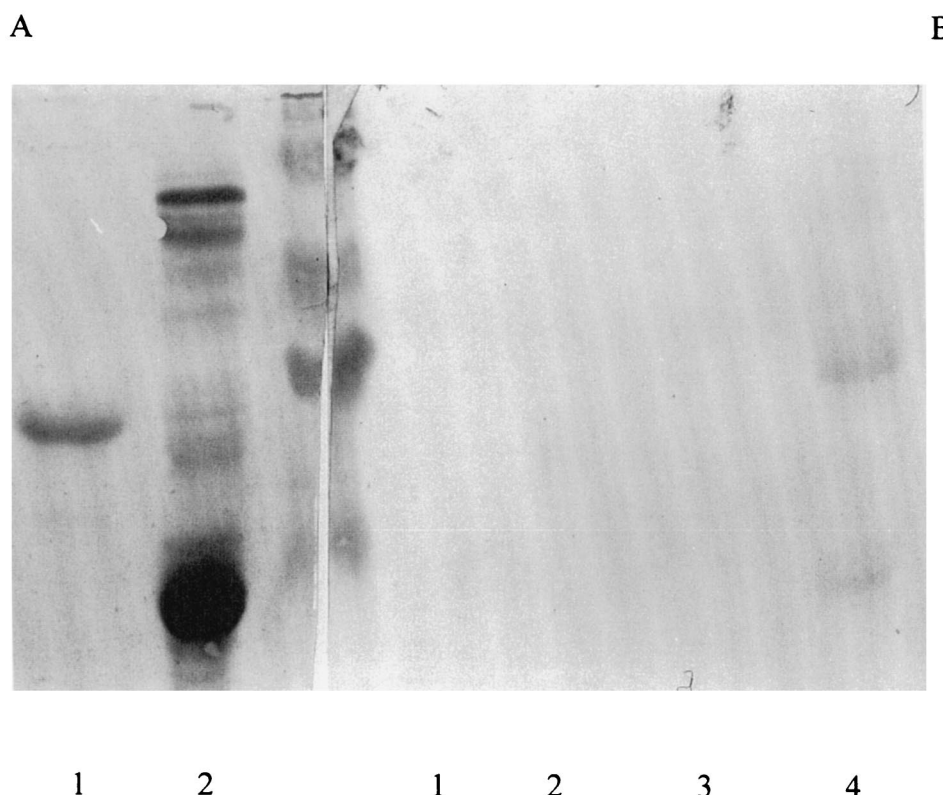


FIG. 4. PAS staining of 30 kd kiwi protein. Panel **A** shows Coomassie Blue staining with 30 kd protein at 10 µg (lane 1) and with positive control milk whey proteins (lane 2). Panel **B** shows PAS staining with 30 kd protein at 5 µg (lane 1), 10 µg (lane 2), 15 µg (lane 3), and with positive control (lane 4). Kaleidoscope standards are shown (Biorad) between two panels.

Isoelectrofocusing

SDS-PAGE and immunoblotting of the anion-exchange fractions showed that fraction 4, migrating at 30 kd, contained the major allergen of kiwi fruit. Isoelectrofocusing was done on this fraction, which gave a mean isoelectric point of 3.5 after Coomassie Brilliant Blue R-250 staining.

Protein modification and amino acid sequencing

The N-terminal amino acid sequence of the 30 kd protein was done on the protein in solution and after SDS-PAGE and Western blotting on Problot membranes. In both cases the result was Leu-Pro-Ser-Tyr-Val-Asp-Trp-Arg-Ser-Ala. A search of the Swiss Protein Bank showed this sequence corresponded to amino acids 127 to 136 of the complete sequence (amino acids 1 to 346 including the activation peptide from amino acid 1 to 126) of actinidin, the major protein component of kiwi.

To confirm this, the 30 kd protein, corresponding to the fourth fraction of the ion-exchange column, was reduced, carboxamidomethylated, and subjected to tryptic digestion. Aliquots of some of the peaks obtained after separation of the tryptic peptides on a RP-HPLC C₁₈ Vydac column were directly subjected to sequence analysis; all the resulting sequences matched internal

stretches of the actinidin molecule (from amino acid 127 to 346). Fig. 5 shows the known sequence of the actinidin chain, with the solid lines underlining the tryptic peptides sequenced. The N-terminal sequence of the 24 kd fraction had Ile-Ser-Ser-Arg-Asn-Gly-Pro-Ile-Arg-Asp-Leu-Asn-Asp as its first 13 amino acids and did not belong to any known protein. The N-terminal sequences of the 28 and 24 kd fractions were identical. The N-terminal amino acid sequence of the 17 kd band could not be determined because the first amino acid of the protein was blocked.

DISCUSSION

This study provides evidence that actinidin is the major allergen of the fruit *Actinidia chinensis*, commonly called kiwi. Theoretically, this should not be surprising because actinidin accounts for about 50% of the soluble proteins of the fruit. However, we did not imagine this when, in a previous study, we identified the major allergen of kiwi as a 30 kd band because actinidin was reported in 1978 as an enzymatic protein of 23.5 kd.¹⁴ The apparent MW of nearly 30 kd obtained from the sodium dodecylsulfate gel for the actinidin molecule is close to the calculated MW from the amino acid sequencing, giving 27.445 d. Identification of the 30 kd component as actinidin was first suggested by its isoelec-



FIG. 5. Actinidin amino acid sequence. Sequenced peptides from tryptic digestion of kiwi 30 kd protein are indicated by *solid lines*.

tric point of 3.5 and the increase in size in the SDS-PAGE experiment with a kiwi extract previously treated with E64 used as a thiol-protease specific inhibitor and definitely proved by sequence determination.

Because this is the major allergen of kiwi, evoking 100% IgE reactivity in the population studied, and the N-terminal sequence was determined from a partial deduction of the amino acid sequence, we called it Act c 1 (as authorized by the IUIS Allergen Nomenclature Subcommittee, accession P00785) according to the current Allergen Nomenclature.¹ This adds another major allergen to the list of 13 food sources, including chicken's egg, cow's milk, cod, salmon, shrimp, peanut, soybean, barley, rice, mustard, celery, apple, and cherry. The identification of major food allergens is of the utmost importance because it adds to our knowledge of food allergy and leads to diagnostic extracts with high sensitivity and specificity, as demonstrated by Aas and Lundkvist¹⁵ with the purified major allergen from codfish, Gad c 1, called at that time allergen M.

We also looked at the 24 and 28 kd allergens, and determination of the N-terminal sequence showed that these proteins did not belong to any protein family already described. A full amino acid deduction is needed for their complete description, but the two components appear identical, and, after actinidin, they are the most important allergens of kiwi.

The question of the 17 kd protein, which was purified in the HPLC system in the third fraction but did not show any allergenic activity, still remains. Considering that a MW of 17 kd corresponds to about 120 amino acids and the size increased in the SDS-PAGE experiment with the protease inhibitor E64, we are led to believe this protein might have an enzymatic function, being either a product of cleavage of actinidin or its prosequence. Because different incubating periods of

the fraction containing the 30 kd protein did not increase the 17 kd band, this protein is probably not a product of digestion of the 30 kd band but the prosequence of actinidin.

Actinidin is a proteolytic enzyme secreted in an inactive form as zymogen, which has a MW of 39 kd and is named actininidin. Several other proteases containing a thiol group, essential for their activity, are secreted in this inactive form, and it has now been demonstrated that cleavage of this peptide is necessary for it to fully express its allergenic activity. A recombinant Der f 1 allergen, with IgE-binding activity corresponding to 20% of the native Der f 1, has been produced, but removal of the prosequence in an acidic solution sharply raised the IgE-binding activity to almost the same level as that of native Der f 1.¹⁶ Thus the 30 kd fragment, actinidin, might bind IgE only after cleavage of the prosequence.

The class of thiol proteases includes several other plant enzymes such as papain (from papaya), ficin (from fig), bromelain (from pineapple),¹⁷ and aleurain (from barley).¹⁸ Other thiol proteases have been identified as animal enzymes such as cathepsin B and H in rats,¹⁹ the protease of *Dermatophagoides pteronyssinus* (Der p 1),²⁰ and a protease from *Dictyostelium discoideum*.²¹ Several similarities are reported between actinidin, bromelain, and papain. They share a similar sequence in active site cysteine and histidine residues and have a similar mode of action. However, differences are found in amino acid composition, isoelectric points, and MWs. For example, a study comparing actinidin and papain found only 48% similarity in their amino acid sequences.¹⁷ Nevertheless, their structures are surprisingly similar, with some main-chain/side-chain hydrogen bonds strongly conserved, which may explain the similar folding of nonrepetitive parts of the structures. These bonds would thus ensure very strong conservation of the tertiary structure as

demonstrated for actinidin and papain in which polypeptide backbones are virtually superimposable.¹⁷

A homology of papain and actinidin amino acid structures with rat liver cathepsin B and H was demonstrated.¹⁹ The sequences are extremely similar in regions that, according to the three-dimensional structure of papain and actinidin, constitute the catalytic site. This is extremely important because if the allergenic epitopes have a similar catalytic site, there may be extensive cross-reactivity between mammalian and plant endopeptidases.

The homology of structures between these thiol proteases has led to suggestions of evolution from a common ancestral protein thus opening the way to speculation on the possible immunologic meaning of similar enzymatic sites or functions in organisms phylogenically so distant. On the other hand, the observation that other allergens are also enzymatic proteins (e.g., phospholipase in insect venom, soybean trypsin-inhibitor, and α -amylase in wheat flour) makes it conceivable that enzymatic activity is particularly efficient in inducing IgE sensitization.

It is easier to argue for a cross-reactivity of kiwi actinidin with other fruit proteases, which has to be confirmed in patients with clinical reactions to allergenic sources containing other thiol proteases. In addition, the identification of actinidin as the major allergen of kiwi should lead to adequate procedures for preparing an extract for in vitro studies in which self-degradation of this protease and alteration of other allergenic components can be avoided as well.

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