

# Characterization of apple 18 and 31 kd allergens by microsequencing and evaluation of their content during storage and ripening

Li-Shan Hsieh, PhD,<sup>a</sup> Malcolm Moos, Jr., MD, PhD,<sup>b</sup> and Yuan Lin, PhD<sup>a</sup>  
Bethesda, Md.

*Patients with tree pollinosis frequently report allergic reactions after ingestion of apples. The severity of apple allergy has been related to the variety of apples and their degree of maturity. To generate a serum pool that is representative of various IgE-binding patterns of apple-allergic sera, serum samples from 34 patients allergic to tree pollens were screened. Only 24 serum samples reacted to the apple extract. Pooled serum was used to identify allergens in apples. An efficient and consistent extraction method for apple fruits was used to compare the immunoreactivities of extracts of different varieties (McIntosh, Red Delicious, Granny Smith, and Golden Delicious) of freshly picked and store-purchased apples. We found that Golden Delicious apples had the greatest amount of the 18 kd allergen, which has been reported to be a potent IgE-binding apple allergen. Store-purchased apples contained higher concentrations of the 18 kd allergen than freshly picked apples. In our study only 37.5% of sera reacted to the 18 kd protein, whereas 75% of the sera reacted to a 31 kd allergen. Other immunoreactive bands in apple extracts included proteins of 50, 38, 16, 14, and 13 kd. The amino-terminal amino acid sequences of the two major allergens, 18 kd and 31 kd, were determined. These sequences shared approximately 50% identity with disease resistance proteins of various plants or Bet v 1 in birch tree pollens. The appearance of various allergens was also investigated in mature apples during storage. The amount of 18 kd allergen increased significantly when apples were stored at 4° C. However, under controlled atmospheric conditions in which oxygen- and carbon dioxide-induced ripening were regulated, the amount of 18 kd allergen remained unaffected. Because ripening and maturation were not associated with increases in 18 kd allergen content, the observed changes might be induced by factors related to disease resistance. (J ALLERGY CLIN IMMUNOL 1995;96:960-70.)*

**Key words:** Apple allergens, food allergy, Bet v 1, IgE-reactive proteins, disease resistance proteins

In Northern Europe, up to 70% of patients with birch pollen allergy demonstrated intolerance to fruits and/or vegetables in contrast to 20% of patients not allergic to birch pollen.<sup>1,2</sup> Most complaints were related to ingestion of apples (*Malus domestica*).<sup>3-5</sup> The severity of symptoms in patients allergic to apples was highly correlated with the variety and maturity of apples.<sup>6</sup> Among apple

## Abbreviations used

|           |                                                          |
|-----------|----------------------------------------------------------|
| ACC:      | 1-Aminocyclopropane-1-carboxylic acid                    |
| SDS-PAGE: | Sodium dodecylsulfate-polyacrylamide gel electrophoresis |

allergens, the occurrence of an 18 kd protein was reported to correlate with the severity of allergic reaction.<sup>6</sup> Vieths et al.<sup>6</sup> also reported that the appearance of this 18 kd protein increased during storage and suggested that this was caused by ripening. Apple ripening is associated with a rise in ethylene.<sup>7</sup> Mature apples (fully developed fruit) with no onset of ripening (e.g., browning, softness, deterioration, or flavor changes) do not produce ethylene. The biosynthesis of ethylene is regulated

From <sup>a</sup>Laboratory of Immunobiochemistry, Division of Allergic Products and Parasitology; and <sup>b</sup>Laboratory of Developmental Biology, Division of Cellular & Gene Therapies, Center for Biologics Evaluation and Research, Food and Drug Administration.

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Reprint requests: Li-Shan Hsieh, PhD, FDA/CBER/DAPP, HFM-422 1401 Rockville Pike, Rockville, MD 20852-1441.

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by two enzymes, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase. ACC synthase converts S-adenosyl-L-methionine to ACC, and ACC oxidase catalyzes the oxidation of ACC to ethylene. In fact, ACC oxidase is induced by the presence of ethylene.<sup>8</sup> Many factors, such as anaerobiosis, infection, and physical wounding, will regulate ethylene release, which occurs when apples are not stored properly. ACC oxidase is often used by plant biochemists as an indicator of ripening. It can therefore be used to determine whether the increase in apple allergens is associated with ripening.

Apple allergens were reported to share structural similarities with those in birch pollens.<sup>2</sup> Studies have shown that apple allergens with molecular weights of 17 to 18 kd and 13 to 14 kd cross-react immunologically with the birch pollen allergens, Bet v 1 and Bet v 2, respectively.<sup>4, 9-12</sup> In a Northern blot study, apple messenger RNA also shared significant homology with Bet v 1 complementary DNA.<sup>13</sup> The nucleotide sequence of Bet v 1 is reported to have 55% sequence identity with a disease resistance-responsive gene in peas.<sup>9</sup> Recently, the amino-terminal amino acid sequence of the 18 kd allergen in apple was reported to have 62% identity with Bet v 1.<sup>14</sup> Therefore the 18 kd protein in apple may be related to a disease resistance protein. A 31 kd protein has also been reported to be a major allergen in many fruits but has not been characterized.<sup>5, 15</sup> Nevertheless, apples are known to contain phenolic compounds, which makes it difficult to isolate proteins in the native state.<sup>16</sup> To study the apple allergen concentrations, an efficient and quick method for comparison was needed. The aims of this study were to compare the appearance of these apple allergens among different varieties of apples and to investigate the change in concentrations of these allergens during storage and after the canning process. Partial amino acid sequences of the 18 kd and 31 kd proteins and their expression after induction of ripening were also studied.

## METHODS

### Sera from allergic patients

Sera from 34 patients allergic to birch, elm, alder, or oak pollens were tested with tree pollen extracts by immunoblotting. The same set of sera were also examined with apple fruit extract. Sera were either purchased from PlasmaLab International (Everett, Wash.) or collected from local volunteers to test for their IgE reactivities to apple extracts.

### Apple fruit and tree pollen extracts

Fresh orchard-grown apples (Red Delicious, Golden Delicious, McIntosh, and Granny Smith) were picked from an orchard in the Western Maryland Research and Education Center of the University of Maryland. Mature apples were picked and shipped at room temperature (within 2 hours) and stored at 4° C without specific treatment until use. Some apples were picked and frozen in liquid nitrogen at -135° C immediately before storage. Apples were also purchased from local grocery stores for comparison. Apple fruits at different growth stages were collected monthly from May to October in 1992, usually 7 to 10 days after the regular spraying schedule, and tested for the appearance of IgE-reactive proteins. Canned apples were purchased from local stores.

Birch, elm, elder, and oak pollen extracts were obtained from Pharmacia AB (Uppsala, Sweden) as lyophilized powder and were reconstituted in a 50% glycerol-water solution before use.

### Extraction of apple allergens

Ten grams of peeled and chopped apple pulp or powdered whole frozen apples (unpeeled) were homogenized with 12 ml of extraction buffer composed of 1 mol/L sucrose, 2% (wt/vol) of polyvinylpyrrolidone (Sigma Diagnostics, St. Louis, Mo.), 2 mmol/L ethylenediaminetetraacetic acid, and 1 mmol/L of diethyldithiocarbamic acid (Sigma Diagnostics) at a pH of 9.5 to 10. Particles were removed by gauze filtration and centrifugation (40,000 g at 20° C for 30 minutes). The supernatant was filtered through a Millex-HA 0.45 gm filter unit (Millipore, Bedford, Mass.) and stored in 1 or 3 ml aliquots at -20° C. Canned fruits were extracted in the same way, except 5 gm of wet weight was used with 6 ml of the extraction buffer. To compare the concentrations of allergens, an equal volume of the freshly thawed extracts was loaded on the electrophoresis gels.

### Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 16% Tris-Glycine NOVEX Pre-Cast gel (Novex, San Diego, Calif.) according to the method of Laemmli<sup>17</sup> under reducing conditions. Proteins were separated at 130 V for 2 hours.

### Transfer and immunoblotting

The separated proteins on the gels were transferred to ProBlott membranes (Applied Biosystems, Foster City, Calif.) at 300 mA for 1 hour in 10 mmol/L 3-cyclohexylamino-1-propanesulfonic acid (Sigma Diagnostics) with 0.05% (wt/vol) dithiothreitol at pH 10.5. Membranes were blocked in phosphate-buffered saline with 0.1% BRIJ 35 (Sigma Diagnostics) (B114) containing 1% nonfat dry milk (Carnation; Nestle Food Company, Glendale, Calif.) for 1 hour and cut into 3 mm strips.

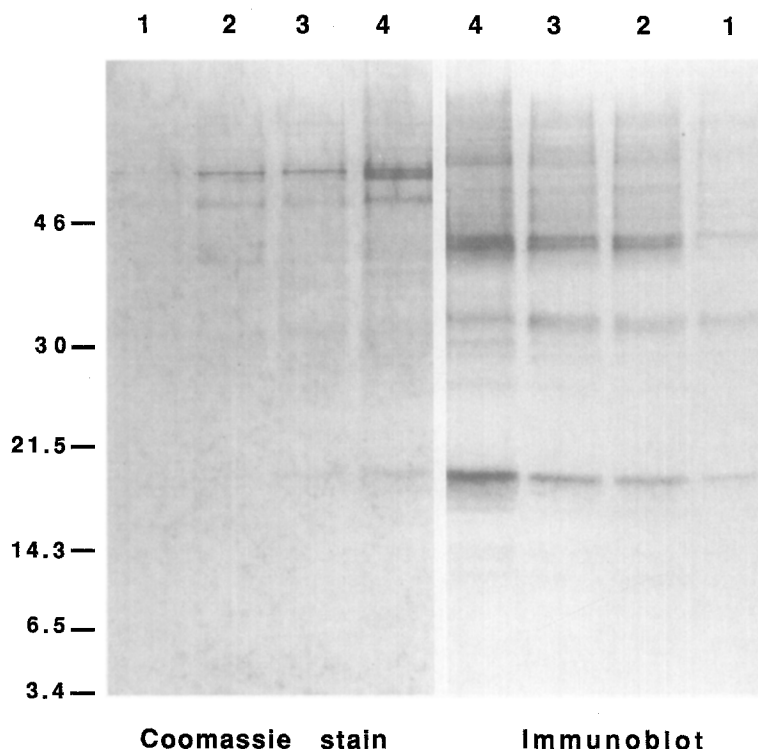


**FIG. 1.** Effect of pH on the efficiency of apple fruit extraction. The pH values are: *lane 1*, 4.74; *lane 2*, 6.77; *lane 3*, 8.36; and *lane 4*, 10.20. Panels are mirror images of blotted membrane. Molecular weight marker is in kilodaltons.

Strips were incubated overnight in patient's serum diluted 1:10. Strips were washed with the same buffer and incubated for 2 hours in phosphatase-labeled goat anti-human IgE( $\epsilon$ ) antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted 1:1000 and then washed with B114 buffer and developed with BCIP/NBT solution (Kirkegaard and Perry Laboratories).

#### Amino acid sequence analysis

Crude sucrose extracts of Golden Delicious apples were dialyzed against 50 mmol/L Tris buffer at pH 7.5, 500 mmol/L KCl, and 3 mmol/L sodium azide. The dialysate was centrifuged (5000 *g*) to remove most of the polysaccharide precipitate. Further purification of the supernatant was performed either by using a Centricon-3 filter (Amicon Inc., Beverly, Mass.) or by fractional precipitation with ammonium sulfate. The removal of polysaccharides made samples easier to concentrate and improved protein staining. Blotted Problott membranes were stained with 0.1% Coomassie brilliant blue G-250 (Sigma Diagnostics) in 50% methanol and 10% acetic acid. The 18 kd and 31 kd protein bands were excised for sequence analysis as described previously.<sup>18</sup> Excised bands were inserted into a Blott cartridge and analyzed in an Applied Biosystems model 477A sequencer equipped with a model 120A on-line PTH amino acid analyzer. The Blott-2 reaction

cycle and the conversion cycle modifications suggested by Tempst and Reviere<sup>19</sup> were used for these analyses. Data analysis was aided by Applied Biosystems model 610A software.

#### Apple proteins in a controlled storage atmosphere

Gala apple (a derivative of Golden Delicious) proteins extracted from apples stored under controlled atmospheric conditions and antibody to ACC oxidase were generously provided by Dr. Theophanes Solomos in the Department of Horticulture at the University of Maryland. The experiment was designed as described by Kanellis et al.<sup>20</sup> Only mature hard apples (before ripening), which hardly evolved ethylene, were used for the experiment. Apple samples were collected and stored at 1° C in a dark chamber under a constant flow of CO<sub>2</sub>-free humidified air or nitrogen supplemented with 1.5% oxygen (1.5% oxygen). The flow rate was selected to ensure that CO<sub>2</sub> accumulation did not exceed 0.3%. Samples were collected from apples: (1) air-stored throughout; (2) air-stored for 95 days, then treated with 1.5% oxygen; (3) air-stored for 55 days, then treated with 1.5% oxygen; and (4) stored in the presence of 1.5% of oxygen throughout and sampled 130 days from the start of storage. A controlled sample was collected 8 days before the onset of ripening.

**TABLE I.** Reactivity of patients' sera to tree pollens and apple extracts

| Patient No. | Age (yr)<br>Sex | Birch (kd) |    |    |    | Elm (kd) |    |    | Elder (kd) |    |    |    | Oak (kd) |    |    |       | Apple (kd) |    |    |    |    |    |
|-------------|-----------------|------------|----|----|----|----------|----|----|------------|----|----|----|----------|----|----|-------|------------|----|----|----|----|----|
|             |                 | >50        | 17 | 14 | 13 | >35      | 24 | 14 | >29        | 20 | 17 | 14 | >30      | 20 | 19 | 13-14 | >38        | 31 | 18 | 16 | 14 | 13 |
| 2           |                 | -          | -  | +1 | -  | +        | -  | +  | +          | -  | -  | +2 | +        | -  | -  | +     | +2         | +3 | -  | -  | -  | -  |
| 5           | 29/F            | +          | +3 | -  | -  | +        | +  | +2 | +          | +5 | -  | +2 | +        | +  | +  | +2    | +          | +2 | +2 | +  | +  | -  |
| 6           | 30/F            | +          | -  | +2 | +  | +        | -  | +2 | +          | -  | +  | +2 | +        | -  | -  | +2    | +          | +3 | -  | -  | +  | +  |
| 7           | 41/M            | +          | -  | +  | -  | +        | -  | +  | +          | -  | +  | +2 | ±        | -  | -  | +     | +          | +  | -  | -  | +  | +  |
| 8           | 26/M            | -          | -  | +  | -  | ±        | -  | +2 | -          | -  | ±  | +2 | ±        | -  | -  | +     | -          | +  | -  | -  | +  | +  |
| 9           | 35/F            | +          | +2 | -  | -  | ±        | -  | -  | ±          | -  | -  | -  | ±        | -  | -  | -     | -          | -  | +2 | -  | -  | -  |
| 10          | 39/M            | -          | +  | -  | -  | +        | -  | -  | +          | -  | -  | +  | +2       | -  | -  | -     | +          | +2 | +2 | -  | -  | -  |
| 11          | 44/M            | -          | +  | -  | -  | -        | -  | -  | -          | -  | -  | -  | -        | ±  | +  | -     | -          | -  | +  | -  | -  | -  |
| 12          | 42/M            | +2         | -  | -  | -  | +3       | +  | -  | +2         | -  | +  | +  | +3       | +  | -  | ±     | +2         | +3 | -  | -  | -  | -  |
| 13          | 36/F            | -          | +  | -  | -  | -        | -  | -  | -          | -  | -  | -  | -        | -  | -  | -     | -          | -  | +  | -  | -  | -  |
| 14          | 39/M            | +          | -  | -  | -  | +        | -  | -  | +          | -  | -  | -  | +        | -  | -  | -     | +          | +  | -  | -  | -  | -  |
| 15          | 39/M            | -          | +  | -  | -  | -        | -  | -  | -          | -  | -  | -  | -        | -  | +  | -     | -          | -  | +2 | -  | -  | -  |
| 16          | 27/M            | -          | -  | +4 | +2 | +2       | +2 | +4 | +2         | -  | +2 | +3 | +2       | -  | -  | +3    | +          | +2 | -  | -  | +2 | +2 |
| 19          | 35/M            | +          | -  | +  | -  | +        | -  | +  | +          | -  | -  | +2 | +        | -  | -  | +     | +2         | +3 | -  | -  | -  | -  |
| 23          | 42/M            | -          | +2 | -  | -  | -        | +  | -  | -          | +  | -  | -  | -        | -  | +  | -     | -          | -  | +  | -  | -  | -  |
| 26          | 30/F            | -          | +  | -  | -  | +        | +  | -  | +          | +  | -  | -  | -        | +  | +  | -     | -          | -  | +2 | -  | -  | -  |
| 27          | 35/F            | +          | -  | +3 | +  | +2       | -  | +3 | +2         | -  | +  | +2 | +3       | -  | -  | +     | ±          | +2 | -  | -  | -  | -  |
| 28          | 32/F            | +          | -  | -  | -  | +2       | -  | -  | +2         | -  | +  | +  | +2       | -  | -  | -     | +2         | +3 | -  | -  | -  | -  |
| 29          | 25/F            | -          | -  | +  | ±  | -        | -  | +3 | -          | -  | +  | +2 | -        | -  | -  | +3    | ±          | +  | -  | -  | -  | -  |
| 30          | 15/M            | +          | -  | ±  | -  | +        | -  | +  | +          | -  | ±  | ±  | +2       | -  | -  | ±     | +          | +3 | -  | -  | -  | -  |
| 31          | 30/M            | +          | -  | ±  | -  | +2       | -  | +  | +2         | -  | +  | +  | +        | -  | +  | -     | +2         | +3 | -  | -  | -  | -  |
| 32          | 25/F            | +          | +2 | +  | +  | +2       | -  | +  | +          | -  | +  | +  | +2       | -  | +  | -     | +          | +  | +  | -  | -  | -  |
| 34          | 66/M            | -          | -  | -  | -  | +        | -  | -  | ±          | -  | -  | -  | +        | -  | -  | -     | -          | +  | -  | -  | -  | -  |
| 35          | 22/M            | +          | -  | +3 | +  | +2       | +  | +3 | +2         | -  | +  | +  | +        | -  | -  | +3    | +          | +2 | -  | -  | +  | +  |

Results of immunoblotting are given as: +, positive band; +2, stronger positive band; +3 or +4, strongest positive band; ±, weak reactive band; and -, no reaction to the band.

## RESULTS

### Effect of pH during extraction

Golden Delicious apples purchased from a grocery store were extracted with the extraction buffer adjusted to different pH values by addition of NaOH. The amount of extractable proteins increased with increasing pH of the buffer. Likewise, more allergens could be seen on the immunoblot when extracted at pH 10.2 as compared with neutral pH (Fig. 1). In this study, all extractions were carried out with buffer with a pH of 9.5 to 10.5.

### IgE-reactivity analysis of tree pollen extracts and apple fruit extract

Of 34 serum samples from patients with tree pollen allergy used in this immunoblotting study, 24 reacted to apple allergens. Reactive bands of these 24 sera to both tree pollens and apple extract are summarized in Table I. Seventy-five percent of patients allergic to apples (18 of 24) reacted to the 31 kd protein, 37.5% (9 of 24) reacted to the 18 kd

protein, 4% (1 of 24) to a 16 kd protein, and 25% (6 of 24) to a 13 to 14 kd protein. Of the patients with hypersensitivity to Bet v 1 (17 kd) in birch pollen extract, 100% reacted to the 18 kd protein of the apple extract. This is consistent with the report that 97.6% of patients with reactivity to Bet v 1 demonstrated IgE cross-reactivity to 17 and 18 kd proteins in apple.<sup>10</sup> Patients with reactivity to Bet v 2 (13 to 14 kd) in birch pollen reacted variably to the 13 to 14 kd protein of apple. Patients with multiple reactivities to tree pollen extracts usually demonstrate multiple reactivities to apple proteins as well. The relationship between other tree pollen extracts and apple extract requires further investigation. Fig. 2 shows five representative serologic reactivity patterns of birch pollen extract and those of apple fruit extract. Patients with exclusive hypersensitivity to Bet v 1 in birch pollen extract reacted solely to the 18 kd protein of the apple extract (Fig. 2, lane 1). Patients with reactivity to other tree pollen allergens besides Bet v 1 reacted to other proteins and to the

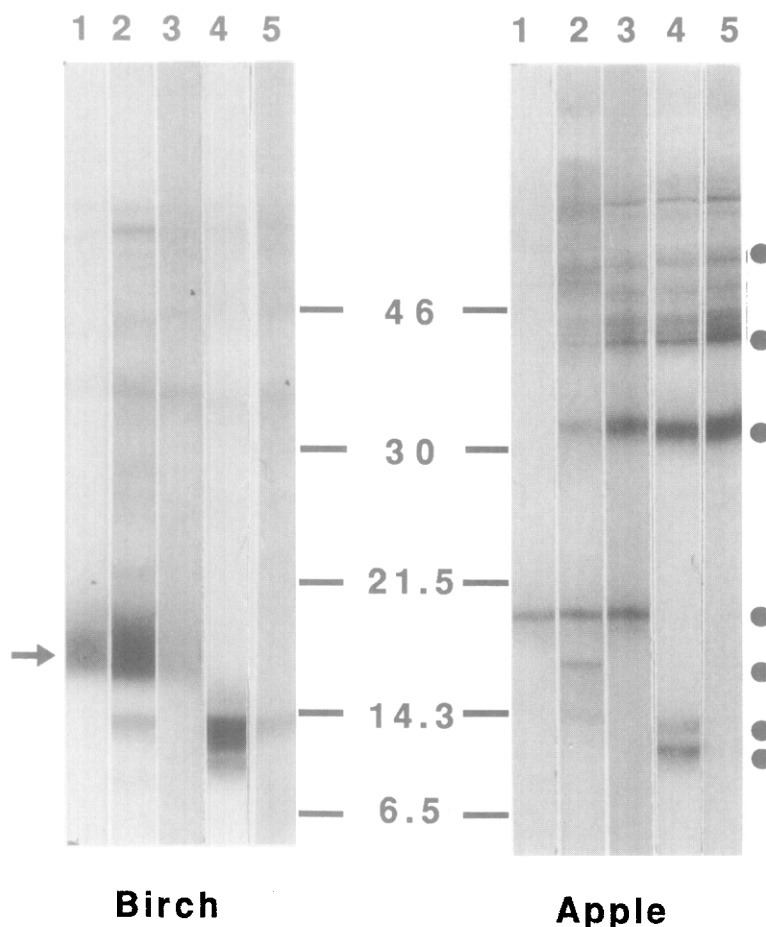


**FIG. 2.** IgE reactivity patterns of birch pollen extract and apple fruit extract. Arrow indicates Bet v 1, and dots indicate apple allergens. Lanes 1 to 5 are different patient sera. Molecular weight marker is in kilodaltons.

18 kd protein in apple (Fig. 2, lanes 2 and 3). Patients who did not have Bet v 1 reactivity but did have other tree pollen reactivity reacted to the 31 kd protein and other proteins in apple (Fig. 2, lanes 4 and 5). Distinctive allergens in apple extracts are 50, 38, 31, 18, 16, 14, and 13 kd and are indicated by dots. A pool of sera from patients allergic to apple, which reacted to all apple allergens, was made up and used for detecting allergens.

#### Characterization of apple allergens

The amino-terminal amino acid sequences of two major apple fruit allergens found in the study, 31 kd and 18 kd, were determined and are listed in Table II. The 18 kd sequence shares 52% identity with disease resistance response proteins DRRG49-c, pI176, and PI49 and 48% to 50% identity with PvPR2, PvPR1, and pathogenesis-related proteins of the kidney bean. It has 52% identity with Bet v 1 as well. The 31 kd sequence

shares 46% identity with the sequence of pathogenesis-related proteins of PR-5, or 46% identity with the amino-terminal sequences of wheat trima-tin, oat avenatin, and barley thaumatin-like protein.

#### IgE reactivity analysis of different varieties and sources of apple

Different varieties of freshly picked apples were examined for their IgE reactivities with a pool of sera with all reactivity patterns. Apples were collected, and extracts were made immediately after harvest. Serum reactivity to each variety is shown in Fig. 3. Red Delicious and McIntosh apples did not contain as high a level of 31 kd protein (Fig. 3, lanes 2 and 3) as did the Golden Delicious and Granny Smith apples (Fig. 3, lanes 4 and 5). All four varieties of apple contained varying amounts of 16 kd and 18 kd proteins. The extracts of freshly picked apples were also compared with extracts of

**TABLE II.** N-terminal amino acid sequences of apple 18 and 31 kd-proteins are compared with pathogenesis-related proteins

|                            |                                                                                                                      |
|----------------------------|----------------------------------------------------------------------------------------------------------------------|
| <b>Apple 18 kd protein</b> |                                                                                                                      |
| Bet v 1 (Europe)           | GVYTFENEYTS <b>L</b> IPPX <b>I</b> L <b>F</b> KAFVXD                                                                 |
| Bet v 1 (cDNA)             | SVFN <b>Y</b> ETETTSVIPAA <b>M</b> L <b>F</b> KAFILD                                                                 |
| PI49 (pea)                 | MGVFN <b>Y</b> ETETTSVIPAA <b>R</b> L <b>F</b> KAFILD                                                                |
| PI176 (pea)                | MGVFN <b>V</b> ED <b>E</b> ITSVVAPAIL <b>Y</b> KALVTD                                                                |
| DRRG49-c (pea)             | MGVFN <b>V</b> ED <b>E</b> ITSVVAPAIL <b>Y</b> KALVTD                                                                |
| PvPR1 (kidney bean)        | MGVFN <b>F</b> EE <b>E</b> ATSIVAPAT <b>L</b> HKALVTD                                                                |
| PvPR2 (kidney bean)        | GV <b>F</b> TFEDQTTSPVAPAT <b>L</b> YKAVAKD                                                                          |
| SAM2 (soybean)             | MA <b>V</b> FTFEDQTTSPVAPAT <b>L</b> YKALVKD                                                                         |
| PR1-1 (parsley)            | MGV <b>F</b> TFE <b>D</b> EINSPVAPAT <b>L</b> YKALVTD                                                                |
|                            | MGV <b>V</b> Q <b>K</b> SE <b>V</b> ETTS <b>S</b> VS <b>A</b> E <b>K</b> L <b>F</b> K <b>G</b> L <b>C</b> L <b>D</b> |
| <b>Apple 31 kd protein</b> |                                                                                                                      |
| Wheat trimatin             | AK <b>I</b> TT <b>T</b> NNXPNTVWPG <b>I</b> L <b>T</b> GF <b>G</b> Q <b>K</b> PQ                                     |
| Antifungal protein R       | AT <b>I</b> TT <b>V</b> N <b>R</b> CSYTVWPGAL <b>P</b> GGGA                                                          |
| Oat avematin               | AT <b>I</b> TT <b>V</b> N <b>R</b> CSYTVWPGAL <b>P</b> GGGVRLD                                                       |
| PR5                        | TT <b>I</b> TT <b>V</b> N <b>K</b> CSYTVWPGAL <b>P</b> GGGVVLD                                                       |
|                            | ---TL <b>R</b> NCPTTVWAGT <b>L</b> AG <b>G</b> PK <b>L</b> G                                                         |

An amino acid of the apple 18 kd protein (boldface type) differs from the report by Vieths et al.<sup>6</sup> X indicates uncertain amino acid. The identical amino acids are boxed. The antifungal proteins R and PR5 are thaumatin-like proteins of barley and *Arabidopsis thaliana*, respectively. The PR5 fragment represents amino acids 27 to 43. This search was performed at the National Center for Biotechnology Information (NCBI) with the BLAST network.

those purchased from stores. A typical pattern of freshly picked apple extract (*lane 1*) compared with the stored-purchased apple extract (*lanes 2 to 4*) is shown in Fig. 4. The amount of 18 kd allergen was relatively higher in the store-purchased apples.

### Heat stability of apple allergens

To test the thermolability of apple allergens, Golden Delicious apple extract was heated in boiling water for 20 minutes before the SDS-PAGE analysis. Processed canned apple and apple sauce extracts were also examined. In the immunoblots there was no detectable allergen in the heated sample and only a trace amount in the canned apple extract. Our results indicated that apple allergens can be destroyed by heating. Thus during canning when heat is applied, most allergens are destroyed.

### Allergen variation during prolonged storage

Orchard-picked apples stored at 4°C were extracted at different storage intervals and analyzed by immunoblotting SDS-PAGE (Fig. 5). Both 31 kd and 18 kd proteins increased during prolonged storage. Increases in both allergens were clearly shown in McIntosh (panel A), Red Delicious (panel B), and Granny Smith (panel C) varieties. An obvious increase in the 18 kd protein was observed after 3 weeks of storage. The amount of 18 kd and 31 kd proteins in Golden Delicious apples (panel D) remained at a high level through-

out the entire storage period. The 16 kd protein level remained low during the storage period in the Granny Smith apples, although it increased transiently in the other varieties.

### Changes in allergens under defined ripening conditions

Equal amounts of Gala apple extracts at each ripening stage were examined with pooled sera and with an antibody to ACC oxidase (Fig. 6). The degree of ripening was altered by the number of days of exposure to air and to low oxygen and was determined by the amount of ACC oxidase. During prolonged air exposure (*lane 1*), ACC oxidase was expressed; in contrast during low oxygen exposure (*lane 4*), ACC oxidase was low. Although the amount of 18 kd and 31 kd allergens fluctuated with each preparation, there was no direct correlation to the amount of ACC oxidase expression during different ripening conditions (Fig. 6).

### DISCUSSION

The apple allergen, 17 to 18 kd, has been reported to share common epitopes with the birch pollen allergen, Bet v 1.<sup>13</sup> Vieths et al.<sup>6</sup> demonstrated that the level of 18 kd allergen in apples correlates with the IgE binding potency in patients allergic to apples. However, apple allergens are difficult to quantify because of the high levels of phenolic compounds.<sup>3</sup> To prevent the interference of phenolic compounds with protein isolations, we

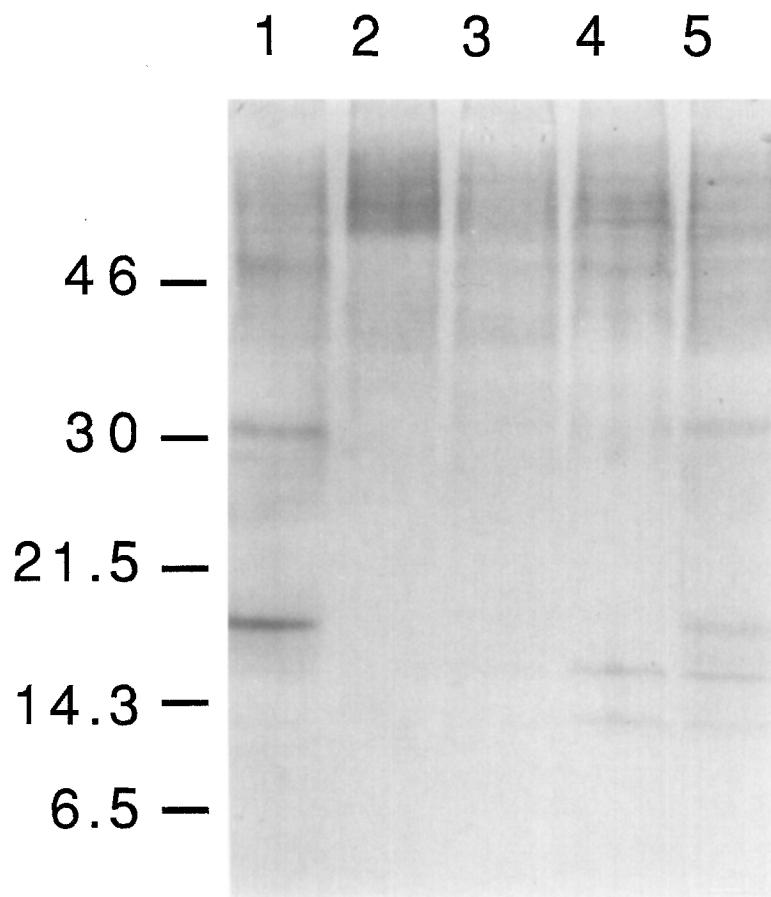


**FIG. 3.** Allergens from freshly picked apples. *Lane 1*, Control and store-purchased Golden Delicious apples; *lane 2*, McIntosh; *lane 3*, Red Delicious; *lane 4*, Granny Smith; and *lane 5*, Golden Delicious. Molecular weight marker is in kilodaltons.

modified the method of Björkstén et al.<sup>16</sup> by adding sucrose and adjusting the pH. The pH effect on allergen extraction is clearly shown in Fig. 1. At a pH of 9.5 to 10, apple proteins can be extracted in consistent amounts for comparison by immunoblots (Fig. 1), though small fluctuations of protein content still existed among individual apples. The pH effect on the extraction efficiency could be due to different physicochemical characteristics of each allergen when extracted in a single-phase solution, such as an aqueous solution, rather than in a mixture of aqueous and organic solutions. In general, comparison of allergen concentrations between varieties is complicated by the absence of well-characterized internal standards. Even though the amounts of allergens in apple can be calculated as a percentage of total proteins in the preparation, absolute quantitation of specific allergens remains difficult. Because plant extracts comprise a complicated mixture of antigenic components,

some allergens may be underestimated because of their limited extraction capacity.

The amount of 31 and 18 kd allergens differed among the varieties of freshly picked apples (Fig. 3). Among freshly picked apples, the amount of 31 kd protein was higher in Golden Delicious and Granny Smith varieties than in Red Delicious and McIntosh varieties. The amount of 18 kd protein in Golden Delicious apples is higher than that in the other varieties tested. This result is consistent with the report of Vieths et al.<sup>6</sup> The relative amounts of other allergens among varieties also varied (Fig. 3). The amount of 16 kd allergen is either equal to or greater than the amount of 18 kd allergen among freshly picked apples. The 18 kd allergen predominated in the control, a store-purchased Golden Delicious apple (Fig. 3, lane 1). The same phenomenon was seen when freshly picked apples and store-purchased Granny Smith apples were compared (Fig. 4). No conclusions about the pre-

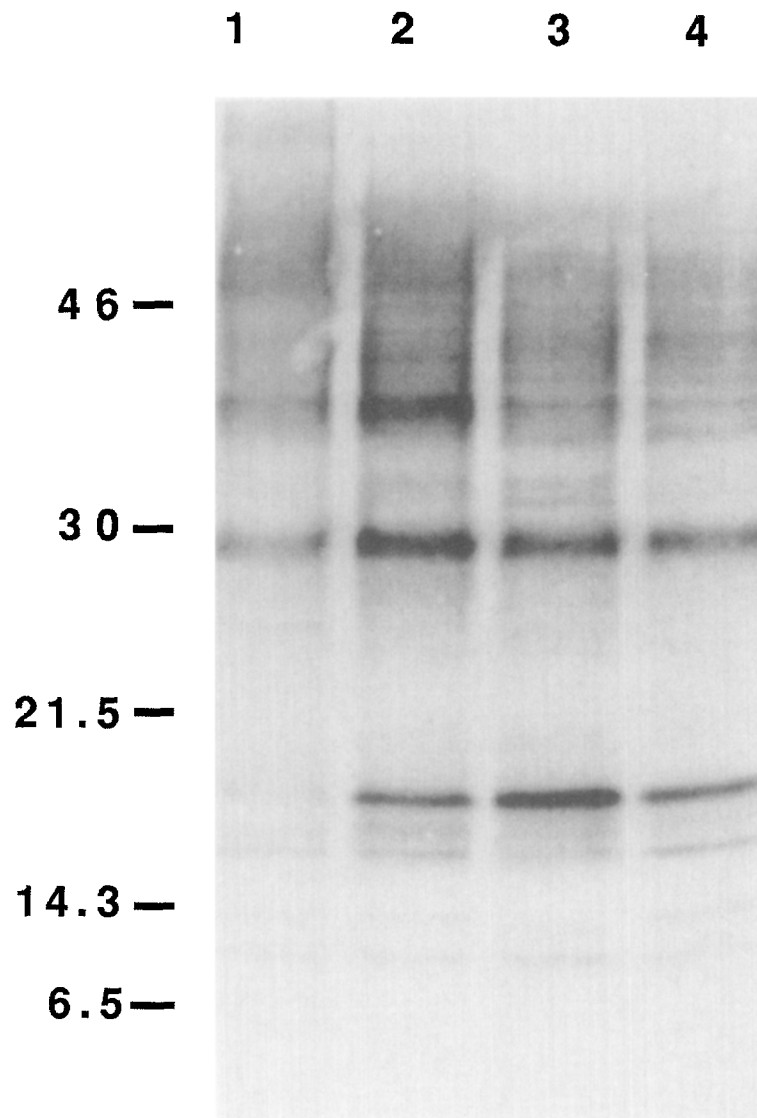


**FIG. 4.** Comparison of freshly picked and store-purchased Granny Smith apples. *Lane 1*, Freshly picked Granny Smith apples; *lanes 2 to 4*, different local store-purchased Granny Smith apples. Molecular weight marker is in kilodaltons.

dominance of the 18 kd protein in store-purchased apples can be drawn from this observation because of a lack of control of storage conditions and lengths of time in stores.

It was noted that during storage at 4° C, the 18 kd allergen of McIntosh, Red Delicious, and Granny Smith apples began to increase in the third week (Fig. 5). Because the extractions were not performed at the same time with the same individual apple, the amount of the protein may vary. However, the tendency for the increase of the 18 kd allergen during storage is clear (Fig. 5). Certainly, other possible triggering factors, such as

temperature, CO<sub>2</sub> buildup, and microbial infection, cannot be excluded.

Because ethylene behaves as a plant hormone that regulates fruit ripening, the ripening process can be stimulated by increasing the levels of ethylene.<sup>7,8</sup> After apples are placed in a low oxygen atmosphere, a surge of ethylene production occurs on their return to air. At the same time, an enzyme, ACC oxidase, is also expressed. We can therefore consider the ACC oxidase level as an indicator of degree of ripening (Fig. 6). The 18 kd and 31 kd protein levels remained relatively constant, whereas the amount of ACC oxidase in-

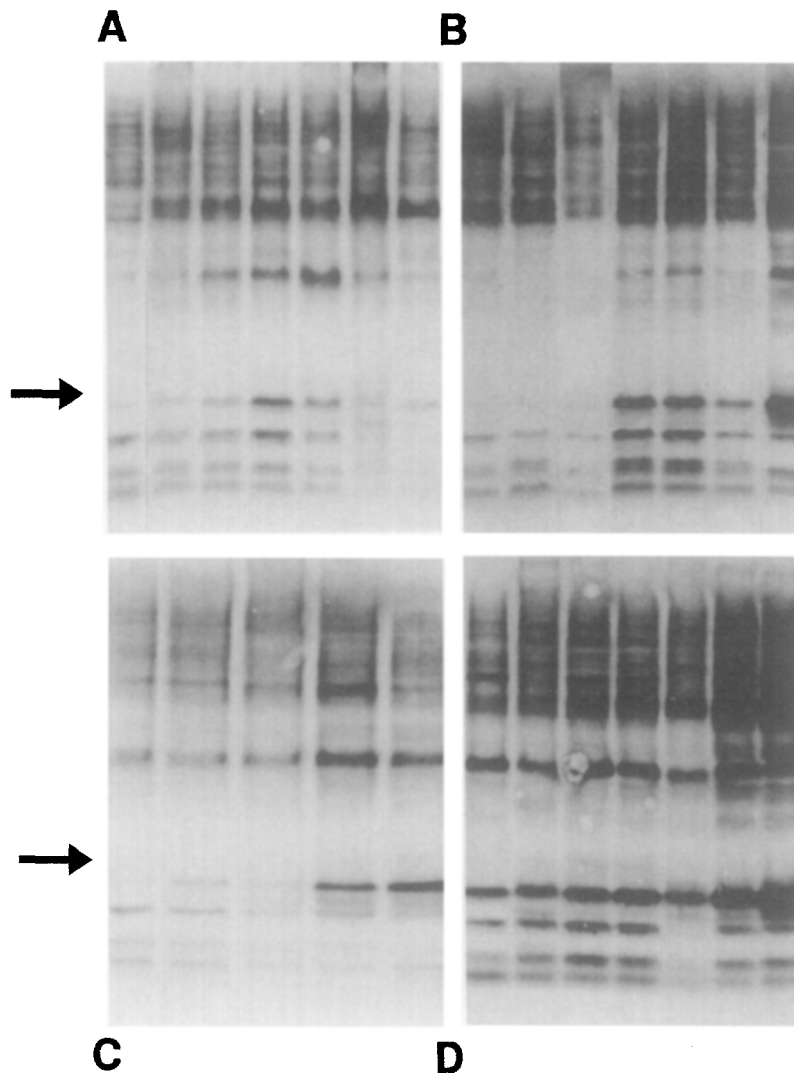


**FIG. 5.** Variation in apple allergens during storage: **A**, McIntosh; **B**, Red Delicious; **C**, Granny Smith; and **D**, Golden Delicious. Arrow indicates the position of 18 kd allergen. The storage time increases from left to right; the first lane is the starting day. The storage days are as follows. **A**: 0, 28, 42, 62, 75, 96, 130 days; **B**: 0, 14, 22, 38, 47, 68, 105 days; **C**: 0, 12, 25, 46, 86 days; and **D**: 0, 7, 13, 26, 48, 82, 113 days.

creased during ripening (Fig. 6). Although fluctuations in the amount of 18 and 31 kd proteins were observed in preparations from each ripening stage, there was still no clear correlation between the level of ACC oxidase and the amount of 18 and 31 kd allergens. However, the reduction of the 16 kd allergen was related to ripening, as clearly demonstrated in the Gala apple (Fig. 6). When apples were harvested 1 week before ripening, they did not contain detectable amounts of ACC oxidase but did contain significant amounts of 31 kd, 18 kd, and 16 kd allergens (data not shown). Furthermore, only the 16 kd allergen was observed during oxygen-regulated ripening if apples were sprayed

to prevent microbial infection. The 18 and 31 kd proteins also cross-react immunologically with a fungally induced protein, PI49, of peas (data not shown). These findings suggest that expression of the 18 and 31 kd allergens may not be affected by ripening but by factors related to disease resistance.

According to the amino acid sequence analyses, the 18 and 31 kd allergens share significant homologies with different types of plant disease-resistant proteins (Table II). Pathogenesis-related proteins are defensive proteins that are expressed when plants are exposed to various stressful conditions, such as microbial or environmental stresses.<sup>21</sup> It is

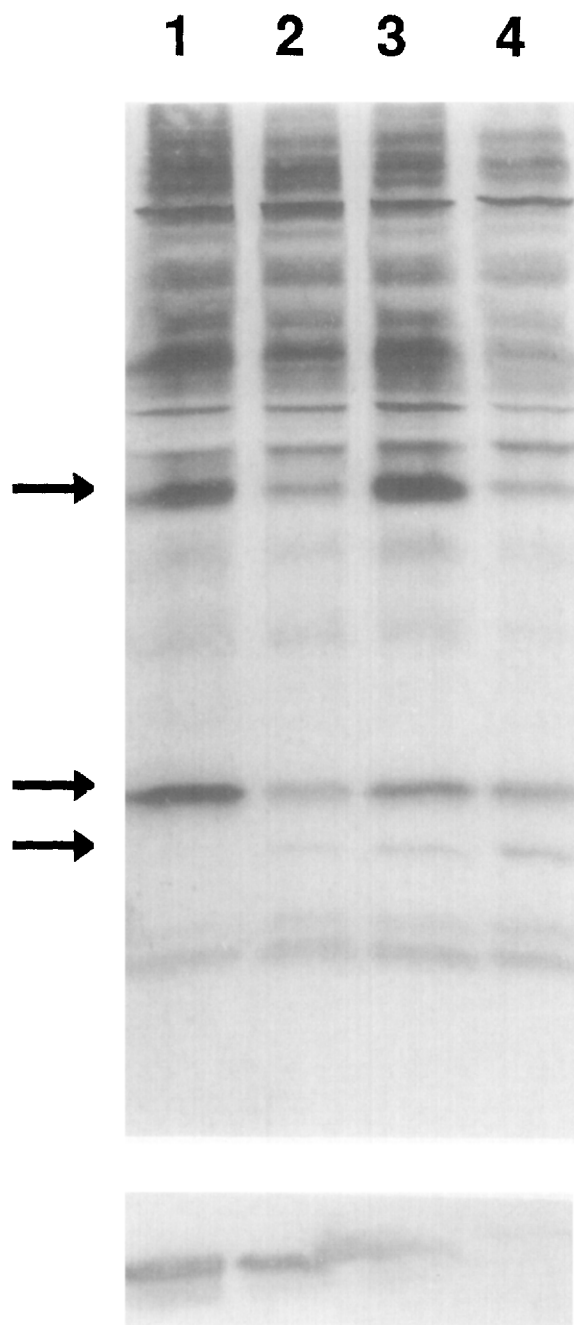


**FIG. 6.** Changes in apple allergens under controlled atmospheric conditions. **The upper panel** is the serologic blotting, and **the lower panel** is the blotting against ACC oxidase antibody. The appearance of ACC oxidase indicates ripening. Arrows indicate the 31, 18, and 16 kd allergens. Lanes 1 to 4 are four ripening conditions, as described in the Methods section.

tempting to speculate that the expression of these proteins is triggered by microbial attack, because the 18 and 16 kd proteins increased only in June and July (data not shown), when microbial attacks are common. According to farm records during the

summer months, fungal infections, such as *Gloeodes pomigena* and *Microthyriella rubi*, are common in apple-growing areas of the eastern United States (*Diseases of Tree Fruits* published by SEA-Extension, USDA). Allergens known to be homologous with disease-resistant proteins are Bet v 1 and Dol m 5.<sup>9,23</sup> The latter is a white-faced hornet venom component, which also shares homology with a pathogenesis-related protein from tobacco leaf. A detailed molecular analysis and epitope mapping will provide further information pertinent to our understanding of the structure-function relationship in apple allergens. In addition, we confirmed that apple allergens are thermolabile and that canned apples and applesauce contain only trace amounts of IgE-reactive allergens. This observation could explain why patients allergic to apples are able to tolerate canned but not fresh apples.<sup>24</sup>

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