

Identification and Characterization of the Allergens in the Tomato Fruit by Immunoblotting

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Key Words

β -Fructofuranosidase · Oral allergy syndrome · Pectinesterase · Polygalacturonase · Superoxide dismutase · Tomato fruit

Abstract

Background: Although the tomato fruit (*Lycopersicon esculentum*) has been widely investigated for breeding purposes, there have been few studies on tomato allergenicity. We attempted to identify the tomato fruit allergens and to compare the concentrations of IgE-binding proteins among the different growth stages with sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. **Methods:** An immunoblot experiment on tomato fruit extracts was performed using sera from 11 patients with oral allergy syndrome (OAS) to tomatoes. Bands reacting with IgE from more than half of the OAS patients' sera were excised and subjected to determination of N-terminal amino acid sequences using the automated Edman degradation method. Moreover, we compared the concentrations of these proteins at each growth stage of the tomato fruit with SDS-PAGE and immunoblotting. **Results:** Four proteins binding with IgE from more than half of the OAS patients' sera were determined to be polygalacturonase 2A (PG2A), β -fructofuranosidase, superoxide dismutase

(SOD) and pectinesterase (PE). The concentrations of PG2A, β -fructofuranosidase and PE were highest in the red ripening stage with both SDS-PAGE and immunoblotting. **Conclusion:** The concentrations of 3 of 4 tomato allergens increased during ripening.

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Introduction

Although the tomato (*Lycopersicon esculentum*) has been widely investigated for breeding purposes, there have been few reports on its allergenicity. According to Bleumink et al. [1], the most allergenic fraction of ripe red tomatoes was fraction G. It was obtained via chromatography of a crude fraction gained by $(\text{NH}_4)_2\text{SO}_4$ precipitation of the dialyzed extract of ripe tomatoes using DEAE-Sephadex and was shown to be a polydisperse mixture of closely related protein-carbohydrate complexes. They also demonstrated with skin tests that the allergenicity of unripe tomatoes was lower than that of ripe tomatoes [2]. Petersen et al. [3] identified the main tomato allergens using 8 sera from subjects with high specific IgE to tomatoes who also had grass pollen allergies. Five of 8 sera reacted to β -fructofuranosidase, and another 5 reacted to profilin (2 of them reacted to both proteins).

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Table 1. Backgrounds of the patients for the immunoblotting experiment of red ripening tomato fruit extract

Serum No.	Age year	Sex	Total IgE IU/ml	CAP-FEIA ¹		OAS to tomato	Other allergic disease		
				AU/ml	class		POL	BA	AD
1	3	M	3,300	7.58	3	+	+		+
2	7	F	68	4.96	3	+	+	+	
3	8	F	1,400	7.72	3	+	+	+	+
4	10	M	1,060	23.8	4	+	+	+	+
5	11	M	530	9.34	3	+	+	+	+
6	13	F	560	19.5	4	+	+		
7	13	F	823	5.49	3	+	+		+
8	14	F	6,400	10.9	3	+	+	+	+
9	14	M	9,200	27.5	4	+	+	+	+
10	20	M	8,800	8.69	3	+	+		+
11	28	M	88	1.39	2	+	+	+	+

POL = Pollinosis; BA = bronchial asthma; AD = atopic dermatitis.

¹ Pharmacia CAP-FEIA System.

In this study, we performed an immunoblotting experiment to identify the allergens of red ripening tomatoes using sera from 11 patients with oral allergy syndrome (OAS) to tomato and assessed whether these allergens increased with ripening.

Materials and Methods

Extraction

Tomato fruits of different stages (green, breaker, pink and red ripening) were obtained from the Wye Research and Education Center of the University of Maryland (Queenstown, Md., USA). Tomato fruits of each stage were freshly harvested just before the experiments. The tomato pericarp was dissected and homogenized for 15 s in cold distilled water [4]. After centrifugation at 2,400 *g* for 10 min, the pellet was collected and resuspended in 1 *M* NaCl at pH 6 with 1 *M* NaOH. After stirring for 3 h at 4 °C, the debris was removed by centrifugation at 2,400 *g* for 10 min. The supernatant was filtered and brought to 75% saturation with ammonium sulfate overnight. The precipitate was collected by centrifugation at 10,000 *g* for 20 min and suspended in 0.15 *M* NaCl at pH 6. This extract was dialyzed against 0.15 *M* NaCl (pH 6) at 4 °C over two nights. The residues were removed by centrifugation at 10,000 *g* for 20 min. The collected supernatant was used throughout.

Transfer and Immunoblotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 16% Tris-glycine precast gel (Tefco Corporation, Machida, Japan) according to Laemmli [5] under reducing conditions. Proteins were separated at 120 V for 2 h. After SDS-PAGE, proteins were transferred to Immobilon™-P membranes (Millipore, Bedford, Mass., USA) as previously reported [6]. For

detection of bound IgE, we used a phosphatase-labeled goat antihuman IgE (ε) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA) diluted 1:1,000 and developed with BCIP/NBT solution (Kirkegaard & Perry Laboratories). The protein concentrations of these samples were determined by a BCA protein assay (Pierce, Rockford, Ill., USA).

N-Terminal Amino Acid Sequence

After blotting, the Immobilon-P membranes were stained with 0.1% amido black (Sigma Diagnostics, St. Louis, Mo., USA) in 50% methanol and 10% acetic acid, destained with 40% methanol and 10% acetic acid and air-dried. Protein bands with IgE-binding activity were excised and subjected to N-terminal amino acid sequencing via automated Edman degradation using an Applied Biosystems model 470A protein sequencer [7]. The resulting phenylthiohydantoin (PTH) amino acid derivatives were identified using the model 120A one-line PTH analyzer and the standard Applied Biosystems program. The N-terminal amino acid sequence of each protein was determined at least in triplicate in two different laboratories.

Human Sera

To identify the tomato fruit allergens in immunoblotting, we used 11 sera from patients with OAS after they had eaten fresh tomato fruits (table 1). OAS was diagnosed by clinicians according to the repeated immediate hypersensitive history (intra-oral and lip irritation) after eating fresh tomatoes. In 3 unspecific cases (patients reporting one hypersensitive episode or an aversion to tomatoes) we performed open oral challenges with fresh tomato fruit, excluding only 1 subject from the study because of no immediate hypersensitive symptoms to tomato ingestion. Control serum pools were obtained from subjects who had no histories of hypersensitive symptoms to fresh tomatoes. The sera were kept at –20 °C until use.

Fig. 1. Immunoblots of red ripening tomato fruit extract using sera from 11 patients with OAS to fresh tomato fruit. The backgrounds of the patients are shown in table 1. Control sera were obtained from subjects who had no histories to hypersensitive symptoms to fresh tomato. The specific IgE-binding bands of more than half of the subjects had relative molecular masses (M_r) of 46, 22, 18, and 14 kD (indicated by closed square). Dots indicate nonspecific binding bands.

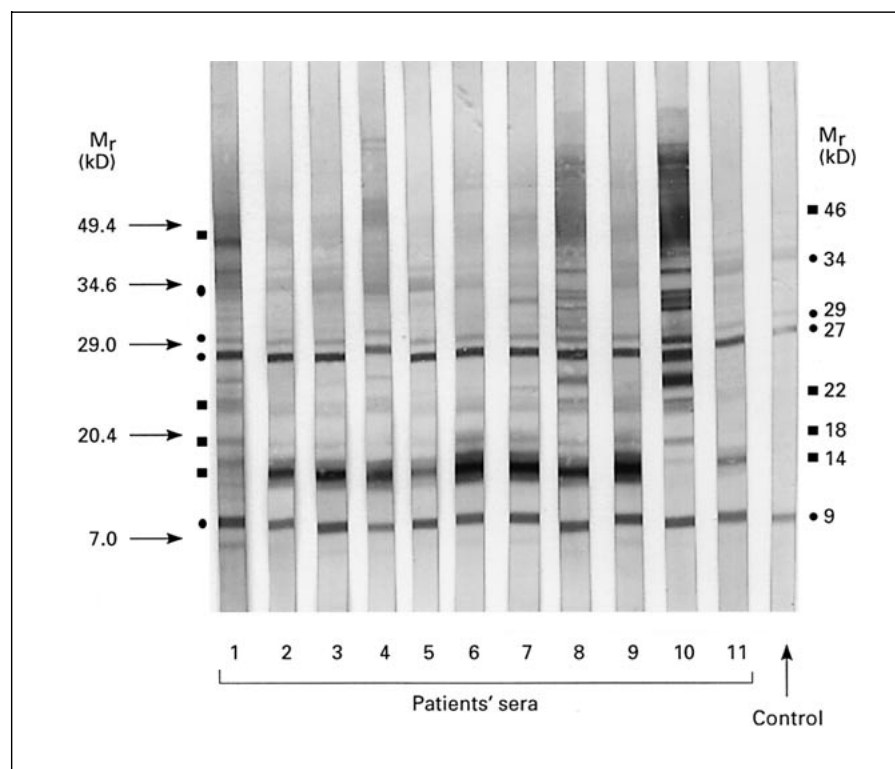


Table 2. IgE-binding activity to red ripening tomato fruit extract

M_r kD	Patients' sera											Control serum pool	Positive %
	1	2	3	4	5	6	7	8	9	10	11		
46	×	×	×	×			×	×	×	×			72.7
34	×	×	×	×	×	×	×	×	×	×	×	×	
32							×						9.1
30	×	×						×		×			36.4
29	×	×	×	×	×	×	×	×	×	×	×	×	
27	×	×	×	×	×	×	×	×	×	×	×	×	
25	×		×	×				×		×			45.5
24	×						×						18.2
22	×	×	×	×		×	×	×	×	×			81.8
18	×	×	×		×	×	×	×	×	×			81.8
16	×												9.1
14		×	×	×	×	×	×	×	×	×	×	×	81.8
9	×	×	×	×	×	×	×	×	×	×	×	×	
<7	×		×	×		×	×	×	×	×			72.7

M_r = Relative molecular mass.

Results

Immunoblotting of Tomato Fruit Extract with Patients' Sera

As shown in figure 1, 13 protein bands with a molecular weight range of 7–50 kD reacted to IgE from patients. Among these bands, 34-, 29-, 27- and 9-kD protein bands exhibited nonspecific binding to goat IgG because they were not only detected when control serum was added, but also when no serum was added. The bands reactive with IgE from more than half of the patients' sera were 46 (72.7%), 22 (81.8%), 18 (81.8%) and 14 kD, (81.8%), as shown in table 2. The IgE-binding activities of these proteins were inhibited completely by preincubating the patients' sera with tomato fruit extracts.

Partial protein sequences of these bands were determined and screened for homology with sequences in the Swiss Prot Data Base. The N-terminal amino acid sequence of some protein bands (32, 30, 29, 23 and 16 kD) could not be determined because the protein amounts were too small. The N-terminal amino acid sequences of the proteins showed almost complete identity with the amino acid sequences of polygalacturonase 2A (PG2A,

Table 3. Comparison of the N-terminal amino acid sequence with known protein sequences

M _r /known protein source/accession/reference	1	5	10	15	20	25	30	Identity %
46 kD ¹ /PG2A Tomato/P05117/[8]	R I K V I N V L S F G A K G D 72		80					93.3
	G I K V I N V L S F G A K G D							
34 kD/PE Tomato/A25010/[9]	I I A N A V V A Q D G T G D Y 1		10					100.0
	I I A N A V V A Q D G T G D Y							
27 kD/Pathogenesis-related protein P23 Tomato/S16264/[10]	A T F E V R N N G P Y T V X A A 1		10					87.5
	A T F E V R N N C P Y T V W A A							
25 kD/ β -Fructofuranosidase Tomato/S31155/[11]	Y A W S N A M L S W Q R T A Y H F Q P Q K N W M N D P N G P 93		100		110		120	100.0
	Y A W S N A M L S W Q R T A Y H F Q P Q K N W M N D P N G P							
22 kD/ β -Fructofuranosidase Tomato/S31155/[11]	A D H V G F S X S T S G G A A S R G I L G P F G V I V I A D 481		490		500		510	96.7
	A D H V G F S C S T S G G A A S R G I L G P F G V I V I A D							
18 kD ¹ /SOD Tomato/AAA34194/[12]	V K A V A V L N S Q E G V S G 2		10					93.3
	V K A V A V L N S S E G V S G							
14 kD ¹ /PE Tomato/A25010/[9]	A Q G R T D P N Q A T G T S I Q F X N I 174		180		190			95.0
	A Q G R T D P N Q A T G T S I Q F C N I							
9 kD/PE-A Tomato/AAB30509/[13]	L S X G Q V E S G L A P X L P 1		10					100.0
	L S X G Q V E S G L A P X L P							

¹ Allergens in the tomato fruit by immunoblotting.

46 kD) [8], pectinesterase (PE, 34 and 14 kD) [9], pathogenesis-related protein P23 (27 kD) [10], β -fructofuranosidase (25 and 22 kD) [11], superoxide dismutase (SOD, 18 kD) [12] and PE-A (9 kD) [13], respectively (shown in table 3). The tomato fruit IgE-binding proteins were found to be PG2A, PE, β -fructofuranosidase and SOD.

Comparison of the Concentrations of the Main Allergens in Tomatoes at Various Stages by SDS-PAGE and Immunoblotting with Pooled Serum from OAS Patients

As shown in figure 2a, the concentration of PG2A (46 kD), visualized by silver staining, clearly increased with ripening, although the applied protein concentration of each sample was identical (3.5 μ g of protein amount/lane). As the amount of protein of the remaining main allergens was too small for identification, we performed immunoblotting with OAS patient sera. Figure 2b shows the immunoblotting of each tomato stage (10.0 μ g/lane) using the serum pool from OAS patients. The concentra-

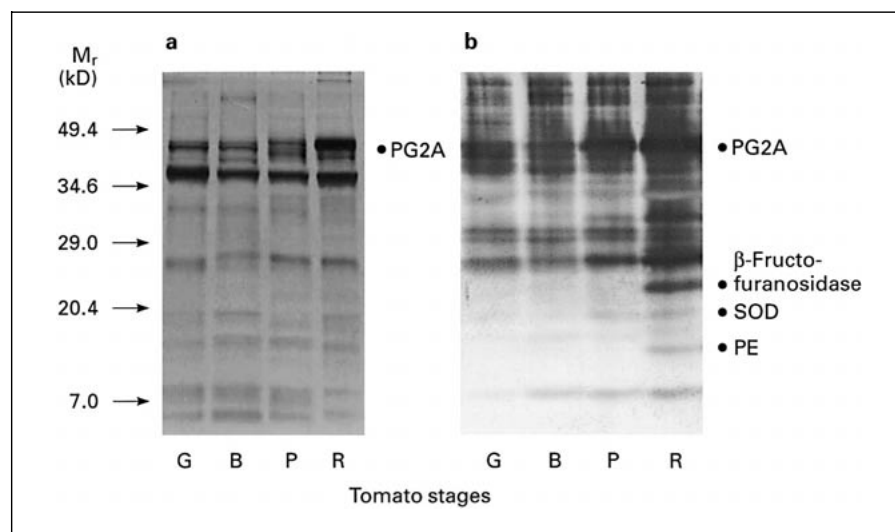
tions of PG2A, β -fructofuranosidase and PE were highest in the red ripening stage by immunoblotting with OAS patient sera.

Discussion

There have been few studies on the allergenicity of the tomato fruit. The aim of this study was to identify the tomato fruit allergens in immunoblotting using sera from 11 subjects with OAS to fresh tomato and to define the relationship between the allergenicity of tomato fruit and ripening using SDS-PAGE and immunoblotting.

Figure 1 and table 2 show the results of the immunoblotting of red ripening tomato fruit extracts. About 9 protein bands in a molecular mass range of 7–50 kD reacted with the IgE of the patients' sera, except for controls. Among them, protein bands with molecular masses of 46, 22, 18 and 14 kD reacted with more than half of the patients' sera. These data suggested that PG2A (46 kD),

Fig. 2. **a** Comparison of protein concentrations among the different growth stages of tomato fruit in SDS-PAGE stained by silver. Each sample showed the same protein concentration (3.5 µg/lane). **b** Comparison of the IgE-binding protein concentrations among the different growth stages in immunoblotting with pool serum from OAS patients. Dots indicate allergens of tomato fruit extract in immunoblotting. G, B, P and R indicate each tomato growth stage; G: green, B: breaker, P: pink, and R: red ripening. Each sample had the same protein concentration (10.0 µg/lane). M_r = Relative molecular mass.



β -fructofuranosidase (22 kD), SOD (18 kD) and PE (14 kD) were the allergens in tomato fruit in this immunoblotting experiment.

Of these allergens, PG2A [4], β -fructofuranosidase [11], and PE [14] were previously reported to be related to ripening and softening of the tomato fruit. Indeed, our SDS-PAGE and immunoblotting studies of different growth stages revealed that each concentration of PG2A, β -fructofuranosidase and PE was highest at the red ripening stage among all growth stages.

On the other hand, Petersen et al. [3] investigated tomato allergens using sera from 8 subjects who had specific IgE to tomatoes and grass pollen allergies. The IgE from 5 of 8 sera reacted with a 16-kD protein, which was identified as profilin. Profilin contributed considerably to the cross-reactivity between tomato fruit and grass pollen. Moreover, they reported that the most prominent IgE-reactive component in tomato extract was β -fructofuranosidase, which showed no sequence homology to any of the grass pollen allergens. After destroying the carbohydrate structures by periodate treatment, the only remaining IgE-reactive component was profilin.

In our experiment, β -fructofuranosidase was one of the dominant IgE-binding proteins in the tomato fruit and the IgE-binding capacities of these allergens decreased after periodate treatment (data not shown). These data are similar to those of Petersen et al. [3] and may support the findings of Bleumink et al. [1, 2] that most allergenic red ripe tomato fractions comprised a polydisperse mixture of closely related protein-carbohydrate complexes. How-

ever, the clinically relevant allergenicity of tomato fruit IgE-binding carbohydrate moieties remains to be elucidated.

Since the 16-kD protein had a low frequency of IgE-binding activity in our study (9.1%), we did not perform a further investigation to confirm whether this 16-kD protein was profilin. As mentioned above, profilin contributes considerably to the cross-reactivity between tomato fruit and grass pollen. Profilins have been reported by Valenta et al. [15] to be prominent allergens that can be isolated from tree pollens (*Betula verrucosa*, birch), pollens of grasses (*Phleum pratense*, timothy grass), and weeds (*Artemisia vulgaris*, mugwort). On the other hand, Arai et al. [16] measured profilin antibodies with enzyme-linked immunosorbent assays using sera from patients allergic to Japanese cedar ($n = 123$) and birch pollen ($n = 12$). The results showed that patients allergic to Japanese cedar pollen were all negative to profilin, while 2 (16.6%) patients allergic to birch pollen were positive. In Japan, the most important pollen allergen causing pollinosis is neither grass nor birch, but Japanese cedar. The different situations between Japan and Europe may be the reason why IgE binding to the 16-kD protein was less frequently recognized by IgE from subjects with tomato-induced OAS in our immunoblotting experiment.

In this study, all of the tomato-induced OAS patients suffered from Japanese cedar pollinosis.

The carbohydrate structures were identified in Cry j 1, which is a major allergen of Japanese cedar pollen [17]. The carbohydrate moieties of Cry j 1 were found to bear

IgE-reactive epitopes. The IgE binding to Cry j 1 could cross-react with the above-mentioned tomato fruit allergens. Indeed, the IgE of some subjects allergic to Japanese cedar pollen without OAS to tomato also reacted to these tomato fruit allergens (data not shown). We speculate that the occurrence of tomato OAS in patients allergic to Japanese cedar pollen does not depend on the recognition of immunoblotting patterns, but on the high values of specific IgE to both tomato fruit and Japanese cedar.

In conclusion, we identified the tomato allergens by immunoblotting using sera from patients with tomato-induced OAS. Three of these allergens increased in con-

centration with ripening in the immunoblotting experiments. However, further investigation is needed to confirm the allergenicity of these IgE-binding proteins in tomato fruits.

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