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cDNA Cloning, Expression and Characterization of an Allergenic 60s Ribosomal Protein of Almond (*Prunus dulcis)*

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ABSTRACT

Tree nuts, including almond (*prunus dulcis)* are a source of food allergens often associated with life-threatening allergic reactions in susceptible individuals. Although the proteins in almonds have been biochemically characterized, relatively little has been reported regarding the identity of the allergens involved in almond sensitivity. The present study was undertaken to identify the allergens of the almond by cDNA library approach.

cDNA library of almond seeds was constructed in Uni-Zap XR lamda vector and expressed in *E. coli* XL-1 blue. Plaques were immunoscreened with pooled sera of allergic patients. The cDNA clone reacting significantly with specific IgE antibodies was selected and subcloned and subsequently expressed in *E. coli*. The amino acids deducted from PCR product of clone showed homology to 60s acidic ribosomal protein of almond. The expressed protein was 11,450 Dalton without leader sequence. Immunoreactivity of the recombinant 60s ribosomal protein (r60sRP) was evaluated with dot blot analysis using pooled and individual sera of allergic patients.

The data showed that r60sRP and almond extract (as positive control) possess the ability to bind the IgE antibodies. The results showed that expressed protein is an almond allergen.

Whether this r60sRP represents a major allergen of almond needs to be further studied which requires a large number of sera from the almond atopic patients and also need to determine the IgE-reactive frequencies of each individual allergen.

Key words: Allergen; Almond (*prunus dulcis)*; cDNA library; 60s ribosomal protein

INTRODUCTION

Tree nuts are energy-rich foods due to their high lipid (45-70% w/w) and protein (20-25% w/w) content. The consumption of almond and walnut has been shown to benefit heart health and favorably affect cholestrol levels.[1,2] However, for people with tree nut allergy, nuts consumption can lead to allergenic reactions that can range from local itching or swelling to generalized urticaria, pharyngeal or laryngeal edema, gastrointestinal involvement, bronchospasm, or cardiovascular collapse and death.[3] Both native and recombinant nut allergens have been identified and characterized.[4] Some allergens, such as lipid transfer proteins, profilins and

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members of the Bet v 1-related family, represent minor constituents in tree nut, but are frequently cross-reactive with other food and pollen homologues. Others, such as legumins, vicilins and 2S albumins, represent major seed storage protein.[4]

Almond ranks first in per capita consumption in the USA and as allergen, it ranks third behind walnut and cashew nut[5,6]. Almonds belong to the Rosaceae family, which also includes apples, pears, peaches, prunes and raspberries. In addition to cultivated almond, *Prunus dulcis*, more than 30 wild or minor cultivated almond species are known to exist. It has been shown that the extract of almond contains approximately 380 different proteins, glycoproteins and low molecular weight compounds (http://www.allergen.org/list.html). Despite the fact that the proteins in almonds have been biochemically characterized,[7-9] relatively little has been reported regarding the identity of the allergens involved in almond sensitivity. Early reports described the purification and biochemical characterization of the major soluble seed storage protein in almond, a 14S globulin (legumin) known as almond major protein (amandin).[10] Under reducing condition, the 42-44 kDa bands showed strong reaction with human almond-allergic sera[11] and the sequencing data from two cDNA clones indicated that they are homologous to pruning 1 and 2.[12,13] Recently two major bands of 45 and 30 kDa were identified by patient IgE immunoblot and N-terminal sequencing as being homologous to a 7S conglutin-γ and a 12 kDa band corresponding to 2S albumin.[11,14,15]

Molecular characterization of the protein allergens are essential, not only for a better understanding of the pathogenesis of atopic diseases, but also for development of more efficacious and specific diagnostic and therapeutic modalities.[4,16] In this report, we employed molecular cloning and expression to identify major allergens of almond seed using sera of almond allergic patients.

PATIENTS AND METHODS

Materials

Restriction enzymes and ligase were purchased from New England Biolabs (Beverley, MA, USA). Polymerase chain reaction (PCR) was performed using the thermal cycler from Perkin Elmer Cetus. DNA amplification reagents were purchased from Bangalore Genei (Bangalore, India). GFX TM DNA and gel band purification kit for purification of PCR products were obtained from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Nitrocellulose membranes were purchased from Schleicher and Schuell (Keene, NH, USA). Peroxidase conjugated antihuman IgG and antihuman IgE antibodies were obtained from Sigma (St Louis, MO, USA).

Serum Samples

Serum samples were obtained from Dr. Suzanne Teuber (Dept. of Internal Medicine, Division of Rheumatology, Allergy and Clinical Immunology, University of California, Davis, USA) and stored at -70°C. The presence of specific IgE against almond was determined with ImmunoCAP from Pharmacia. The sera were selected based on clinical histories of almond allergen and significant ImmunoCAP results, regardless of age, race and gender. Pooled and individual sera of almond allergic patients were used in this study.

cDNA Library

Almond seeds in late maturation were chopped, frozen and ground. Total RNA was extracted in TRIzol (GIBCO BRL Life Technologies Inc., Rockville, Md., USA) and mRNA was isolated using a PolyA tract kit (Promega, Madison, Wisc., USA). The cDNA library was constructed using the Uni-ZAP XR Gigapack Cloning Kit (Stratagene Inc., Cedar Creek, Tex., USA) and cloned into the lambda Uni-ZAP XR expression vector. The library was amplified in *Escherichia coli* strain XL-1 Blue MRF' and screened with human IgE antisera.

Antibody Screening of cDNA Library and Selection of Clone

Immunoscreening of a λZAP cDNA library was performed as follow: *E. coli* XL-1 blue were infected with 10×10^6 phages containing cDNAs and plated onto NZY-agar plates. Expression of fusion protein was induced by overlapping nitrocellulose filters impregnated with 10 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and followed by incubation of the plates for 4 h at 37° C. Filters were washed first with Tris-buffer saline (TBS) (10 mM Tris, pH 8·0, 150 mM NaCl) containing 0.05% v/v Tween 20. They were then blocked with 5% skimmed milk powder in TBS at room temperature for 1 h and were incubated with rabbit or goat anti-human IgE. Filters were washed in TBS containing 0·05% (v/v)

Tween 20 (TBS-T), and reacted with HRP conjugated antihuman IgE. The immunopositive clones were picked, plaque purified and stored in SM buffer (100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 50 mM Tris-HCl, pH 7.5) supplemented with 2% chloroform at 4°C.

Sequencing the Gene

Inserts from selected phage clones were amplified with M13 forward and reverse primers by PCR. PCR cycling conditions were 95°C/3' and 28 cycles of 95°C/30 sec, 55°C/1', 72°C/2' followed by a terminal extension cycle at 72°C/10'. Both strands of the PCR products were sequenced on an ABI 3100 Genetic Analyzer (Foster City, CA., USA) using capillary electrophoresis and Version 2 Big Dye Terminatorrs as described by the manufacturer. The deduced amino acid sequence was determined using the Translate Tool software in the Expasy Molecular Biology Server (http://www.expasy.com). The deduced amino acid sequence was submitted to the database of National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) server for nucleotide and amino acid homology searches (http://www.ncbi.nlm.nih.gov/BLAST). Leader peptide prediction was performed using the SignalP V1.1 World Wide Web Prediction Server (www. cbs.dtu.dk/services/ SignalP/).

Cloning, Expression and Purification of cDNA Encoded 60s Ribosomal Protein

cDNA coding sequences were modified by the addition of a *SalI* site at the 5' end and a *HinIII* site at the 3' end by PCR using *PfuCurbo* DNA polymerase (Stratagene Inc., Cedar Creek, Tex., USA) followed by digestion with *Sal I* and *Hind III* and ligation to maltose-binding protein (MBP) fusion expression vector pMALc2 (New England BioLabs Inc., Beverly, Mass., USA), into which a thrombin cleavage site had been engineered. The cDNA insert was produced by PCR amplification of the sequence extending from the amino acid 21 following the 20 amino acid leader peptide through to the last codon prior to the stop codon using the 60s forward (5'ACGCGTCGAC GAAGATCTCAAGGACATCCTTG3') and reverse (5'CCCAAGCTTGCAACCACTCTAGTCAAAGAG3') primers. For expression, competent E. coli BL21 (DE3) cells (Novagen Inc., Madison, Wisc., USA) were transformed. Positive clones were identified by PCR screening using cDNA 60s ribosomal protein sequence-specific primers. Single colonies were grown and induced with IPTG. The cells were harvested after four hours, lysed with mild sonication and centrifuged at 10,000 g for 30 min. The lysate supernatant was passed over a 4 ml amylose affinity column and the fusion protein was eluted with 10 mM maltose. A yield of 10 mg of soluble fusion protein per liter of cultured cells was routinely recovered after column or batch purifications. Recombinant 60s ribosomal protein (162 µg) was digested with 1 U of thrombin (Sigma, St. Louis, Mo., USA) overnight at room temperature in thrombin cleavage buffer (20 mM Tris-HCl, pH 8.4, 0.15 M NaCl, 2.5 mM CaCl2) and pure protein was analysed by SDS-PAGE.

Subcloning, Expression and Purification of 60s Ribosomal Gene

The cDNA insert was amplified using the modified 60s ribosomal protein forward primer (carrying an *Sal 1* site) and 60s ribosomal protein (60sRP) reverse primer carrying an *Hind III* site. The amplified product was digested with *Sal 1* and *Hind III*, and the resulting fragment was inserted into pMALc2 expression vector, which was digested previously with the same restriction enzymes. The resulting plasmid was designated as pMAL-60sF2. *E. coli* strain BL21 was transformed with plasmid pMAL-60sF2. The transformed cells were grown in LB rich medium containing 100 mg/ml ampicillin at 37°C until the OD_{600} reached 0·5. IPTG was then added to a final concentration of 0·3 M, and the culture was grown for an additional 4 h at 37°C with shaking. Cells were harvested by centrifugation at 4000 g for 20 min and resuspended in 20 ml column buffer (20 ml of 1.0 M Tris-HCl, pH 7.4, 11.7 g NaCl, 2 ml of 0.5 M EDTA, 720 µl 2-ME, dH2O up to 1 lit). The cells were sonicated on ice using microfluidizer for 100 cycles, and centrifuged at 9000 g for 30 min at 4°C. The supernatant was incubated overnight at 4°C with amylase gel. The resin bound to protein was packed into a column and washed with five bed volumes of column buffer. Protein was eluted with column buffer containing 10 mM maltose. Fractions containing purified r60s ribosomal protein were pooled and the purity of the recombinant protein was confirmed on 10% SDS-PAGE.

Gel Electrophoresis (SDS-PAGE), Immunoblotting and Inhibition

Recombinant 60sRP and almond extract were boiled in reducing sample buffer and subjected to electrophoresis. Immunoblots were rinsed for 2 min in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.2% Tween-20 (TBS-T), blocked for 1 h at room temperature in TBS-T containing 2% bovine serum albumin (Sigma) and then incubated overnight at 4°C with diluted patient sera (1:5 in TBS-T). Membranes were then washed once for 15 min, and 3 times for 5 min in TBS-T prior to being incubated overnight at 4° C with ^{125}1-labeled anti-human IgE (Hycor Biomedical Inc., Garden Grove, Calif., USA) diluted 1:10 in a mixture of PBS, 5% nonfat dry milk and 0.05% Tween-20. Three final 10-min washes in PBS-0.05% Tween-20 were performed, and IgE almond allergen reactivity was identified after 1-week exposure at -70° C to Kodak Biomax X-ray film (Kodak, Rochester, N.Y., USA). For inhibition experiments, 10 µg of r60sRP were preincubated with human antisera (1:10 dilution) overnight at 4°C and then incubated with NC strips containing blotted almond extract overnight at 4°C. The strips were then incubated with the appropriate labeled second antibody, as described above, and exposed to X-ray film.

Immunodot-blot

The allergenicity of r60sRP was determined *in vitro* by dot-blot immunoassay using a panel of sera from eight almond sensitive patients. Briefly, different concentrations (1, 5 and 10 µg) of almond extract (positive control), r60s fusion protein and BSA (negative control) were applied onto Hybond C nitrocellulose membrane (Amersham Life sciences, UK). Dot blots were blocked with 5% non-fat milk powder in PBS-T (PBS with 0.05% Tween 20). The blots were incubated separately with 1:5 (v/v) dilution of sera of allergic patients over night at 4°C. After three washes with PBS-T, the blots were incubated with peroxidase conjugated mouse antihuman IgE for 1 h at RT and then developed with ECL Chemiluminescent detection (Amersham Biosciences).

RESULTS

Identification and Isolation of a cDNA Clone

The cDNA library was screened with rabbit anti almond extract IgG and 90 positive clones were identified. The sequencing data did not show any allergenic protein. When cDNA library was screened with a serum pool from eight atopic individuals, Two positive clones were identified (Figure 1). The pure clones were obtained by rescreening the allergen-producing phages three times and amplified with PCR (Figure 2). These clones were not detected when normal serum or rabbit anti almond extract were used.

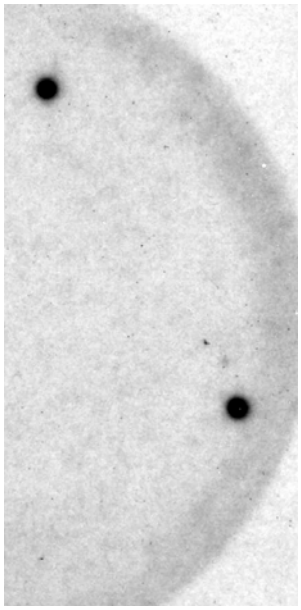

Figure 1. IgE reactivity with allergen producing phage plaques. The phage plaques were lifted onto nitrocellulose membrane and exposed to pooled human atopic sera, and then incubated with ^{125}I-labeled anti-human IgE.

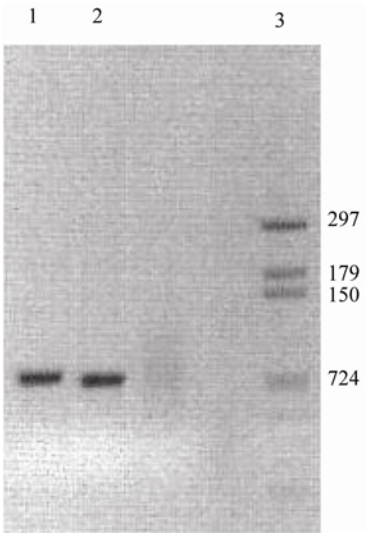

Figure 2. PCR of purified human IgE-reactive plaques. Lanes 1 and two, purified plaques and 3 markers.

Sequencing of the cDNA Clone and Its Deduced Amino Acid Sequence

The DNA sequence of positive clones were identified and both clones were the same and the nucleotide shown (Figure 3). The insert is 1184 bases in length that contains a full length coding sequence of 339 bases with one start and one stop codon. The protein encoded by the gene is 113 amino acid residues in length and its molecular mass is calculated to be 11,415 kDa. Homology analysis of the protein sequence with all known sequences in NCBI revealed that it belongs to the *prunus dulcis* 60s ribosomal acidic protein (60sRP).

Expression of the Gene Encoding Recombinant Allergen in *E. Coli*

To further study the allergenicity of the cloned gene product, the recombinant allergen was synthesized as a bacterial maltose binding (MBP-60sRP) fusion protein in *E. coli* by using the pMAL-c2 gene expression system. Forward primer was designed to eliminate first 20 amino acids as leader sequence. Figure 4A shows a Coomassie blue stained gel of the r60sRP, expressed as maltose binding protein, in different time and Figure 4B shows purified expressed recombinant protein as a single band. The predicted size of the recombinant MBP-60sRP was 53,400 kDa (11.4 kDa for the 60sRP and 42 kDa for the attached MBP). Figure 5 shows digestion of r60sRP allergen from attached MBP.

(A)

Nucleotide sequences:

ATGAAGGTTGTTGCAGCATACTTGTTGGCTGTGTTGGGGGGCAATACCACCCCTTCTGCCGAAGATCTCAAGGACATCCTTGGCTC
TGTTGGAGCTGAGACTGATGATGATAGGATTCAGCTTCTTCTTTCGGAAGTGAAGGGTAAGGACATTACAGACTTATTGCTTCTGGAA
GGGAGAAGTTGGCCTCTGTACCATCTGGTGGTGGTGCTGTTGCAGTGGCTGCACCTGGTGCTGGTGCTGGTGCCGCTGCTCCTGCTGC
AGCTGAGCCAAAGAAAGAAGAGAAGGTTGAGGAGAAAGAGGATACCGATGATGATATGGGCTTCAGTCTCTTTGACTAG

(B)

Amino acid sequences:

M K V V A A Y L L A V L G G N T T P S A E D L K D I L G S V G A E T D D D R I Q L L L S E V K G K D I T E L I A S G R E K L A S V
P S G G G A V A V A A P G A G A G A A A P A A A E P K K E E K V E E K E D T D D D M G F S L F D **Stop**

Figure 3. Coding sequences of the cDNA clone coding for *prunus dulcis* 60s ribosomal proteins and its deduced amino acid sequence. (A) Complete coding nucleotides (339 base, Genbank accession code: DQ836316) of cDNA clone of 60sRP with initiation and stop codons. (B) The deduced 113 amino acid sequences. The boxed 20 amino acid sequences represent the presumptive signal sequences.

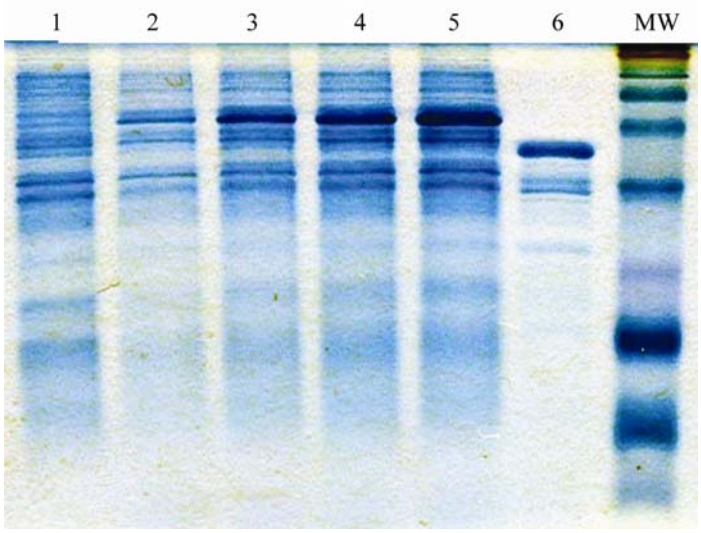

Figure 4. 10% SDS-PAGE stained with colloidal Coomassie blue showing expression of r60sRP at different times from a cDNA clone. Lane M, molecular weight marker in kDa; lane 1, uninduced *E. coli* cell lysate; lanes 2 to 5, induced *E. coli* 1, 2, 3 and 4 hours respectively. Lane 6 maltose binding protein.

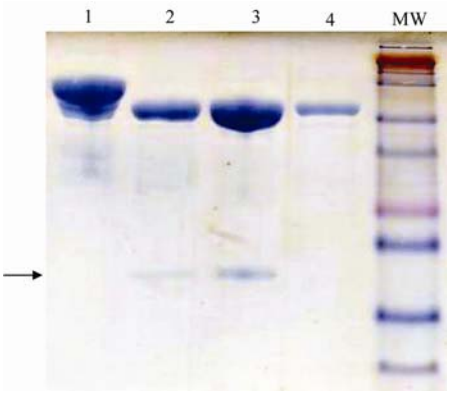

Figure 5. Digestion of purified r60sRP fusion protein with thrombin. Tris-glysin SDS-PAGE (4-20% gradiant) gel was used. Lane 1, 20 µg undigested column purified fusion protein (MBP-60sRP); lane 2 and 3 digested MBP-60sRP fusion protein (10 and 20 µg respectively) and lane 4 pure MBP. Arrow indicates purified 60sRP separated from MBP.

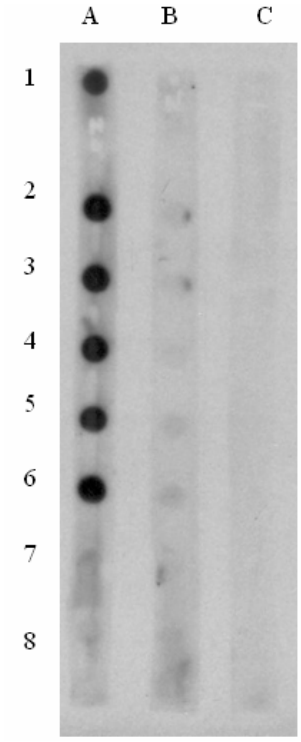

Figure 6. Allergenicity of r60sRP by dot-blot immunoassay. Dots 1, 2 and 3 represent 1, 5 and 10 µg almond extract respectively (as positive control). Dots 4, 5, and 6 represent 1, 5 and 10 µg 60s fusion protein respectively, and dots 7 and 8 represent 5 and 10 µg BSA (negative control). In lane A, pooled sera of almond allergic patients was added (1:10 dilution); in lane B normal serum was added and in lane 3 no second antibody was used.

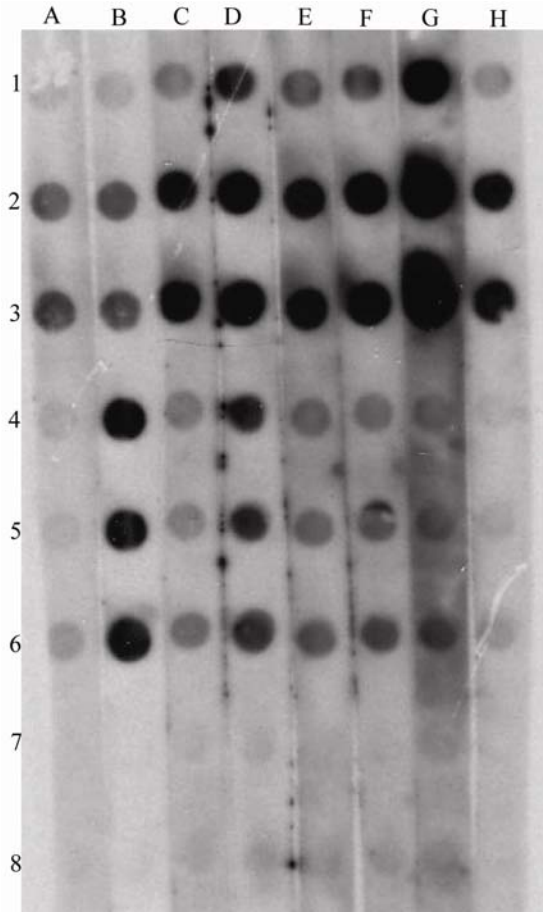

Figure 7. Allergenicity of r60sRP by dot-blot immunoassay using eight sera of individual patient. Numbers are the same as mentioned in the legend of previous figure. A to H represent sera of eight almond sensitive patients.

Immunoreactivity of r60sRP

Dot blot analysis demonstrated that the r60sRP allergen and almond extract (as positive control) possess the ability to bind the IgE antibodies, whereas BSA as a negative control had no reactivity with mixed allergic sera (Figure 6). Out of eight sera of almond allergic individuals, four sera contained IgE antibodies recognizing the allergen (Figure 7).

DISCUSSION

In this report, we described the molecular cloning and characterization of a cDNA clone coding for a protein of almond (*prunus dulcis*). Immunoscreening of

the cDNA library of almond seed, led to identification and expression of a 11.4 kDa protein with sequence homology to 60sRP. Significantly higher level of specific IgE antibody to the purified protein observed in sera of almond sensitive patients than control sera suggested that it may be a novel allergen/antigen of almond seed.

The presence of IgE antibodies to r60sRP in 50% of the almond sensitive patients indicated that it may be a major allergen of almond according to the allergen nomenclature guidelines given by the IUIS (IUIS, allergen nomenclature subcommittee, http://www.allergen.org/Editoral).

To our knowledge, this is the first report that 60sRP has been identified as an allergen. Protein allergens are a group of antigens that are capable of induction of and binding to IgE antibodies under natural conditions. It is still not clear whether any protein allergen contains unique structures which account for the allergenicity, although recent studies indicate that allergens are different from common antigens in terms of their induction of T cell subsets, cytokines, and Ab isotypes.[17-19]

In recent years, a number of plant, animal and fungal allergenic proteins have been identified by biochemical and molecular genetic means, reflecting the hope that the detailed information provided by such studies will lead to the development of hypoallergenic foods, new therapeutic and diagnostic tools and the increased understanding of the properties that render some proteins more allergenic than others.[20-23] Availability of the recombinant allergen would facilitate the analysis of its epitopes as well as the antigenicity and allergenicity of the allergen. Because the protein was synthesized in *E. coli* in which no glycosylation occurs, it can be concluded that at least some of the IgE-binding properties of the 60sRP allergen are inherent in its amino acid sequence. Dot blot analysis demonstrated that the r60sRP allergen and almond extract (as positive control) possess the ability to bind the IgE antibodies. Whether the r60sRP represents a major allergen of almond needs to be further studied, which requires a large number of sera from the almond atopic patients and also need to determine the IgE-reactive frequencies of each individual allergen.

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