

Case report

A 17-kDa allergen detected in pine nuts

Background: Few cases of allergy to pine nuts have been described. We report a case of anaphylactic reaction to pine nuts. The patient needed to be treated in the emergency room due to a systemic reaction immediately after eating pine nuts.

Methods: The patient was studied by prick tests and prick by prick tests. Specific IgE was measured by CAP and by SDS–PAGE/immunoblotting by a diffusion method.

Results: The patient showed positive prick by prick tests to pine nuts (12 mm of maximum wheal diameter). Specific IgE was positive (0.79 kU/l). The patient's serum recognized several proteins by immunoblot. However, a 17-kDa allergen band was detected with high intensity. This protein was found to be sensitive to reducing agents, losing its IgE-binding properties after reduction.

Conclusions: The patient presented an IgE-mediated reaction and detected a 17-kDa protein from pine nuts not previously described.

**J. M. García-Menaya,
M. A. Gonzalo-Garijo**

Allergy Department, Hospital Infanta Cristina, Badajoz

**I. Moneo, B. Fernández,
F. García-González, F. Moreno**

Immunology Department, Centro de Investigación
Clínica, Instituto de Salud Carlos III, Madrid, Spain

Key words: allergen; anaphylaxis; food allergy; pine nut.

Dr Jesús Miguel García-Menaya
Sección de Alergología
Hospital Infanta Cristina
Avenida de Portugal s/n
06080 Badajoz
Spain

Accepted for publication 7 October 1999

Pine nuts (piñon nuts, pignons, pignolas, pinoccios, or pine kernels) are the seeds of *Pinus pinea*, the European pine. This is a common tree in southern Europe, especially in Italy, southern France, and Spain. Nowadays, there is a renewed interest in pine nuts as a food. They are used variously in salads, cakes and other pastries, and sauces, and even as an oil substitute. The first authors to report a hypersensitivity reaction to pine nuts were Santos & Unger in 1958 (1). Thereafter, other cases have been reported (2–5). Only two authors have characterized the allergens by SDS–PAGE and immunoblotting, describing 50- and 66–68-kDa proteins as the main allergens (3, 4).

Material and methods

A 22-year-old man, without previous history of atopy or drug allergy, presented with difficulty in breathing and swallowing, profuse sweating, abdominal pain, and visual disturbances 15 min after eating several pine nuts. In the emergency room, his blood pressure was imperceptible. After treatment with subcutaneous epinephrine and intravenous corticosteroids and antihistamines, he progressively improved, and was discharged 24 h later.

Methods

Human serum. The serum of our patient was used for the *in vitro* studies. Sixteen sera from atopic patients

with unrelated clinical symptoms were used as a control.

Allergens. Pine-nut extracts of six different brands obtained from different local stores were prepared. The pine kernels were ground, and 1 g of each flour was shaken for 1 h with 9 ml of PBS (phosphate-buffered saline) at room temperature. After centrifugation for 10 min at 10 000 g, the supernatant was stored frozen at –20°C.

Skin tests. Skin prick tests were performed with common allergens (mites, pollens, molds, and epithelia) and food (egg, milk, cereals, nuts, fish, fruits, meats, mollusks, crustaceans, and vegetables) of commercial origin (ALK Abelló, Madrid, Spain). Saline was used as a negative control and 10 mg/ml histamine phosphate as a positive control. Results were read after 15 min. Reactions were considered to be positive if the largest diameter of the wheal was 3 mm over the negative control. Prick by prick tests with pine-nut extracts were performed in the patient and in 10 control subjects.

Total and specific IgE. Total serum IgE was determined by the Alastat method (DPC, Los Angeles, CA, USA), according to the manufacturer's instructions. Specific IgE against pine nuts was

determined by CAP (CAP System, Pharmacia, Uppsala, Sweden).

SDS-PAGE. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the discontinuous system of Laemmli (6) with 18% acrylamide resolving gels and 4% stacking gels. The extracts were diluted 1:1 in nonreducing sample buffer. The same dilution of the extract in sample buffer containing 5% 2-mercaptoethanol was also used. The electrophoresis was performed at 150 V, and the gel was dyed with Coomassie brilliant blue.

Specific IgE detection by immunoblotting. After SDS-PAGE, the proteins were transferred by a diffusion method as described (7). The nitrocellulose sheets were incubated overnight with 10 ml of 1/20 dilution of serum from the patient in incubation buffer. After three washes, the membranes were incubated for 3 h with a 1:1000 dilution of monoclonal anti-IgE antibody (Ingenasa, Madrid, Spain). After washing, the membranes were incubated for 1 h with a 1:2000 dilution of anti-mouse-IgG labeled with alkaline phosphatase (Bio Source International, Camarillo, CA, USA). Finally, after new washes, nitroblue tetrazolium/5-bromo-4 chloro-3 indolyl phosphate (Amresco, Solon, OH, USA) was added. For study of the control sera, a Mini-Protein II Multiscreen (Bio-Rad Laboratories) was used. The human serum (0.1 ml) was diluted 1:5 in incubation buffer and shaken for 18 h. Then, the membrane was taken out of the device, and the assay was performed as described above.

Continuous elution fractionation. Preparative SDS-PAGE of the crude extract was performed with a Mini-Prep Cell (Bio-Rad Laboratories, Hercules, CA, USA) by the method of Mehrotra et al. (8) with a 16% resolving gel and a 4% stacking gel.

Other transfer procedures. As an alternative to the transfer by diffusion, electrophoresed proteins were

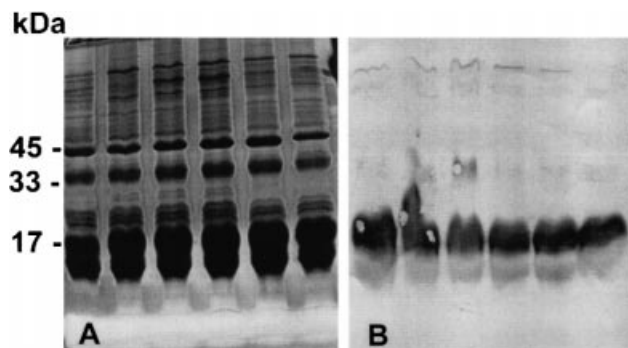


Figure 1. SDS-PAGE performed with six different brands of pine nuts (A) and immunoblotting of serum of patient (B).

transferred by the method of Towbin et al. (9) without modification. A semidry method using the transfer buffer described by Dunn (10) was also performed with a semidry transfer cell (Bio-Rad) for 1 h at 11 V and 0.3 A.

Results

Standard prick tests proved to be positive with grass pollen, but negative with all the other common allergens tested. Prick by prick tests with pine nuts were positive (wheal of 12 mm), and they were negative in controls. Total serum IgE was elevated (698 UI/ml). Specific IgE against pine nuts was weakly positive (0.79 kU/l) in the patient.

SDS-PAGE was performed with six different brands of pine nuts in order to determine whether the low CAP value could be influenced by an antigen of different origin. The electrophoresis revealed multiple proteic bands with the same molecular mass in the different extracts. The main bands were 15 and 17 kDa (Fig. 1). After transfer to a nitrocellulose membrane by the diffusion method, several bands were detected, the main one being about 17 kDa. No differences in the blot were observed among the six brands of pine nuts (Fig. 1).

The main 15- and 17-kDa proteins were reduced by 2-mercaptoethanol (Fig. 2). The main band was not observed in the blot when treating the crude extract with this agent. This fact suggested that S-S bridges are essential for IgE recognition (Fig. 2). The crude extract was fractionated by a continuous elution SDS-PAGE method, and the different fractions obtained were studied

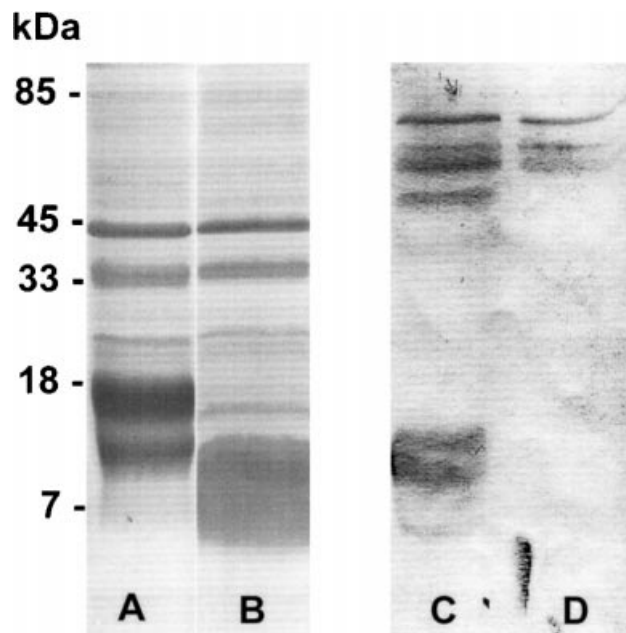


Figure 2. SDS-PAGE of one extract from pine nuts under nonreducing conditions (lane A) and after reducing (lane B). Immunoblotting with patient's serum under nonreducing conditions (lane C) and after treating sample with reducing agent (lane D).

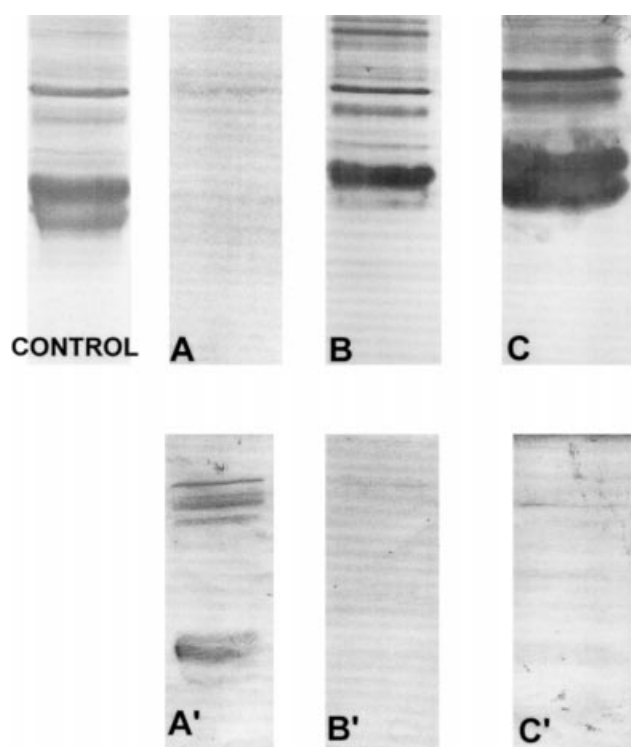


Figure 3. Immunoblotting performed after transfer by diffusion, electric, and semidry electric transfer from SDS-PAGE (lanes A', B', and C'). Gel was stained with Coomassie brilliant blue after transfer in order to determine efficiency of each procedure (lanes A, B, and C). Control: SDS-PAGE of crude extract stained after electrophoresis without any transfer procedure.

by SDS and immunoblot (data not shown). One of the fractions containing an isolated 17-kDa protein recognized by IgE was studied after reduction. This allergen was treated with 2-mercaptoethanol, and three bands of lower mass were obtained, suggesting that this allergen was composed of different chains (data not shown).

To determine why other authors have not found this strongly IgE-binding 17-kDa protein, we evaluated the efficiency of different transfer methods. Fig. 3 shows that only with our diffusion method did the 17-kDa

protein disappear from the gel. Furthermore, only after diffusion did the serum from the allergic patient detect this 17-kDa allergen. Immunoblotting after electric transfer without methanol in the transfer buffer was performed. No band was seen in the blot, although when the transfer control was carried out, the 17-kDa protein had partially disappeared from the gel (data not shown).

Discussion

Pine nuts have been known to be allergenic for years, but few studies describing the allergens are available. In our study, the most prominent proteic bands in the SDS-PAGE had masses of about 15 and 17 kDa in the six different brands of pine nuts assayed. However, previous reports described pine-nut allergens with masses of 50 and 66–68 kDa (3, 4). We could not obtain transfer of our main protein by common electric methods (Fig. 3). Therefore, the 17-kDa allergen was not seen in the immunoblot, although some bands of higher masses were observed, as previously described by De Las Marinas et al. (3) and Koepke et al. (4). Moreover, we found that our 17-kDa protein had disappeared from the gel after transfer, but this information was not recorded in the other studies. Therefore, we believe that when one works with a new allergen, the gel should always be stained.

The IgE-binding properties of this 17-kDa protein disappeared after reduction, suggesting that the responsible epitopes were conformational. This fact could explain why this protein can lose its epitopes after binding to a solid phase, thus leading to a low CAP value, as shown by us. The discrepancy observed between CAP and blotting could also have been caused by the presence of hidden epitopes made accessible to the antibodies by the denaturation induced by the SDS used in the electrophoresis. In any case, the strong IgE-binding properties of this 17-kDa protein correlated with the severe clinical symptoms of our patient.

References

1. SANTOS JM, UNGER L. Severe allergic reaction to pignolia nut. *Ann Allergy* 1958;**16**:459–461.
2. FINE AJ. Hypersensitivity reaction to pine nuts. *Ann Allergy* 1987;**59**:183–184.
3. DE LAS MARINAS D, VILA L, SANZ L. Allergy to pine nuts. *Allergy* 1998;**53**:220–222.
4. KOEPKE JW, WILLIAMS PB, OSA SR, DOLEN WK, SELNER JC. Anaphylaxis to piñon nuts. *Ann Allergy* 1990;**65**:473–476.
5. ARMENTIA A, QUINTERO A, FERNÁNDEZ-GARCÍA A, SALVADOR J, MARTÍN-SANTOS JM. Allergy to pine pollen and piñon nuts: review of three cases. *Ann Allergy* 1990;**64**:49–53.
6. LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970;**227**:680–685.
7. MONEO I, ALDAY E, SANCHEZ-AGUDO L, CURIEL G, LUCENA R, CALATRAVA JM. Skin prick tests for hypersensitivity to alpha-amylase preparations. *Occup Med* 1995;**45**:151–155.
8. MEHROTRA J, MITTAL A, DHINDSA MS, SINHA S. Fractionation of mycobacterial integral membrane proteins by continuous elution SDS-PAGE reveals the immunodominance of low molecular weight subunits for human T cells. *Clin Exp Immunol* 1997;**109**:446–450.
9. TOWBIN N, STAEGELIN T, GORDON J. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets. Procedure and applications. *Proc Natl Acad Sci U S A* 1979;**76**:4350–4354.
10. DUNN SD. Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. *Anal Biochem* 1986;**157**:144–153.