

31699

Allergy to castor bean

II. Identification of the major allergens in castor bean seeds

S. C. Thorpe, BSc, D. M. Kemeny, PhD, R. C. Panzani, MD,
B. McGurl, PhD,* and M. Lord, PhD* London and Coventry, England, and
Marseilles, France

Castor bean proteins were separated and identified by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose paper. The capacity of the castor bean proteins to bind human IgE was probed with sera from castor bean-sensitive patients and radiolabeled anti-IgE. It proved difficult to identify allergens with isoelectric focusing. However, three allergens were identified when proteins were first separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis: the 2S storage albumin, the 11S crystalloid proteins, and a third protein doublet with molecular weights of 47 and 51 kd. Specific IgE antibody to the 2S storage albumin, measured by RAST, was detected in most (96%) castor bean-sensitive patients, confirming it as the major allergen. We would like to suggest that the 2S albumin be named Ric c I, that the crystalloid proteins be named Ric c II, and that the 47/51 kd doublet be named allergen 3. (J ALLERGY CLIN IMMUNOL 1988;82:67-72.)

Castor bean (*Ricinus communis*) represents both a potent occupational sensitizer and an unusual environmental allergen, affecting residents living close to the castor oil mills.^{1,2} In recent years hygiene controls have reduced the frequency of sensitization, at least in some countries.

Attempts to identify the castor bean allergen date back to the 1940s when Spies and Coulson³ isolated a protein fraction that possessed potent skin test activity when it was tested in sensitized patients. The fraction, which they called CB 1A, was water soluble, heat stable, and of low molecular weight. More recently, Youle and Huang⁴ demonstrated that CB 1A and the 2S storage albumin had similar electrophoretic mobilities when they were run on SDS-PAGE and produced precipitating antibodies when they were injected into rabbits. Youle and Huang⁴ concluded that the CB 1A fraction isolated by Spies and Coulson³ was the 2S storage albumin.

The storage albumins are present in the storage bodies of the castor bean seeds and constitute 10% of the dry protein weight. The seeds also contain another storage protein, the crystalloid protein which is present in abundant quantities.⁵ In addition, the castor

Abbreviations used

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IEF: Isoelectric focusing
kd: Kilo daltons
PBS: Phosphate-buffered saline
pI: Isoelectric point

bean lectins, *Ricinus communis* type 1 (RCA₁) and the toxin, ricin (*R. communis* type 2), are found together in the protein bodies of the seed endosperm tissue. In this study, we separated and identified castor bean proteins by IEF and SDS-PAGE and probed their capacity to bind human IgE antibodies with the immunoblot technique. The quantity of human IgE antibody to the major allergen was then measured with RAST.

MATERIAL AND METHODS

Selection of patients

Serum samples (45) were collected from a group of Marseilles residents who had consulted their doctor complaining of symptoms of asthma and rhinitis believed to be due to sensitization to castor bean dust. All samples were scratch test positive (defined as >1 cm diameter wheal size) to a 1/100,000 dilution of castor bean extract (kind gift of Dr. Layton, Western Regional Research Laboratory, Albany, Calif.). Separated serum samples were lyophilized and stored at 4° C until the serum was reconstituted with distilled water for use. Castor bean-specific IgE antibody levels were

From the Department of Medicine, United Medical and Dental Schools, London, and *Department of Biological Sciences, University of Warwick, Coventry, West Midlands, England.

Received for publication April 24, 1987.

Accepted for publication Jan. 20, 1988.

Reprint requests: D. M. Kemeny, MD, Department of Medicine, UMDS, Guy's Hospital, St Thomas's St., London SE1 9RT, England.

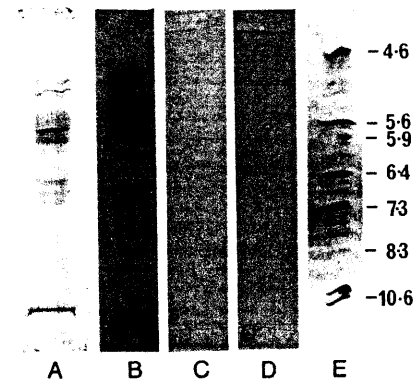


FIG. 1. A, Coomassie blue stain of castor bean proteins separated by IEF. The most heavily stained band is at the origin (*cathode*). B, An immunoblot of an IEF gel of the castor bean-positive pool demonstrating 10 radiostaining bands. C, An IEF immunoblot with a pool of sera from grass pollen-sensitive patients. D, An IEF immunoblot with IgE myeloma diluted to elicit 2500 IU/ml. E, IEF market proteins.

measured with RAST^{1,2} and 10 patients with high levels of specific IgE antibodies (RAST class 4) were pooled (castor bean-positive pool). A pool of sera from 10 grass pollen-positive patients (RAST class 4) was used as a negative control (grass pollen-positive pool).

Preparation of castor bean extract

Castor bean seeds (variety Impala) were purchased from Thompson and Morgan Ltd., Ipswich, U.K. The seeds (20 gm) were ground to a fine powder and extracted in phosphate buffer (100 ml) for 3 hours at room temperature in a fume cupboard. The extract was centrifuged in sealed containers for 15 minutes at 2000 g, the upper fatty layer was removed, and the unheated extract was aliquoted and stored at -20 °C. The unheated extract was used for SDS-PAGE and IEF protein-separation techniques. A portion of the extract was heated for 1 hour in a boiling-water bath to detoxify the castor bean toxin, ricin, and was used to coat cyanogen bromide-activated paper disks for the RAST experiments.

Purification of 2S storage albumins

Unheated castor bean extract was used for purification of the 2S storage albumins. The protein was extracted into 90% saturated ammonium sulfate solution at 4 °C overnight, and the precipitated protein was centrifuged at 2000 g for 15 minutes. The precipitate was resuspended in 0.05 mol/L of imidazole HCl buffer, pH 7.0, and run down a Sephadex G-50 superfine column (2.5/45 cm). Fractions were collected at 1-minute intervals, and their protein concentration

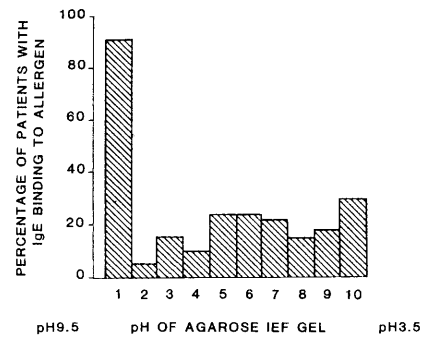


FIG. 2. The proportion of sera demonstrating binding to the allergens separated by IEF. Most (91%) demonstrated binding to allergen 1.

was determined from their absorbance at 280 nm. The second protein peak was pooled, and the protein was reprecipitated in 90% ammonium sulfate as before and rechromatographed on Sephadex G-50 to produce an extremely pure preparation of the 2S albumins.

IEF

Flat-bed IEF was carried out on a Multiphor apparatus (LKB, Ltd., Croyden, Surrey, U.K.) with agarose gels that permitted passive transfer of the separated proteins onto nitrocellulose paper. The gels were composed of 1% (wt/vol) agarose type Z (LKB, Ltd.), 2% (vol/vol) ampholine solution, pH range 3.5 to 9.5 (LKB, Ltd.), and 12% (wt/vol) sorbitol (BDH), and were cast onto gel bond (BDH) with a glass plate heated to 60 °C. Castor bean proteins, at a concentration of 20 µg total protein, were applied on filter paper wicks (LKB, Ltd.) at the cathodic end of the gel and focused for 1 hour at a power setting of 6.3 W. The gel was cooled to 10 °C. The anodic electrode solution was 0.1 mol/L of orthophosphoric acid, and the cathodic solution was 0.1 mol/L of sodium hydroxide. Gels were either fixed and protein stained in 0.5% Coomassie blue (Sigma Chemical Co., St. Louis, Mo.) in 40% methanol and 10% acetic acid solution, or pressed against dampened nitrocellulose paper (45 µm) for 5 minutes for subsequent immunoblotting. The nitrocellulose paper was then cut into 1 cm strips and incubated with PBS containing 3% bovine serum albumin and 0.5% Tween 20 for 1 hour at room temperature to block unreacted protein binding sites.

SDS-PAGE

SDS-PAGE was carried out by the method described by Laemmli⁸ with a 5% stacking gel and a 12% running gel, and the proteins were run out over 12 cm. Separated proteins were stained with Coomassie blue, as for IEF, or were electrophoretically transferred onto nitrocellulose paper with the Western blot technique.⁹ The transfer was carried out at 250 mA at 4 °C for 2 hours with the "Trans-Blot" (Bio-

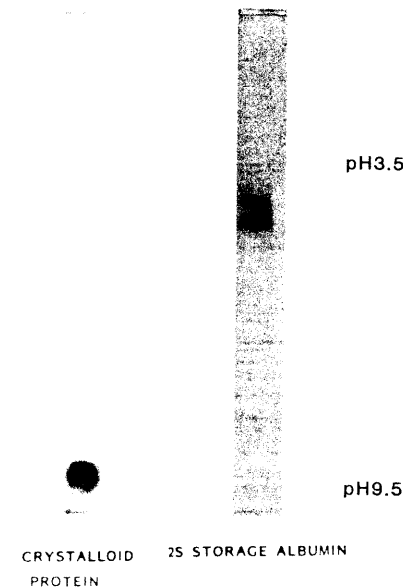


FIG. 3. Immunoblot of pure proteins after focusing for 15 minutes; 2S albumin has a very acidic pI and has migrated toward the acidic end of the gel, whereas the crystalloid proteins have failed to migrate from the origin.

Rad, Richmond, Calif.) apparatus. The nitrocellulose paper was blocked with 3% bovine serum albumin as above.

Immunoblot

Nitrocellulose blots carrying the separated castor bean proteins were incubated with 0.1 ml of castor bean-positive pool in 2 ml of PBS containing 0.5% Tween 20 and mixed overnight at room temperature. Unbound serum proteins were washed off with five changes of 3 ml of 0.9% saline containing 0.1% Tween 20 and subsequently incubated overnight at room temperature with ¹²⁵I radiolabeled goat anti-IgE (kind gift from Prof. K. Ishizaka). The specific activity of the anti-IgE was 5 µCi/µg, and 50 ng were added to 2 ml of PBS/horse serum 10%/Tween 20. Unbound ¹²⁵I-anti-IgE was washed off as before, and the paper strips were dried and incubated with X-ray film (Kodak X-Omat S, Kodak Ltd., Hemel Hempstead, Herts, U.K.) for 3 to 14 days at -70 °C. The films were then developed with standard materials and techniques.

RAST with 2S storage albumin

RAST and RAST inhibition were carried out as described previously.^{9,10} RAST inhibition was used to determine the purity of the isolated 2S storage albumins and the degree

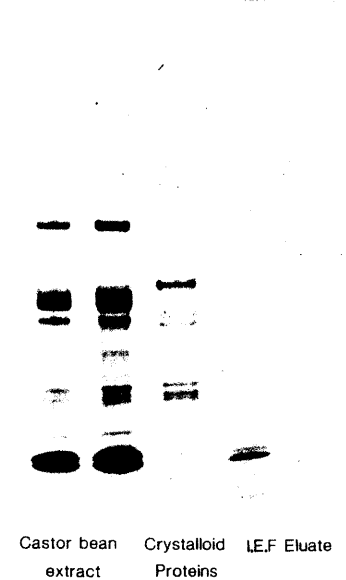


FIG. 4. SDS-PAGE of the protein material eluted from the cathodic region of the IEF gel (allergen 1) demonstrating it to comprise mainly of 2S storage albumin.

of cross-reactivity between the albumins and a crude crystalloid fraction. The castor bean-positive pool (RAST class 4) was used at a 1/10 dilution. RAST inhibition was performed with disks coated with 1.0, 0.1, and 0.01 mg/ml of 2S albumin against a range of dilutions of inhibitor (100 to 0.1 µg/ml). The grass pollen-positive pool was used as a negative control. At a concentration of 0.1 mg/ml of 2S (10 ml extract/1 gm Whatman 541 [Whatman Ltd., Maidstone, England] paper disks), there was a thousandfold less inhibition with the crude crystalloid fraction compared with the 2S albumin. Serum samples from castor bean-sensitive patients were assayed for specific IgE antibodies to 2S storage albumins by RAST at a 1 in 10 dilution in horse serum. Results were expressed in arbitrary units per milliliter by reference to an "in-house" castor bean standard curve and Phadebas reference reagents. A positive result was defined by a class 1 or greater Phadebas RAST score (Pharmacia Ltd., Milton Keynes, U.K.).

RESULTS IEF

When castor bean extracts were separated on agarose IEF gels, a complex pattern of protein staining bands was observed across the pH range, 3.5 to 9.5 (Fig. 1, A). Most noticeable was a darkly staining

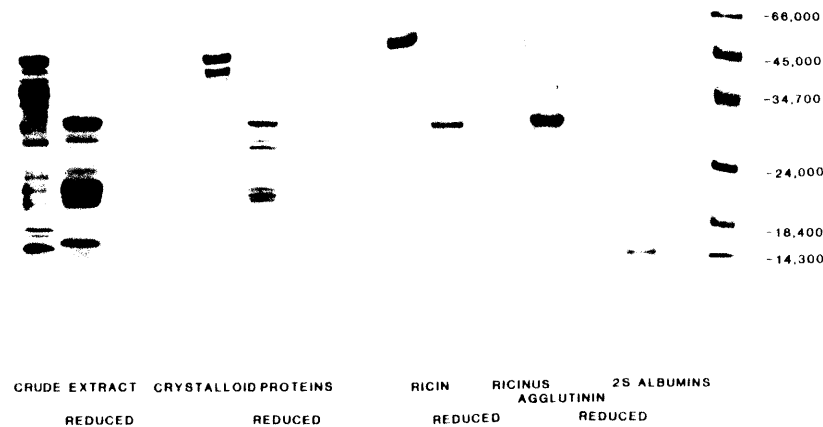


FIG. 5. SDS-PAGE of crude castor bean extract and purified proteins. Crystalloid protein has a molecular weight of 50 kd unreduced, and 30 and 20 kd, reduced.

band at the cathodic origin and a heterogeneous group of proteins between pH 4.0 and 6.0. When the separated components were probed with sera from castor bean-sensitive patients, a total of 10 protein bands demonstrated binding of specific IgE (Fig. 1, B) and, accordingly, were numbered 1 to 10 from the cathodic end. Individual sera from 45 patients were tested in the immunoblot system, and 91% demonstrated binding to allergen 1, whereas <30% demonstrated binding to any of the other protein bands (Fig. 2). Serum from a pool of grass pollen-sensitive patients (Fig. 1, C), whose total IgE levels were comparable with the castor bean-allergic patients, and IgE (Fig. 1, D) myeloma serum did not bind to any of the protein bands.

In an attempt to identify allergen 1, a time-course experiment was performed. Crystalloid and 2S storage albumin were focused at time intervals between 5 and 75 minutes and then blotted onto nitrocellulose paper. Crystalloid protein appeared to move very slowly from the cathodic end of the gel, whereas 2S storage albumin migrated rapidly across the gel and had disappeared off the acidic end within 15 minutes (Fig. 3). The pI of crystalloid protein has been determined by flat-bed polyacrylamide IEF elsewhere¹¹ and is reported to lie between pH 6 and pH 8 in contrast to our observations. There may be precipitation of the crystalloid protein or it may interact with the agarose gel, thus maintaining its position at the cathodic end of the gel.

To test this, whole castor bean extract was focused for 1 hour, the region around the cathodic origin was carefully removed, and the proteins were eluted in distilled water. The eluate was concentrated by freeze-drying and was reduced and separated by SDS-PAGE. The main protein found was the 2S storage albumin (Fig. 4). It therefore appears likely that the crystalloid proteins had interacted with both the agarose, since they could not be eluted from the first dimension gel, and the 2S albumin, or alternatively, the crystalloid proteins, could either precipitate in the IEF gels or be denatured by the pH or ampholyte and remain insoluble.

SDS-PAGE

The castor bean extract was separated by SDS-PAGE. Comparison of the relative mobilities of the protein staining bands in the crude extract with those of purified proteins made it possible to identify the 2S albumins and crystalloid proteins (Fig. 5). The unreduced crystalloid proteins appear as a group of at least six bands in the 50 kd region but, under reducing conditions, two heterogeneous subgroups are observed with molecular weights of approximately 30 and 20 kd. The 2S storage albumin has a low molecular weight of 11 kd unreduced, and 6 and 8 kd under reducing conditions.

Immunoblots of the crude castor bean extract with the castor bean-positive pool demonstrated strong radiostaining to three major bands. The 2S albumin

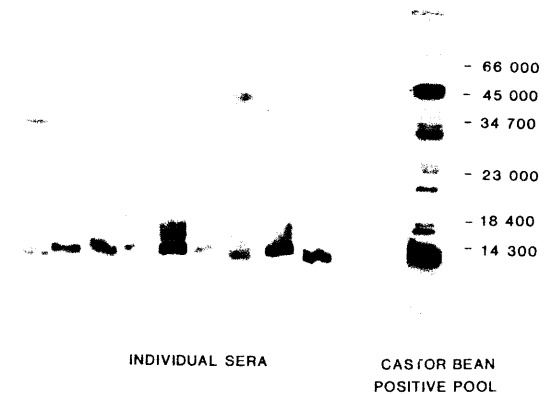


FIG. 6. Immunoblot of castor bean proteins separated by SDS-PAGE. IgE binding to three bands were observed: 2S albumins, crystalloid proteins, and a doublet of 47 and 51 kd. IgE binding of individual sera (45 were tested and 10 are presented here) demonstrates the most binding to the 2S albumin. There was no binding of IgE in the grass-positive pool or IgE myeloma.

bound IgE in both reduced and unreduced forms. Similarly, the reduced crystalloid protein bound IgE but not the unreduced protein. Protein staining of the acrylamide gel after electrophoretic transfer demonstrated that the unreduced crystalloid protein was unable to transfer to the nitrocellulose paper in sufficient quantities to bind human IgE antibodies (results not presented). A third protein doublet with a molecular weight of 47 and 51 kd also demonstrated binding of human IgE. Individual sera from castor bean-sensitive patients demonstrated radiostaining to these different protein bands to varying degrees. Grass pollen-sensitive patient serum and IgE myeloma serum demonstrated no binding to the protein bands (Fig. 6).

RAST with 2S albumin

Most (96%) castor bean-sensitive patients tested had specific IgE antibody to the pure 2S storage albumin, confirming it as the major allergen. Levels of IgE antibody to whole castor bean extract and 2S storage albumin correlated closely in individual sera (Fig. 7) ($r = 0.63$) and suggest that most of the IgE antibody is directed against the 2S storage albumin.

DISCUSSION

The potent sensitizing nature of castor bean dust has been recognized for many years,¹ but the precise identity of the allergens remains obscure. With an aqueous extract and purified proteins from castor beans, we were able to separate the proteins electro-

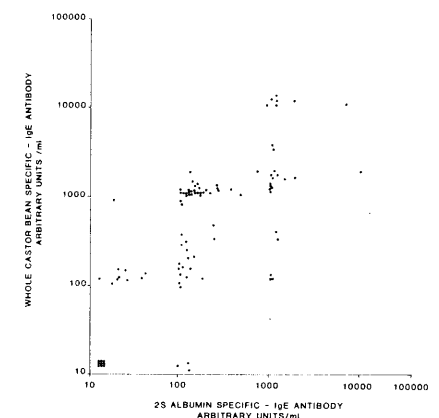


FIG. 7. The quantity of specific IgE to whole crude extract and 2S albumin correlate well in individual sera; $r = 0.63$.

phoretically and probe for their ability to bind IgE antibodies in serum from castor bean-sensitive patients. Agarose IEF immunoblots identified 10 protein bands in the whole castor bean extract that were able to bind specific IgE antibodies. The major radiostaining band appeared close to the origin at the basic end of the gel. Most patients possessed IgE to this protein. This band was removed from the gel, and the pro-

tein was extracted, concentrated, and separated on SDS-PAGE. From its position on the gel, it was identified as the 2S storage albumin, which disagrees with time-course experiments with purified 2S albumins, which demonstrated they have a very acidic pI. A possible explanation is that there is some sort of interaction between the 2S albumin and other components in the crude extract that prevents them from moving away from the origin. The very poor solubility of the crystalloid proteins in water probably explains their absence from the eluate of the radiostaining band on the IEF gel. Thus, the agarose IEF immunoblots provided confusing results and did not appear to be suitable for identifying castor bean allergens.

A clearer picture was obtained by probing blots of proteins separated by SDS-PAGE with individual sera from castor bean-sensitive patients. Three important allergens were identified: the 2S storage albumins, the 11S crystalloid proteins, and a protein doublet with a molecular weight of 47 and 51 kd. It is worth noting that identification of allergens by the SDS-PAGE immunoblot technique is also subject to error, since some allergens, such as unreduced crystalloid proteins, may not transfer properly. With RAST, 2S albumin-specific IgE antibody was detected in most (96%) individual sera tested, and the levels of specific IgE antibody to 2S albumin and the whole crude extract were found to correlate in individual sera, indicating that the 2S albumin is the major castor bean allergen. According to the recently agreed World Health Organization/International Union of Immunological Societies nomenclature,¹² we would like to propose that these allergens be called Ric c I (2S albumin), Ric c II (crystalloid proteins), and allergen 3 (47/51 kd doublet).

Biochemical evaluation of purified major allergens in other inhalant allergies (grass and ragweed pollen and dust mite allergy) have revealed no distinctive amino acid sequence.¹³ They do, however, share similar physical properties. In most cases, major allergens are freely soluble in water, of similar size (10 to 40 kd), and are neither toxic nor irritant to nonallergic people. The 2S albumins from castor bean share similar properties with Amb a I from ragweed pollen, Lol p I from grass pollen, and Der p I from *Dermatophagoides pteronyssinus* in having a low molecular weight (11 kd), high aqueous solubility, and no toxicity. Although the 2S albumins represent only a minor portion of the total dry weight of protein in castor bean seeds, a high proportion of the material is not soluble in water, and they are more abundant in

aqueous extracts. This is also true of Der p I from *D. pteronyssinus*, which constitutes <1% of dry weight of crude culture but represents 15% of total protein in aqueous extracts.¹⁴ Crystalloid proteins have unusual physical characteristics for allergens. They have a larger molecular weight in unreduced form, and a very poor solubility in water. The third protein doublet is resistant to reducing agents, and attempts to purify it are presently being carried out.

We would like to thank Mrs. S. Lockwood for typing this manuscript.

REFERENCES

1. Ordman D. An outbreak of bronchial asthma in South Africa, affecting more than 200 persons, caused by castor bean dust from an oil processing factory. *Int Arch Allergy Appl Immunol* 1955;7:10.
2. Panzani RC. Respiratory castor bean dust allergy in the south of France with special reference to Marseilles. *Int Arch Allergy Appl Immunol* 1957;11:224.
3. Spies JR, Coulson EJ. The chemistry of allergens: isolation and purification of active protein-polysaccharide fraction CB-1A from castor beans. *J Am Chem Soc* 1943;65:1720.
4. Youle RJ, Huang AHC. Evidence that the castor bean allergens are the storage proteins in the protein bodies of castor bean. *Plant Physiol* 1978;61:1040.
5. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350.
6. Ceska M, Lundkvist U. A new and simple radioimmunoassay method for the determination of IgE. *Immunochimistry* 1972;9:1021.
7. Kemeny DM, Lessof MH, Trull AK. IgE and IgG antibodies to bee venom measured by a modification of the RAST. *Clin Allergy* 1980;10:413.
8. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680.
9. Thorpe SC, Kemeny DM, Panzani R, Lessof MH. The relationship between total serum IgE and castor bean-specific IgE antibodies in castor bean-sensitive patients from Marseille. *Int Arch Allergy Appl Immunol* 1987;82:3-4.
10. Kemeny DM, Harries MG, Youlten LJF, Mackenzie-Mills M, Lessof MH. Antibodies to purified bee venom proteins and peptides. I. Development of a highly specific RAST for bee venom antigens and its application to bee sting allergy. *J ALLERGY CLIN IMMUNOL* 1983;71:505.
11. Gifford DJ, Bewley JD. An analysis of the subunit structure of the crystalloid complex from castor bean endosperm. *Plant Physiol* 1983;72:376.
12. Marsh DG, Goodfriend L, King TP, Löwenstein H, Platts-Mills TAE. Allergen nomenclature. *J ALLERGY CLIN IMMUNOL* 1987;80:639.
13. King TP. Chemical and biological properties of some atopic allergens. *Adv Immunol* 1976;23:77.
14. Chapman MD, Platts-Mills TAE. Purification and characterization of the major allergen in *Dermatophagoides pteronyssinus* antigen P₁. *J Immunol* 1980;125:587.