

Characterization and partial purification of the Croatian national standard *Dermatophagoides pteronyssinus* allergen extract

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Lyophilized *Dermatophagoides pteronyssinus* (*Der p*) allergen extract (AE) and partially purified *Der p* extract (PAE) were prepared and characterized. Partial purification of AE was performed by gel filtration on Sephadex G-100 and Sephacryl S-300. Crossed immunoelectrophoresis (CIE) disclosed the same precipitating lines in AE and PAE preparations. The relative potencies of AE and PAE were determined and compared with the WHO International Standard for *Der p* by the RAST inhibition method. The potencies were 6.5×10^5 IU and 1.5×10^6 IU, respectively. Biologic standardization by quantitative skin testing was performed with AE (20 selected patients) and PAE (12 patients). Median C_h was calculated by linear regression analysis (log-log model). One ampoule of AE contained 65 300 BU and 1 ml (vial) of PAE contained 166 000 BU. *Der p* AE could serve as a Croatian national standard for further production of *Der p* allergenic extracts.

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Diagnosis and immunotherapy of atopic diseases depend on standardized allergen preparations. The International Union of Immunologic Societies has established international standard (IS) preparations for various aeroallergens. These IS preparations are used as reference preparations for establishment of national and in-house standards. It is recommended that every country prepare for its own population national standards of typical local allergens (11).

Allergens derived from house-dust mites are a frequent cause of respiratory atopic diseases (23, 27). Allergens from the genus *Dermatophagoides* have been identified and purified (15, 16, 24). *D. pteronyssinus* (*Der p*) is considered to contain a major allergen of house dust (3, 5, 17, 18). It was one of the first three candidates for IS preparation (20). The first IS for *Der p* was prepared in 1985 (13, 14). Potency was declared in international units (IU), and one ampoule contained 100 000 IU (26).

In the present work our aim was to prepare a national standard for *Der p*. To achieve this, we prepared two samples; one of them was freeze-dried allergenic extract of *Der p* and the other, partially

purified *Der p*. Freeze-dried allergenic extract could serve as the national standard for preparations of *Der p*; partially purified preparation is more appropriate for immunotherapy of allergic diseases. Allergen potencies against the WHO IS preparation were determined by RAST inhibition. Biologic activity was assayed by skin prick test.

Material and methods

International standard

The WHO IS for house-dust mite (*D. pteronyssinus*) extract (1st IS, established 1984, 82/518, 100 000 IU/ampoule) was obtained by courtesy of WHO.

Allergen extract (AE)

The source material was commercially available pure culture of whole mite bodies (CSL-Commonwealth serum laboratories, Melbourne, Australia), supplied as dried house-dust mite. This dried house-dust mite *Der p* was extracted in the ratio 1:100 w/v in phosphate-buffered saline (PBS). The extraction was

performed for 24 h at 4°C with constant mixing. After centrifugation at 35 000 *g* for 30 min, the supernatant was twice dialyzed for 24 h against 0.005 mol/l ammonium bicarbonate and filtered (Millipore 0.22 µm) in sterile conditions. One-milliliter portions of the filtrate were freeze-dried in ampoules. In freeze-dried material (AE) the moisture, dry residue, and protein content were determined. Ampoules were stored at -20°C.

Purification of allergen extract (PAE)

Allergen extract (60 ml) before freeze drying was concentrated to 10 ml by ultrafiltration on Amicon Diaflo UM-2 ultrafilter. After two dialyses for 24 h against 2 l of borate-buffered saline (BBS), pH 8, the concentrated extract was applied to a column of Sephadex G-100, 1.5 × 90 cm (Pharmacia, Uppsala, Sweden), equilibrated, and eluted with BBS. The optical density at 280 nm was measured in each fraction; in several fractions, allergenic potency was tested by the RAST inhibition (see Figs. 1 and 2, respectively). The fractions with high allergenic activity were pooled, concentrated by ultrafiltration, and applied to a column packed with Sephacryl S-300, 1.5 × 90 cm (Pharmacia), equilibrated, and eluted with BBS. Optical density at 280 nm and allergenic activity of the eluted fractions were measured again, as specified earlier. The fractions with allergenic activity were pooled and concentrated by ultrafiltration. After dialysis for 48 h (three times against 2 l of PBS), the pooled and concentrated material was filtered through Millipore filters (0.22 µm) and dispensed into sterile vials (1 ml per vial). Protein content before and after each step was determined by two different methods, these of Lowry et al. (21) and Kjeldahl, respectively.

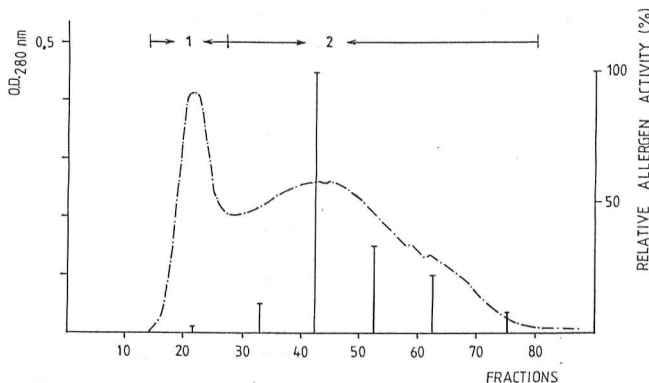


Fig. 1. Elution diagram from Sephadex G-100 column chromatography of *Der p* allergen extract (AE). Fractions were pooled as denoted by solid bars. Relative allergen activity was determined by RAST inhibition.

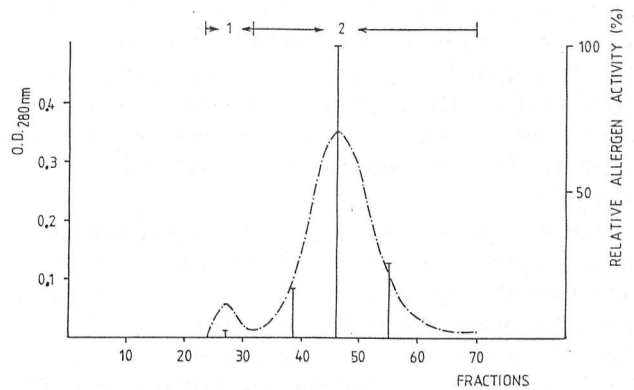


Fig. 2. Fractionation of partially purified *Der p* AE (Fig. 1, peak 2) on Sephacryl S-300. Obtained fractions were pooled as indicated by vertical bars.

Rabbit antisera

Antiserum to *Der p* extract was prepared by immunizing rabbits with the mixture of *Der p* extract and complete Freund's adjuvant (1:1) for the first injection. The dose of 2 ml was divided into two i.m. 0.9-ml injections and two 0.1-ml s.c. injections into the front foot pads. Then booster injections containing *Der p* extract and incomplete Freund's adjuvant (1:1) were given in analogous manner every 3 weeks for 3 months. The animals were bled 8 days after the last injection.

Crossed immunoelectrophoresis (CIE)

CIE was performed in 1% agarose (Pharmacia) in 0.05 M diethylbarbitur-sodium buffer (Merck, Darmstadt, Germany), pH 8, as described by Løwenstein (19).

Patients

Twenty patients (15 men, 5 women) ranging in age from 18 to 65 years (mean 32) were recruited after giving informed consent. They fulfilled the following criteria (10): 1) typical symptoms of rhinitis or bronchial asthma caused by *Der p*; 2) no hyposensitization treatment; 3) no antiallergic drugs at least 2 days before testing; and 4) RAST (Institute of Immunology, Zagreb, Croatia) of classes 3 to 4 (identical to the Pharmacia Phadebas scoring system), positive with *Der p*; negative with other allergens.

Biologic standardization

Biologic standardization of *Der p* (AE and PAE) was performed by the modified procedure of Dreborg & Grimmer (10). The skin prick test was performed with six fivefold dilutions of allergen extract parallel with negative control, histamine HCl, 1 mg/ml (H1)

and histamine HCl, 10 mg/ml (H10). Dilutions of *Der p* (AE and PAE) were prepared immediately before testing in 50% glycerol, which served as a negative control. The highest concentration of allergen contained 0.12 mg of protein per ml (*Der p* 6), and then five fivefold dilutions were made (*Der p* 5 to *Der p* 1).

The skin prick test was performed with the lancet point length of 1 mm in quadruplicate on two radial and two ulnar test sides of both arms. The wheal diameters were recorded 15 min after the test (7, 10). The wheal size for each concentration was calculated as a mean value for the four tested places. The wheal sizes less than 2 mm were not taken into account. For each allergen concentration, the relative diameter (percentage of patient's own histamine 10 wheal diameter) was calculated. The concentration of allergen inducing a wheal response of the same size as histamine 10 (C_h) was calculated by regression line analyses using the log-log model. The median C_h obtained in all tested patients was defined as having biologic activity of 10000 biologic units per ml (BU/ml) (7, 10).

Human serum

A reference standard serum pool was made by equal volumes of sera from 20 patients selectively sensitized to *Der p*. The level of specific IgE was determined in all patients' sera by RAST (specific IgE RIA, Institute of Immunology, Zagreb, Croatia).

Reference disks

The reference disks were prepared by coupling extracts of *Der p* to CNBr-activated paper disks, following the method of Ceska et al. (4).

RAST inhibition test procedure

The test procedure was carried out as previously described (28) with some modifications. Briefly, 100 μ l of serially diluted WHO IS of *Der p*, our freeze-dried *Der p* extract AE, and our partially purified *Der p* extract PAE were preincubated for 3 h at room temperature with 100 μ l of a twofold diluted sera pool. PBS (0.1 M, pH 7.5, with 0.01% $\text{Na}_2\text{S}_2\text{O}_3$, 0.1% BSA, and 1% Tween 20) was used as a diluent. Control was twofold diluted sera pool further diluted with the same volume of PBS. Then the incubation mixtures were used for RAST on reference disks. All assays were conducted in duplicate. RAST inhibition data were analyzed according to the parallel line bioassay designed to calculate relative allergenic activity (12) and expressed in international units (IU/ml; IU/mg of protein). Only measurements having correlation coefficients higher than 0.9 and

allergen dilutions having the percentage of inhibition between 10 and 90 were taken into account.

Results

In vitro characterization of *Der p* allergen extract (AE)

One ampoule of our freeze-dried *Der p* AE contained 2.7 mg dry residue, 0.8% moisture, and 0.3 mg proteins. The relative potency of *Der p* AE versus WHO IS was determined by RAST inhibition assay. Calculated by the parallel line bioassay, the relative potency of our *Der p* AE was 6.5×10^5 IU/ampoule or 2.16×10^6 IU/mg protein (Table 1). Plotted on a semilog scale, the inhibition curves were linear for all tested *Der p* AE dilutions ($r = 0.97$). The precision of the RAST inhibition assays was evaluated in three separate experiments. The coefficient of variation was found to be 2.1–15.8% for interassay and 5.4–15.9% for intra-assay variations.

Partially purified *Der p* allergen extract (PAE)

The first step in *Der p* AE purification was gel filtration on Sephadex G-100. The elution pattern based on optical density at 280 nm, exhibiting two peaks, is presented in Fig. 1. An assay of allergenic activity by RAST inhibition showed that allergenic activity was associated with the material eluted in peak 2. Fractions containing allergenic material were pooled, concentrated, and further purified on Sephacryl S-100 (Fig. 2). By RAST inhibition, the major part of allergenic material was found in peak 2. This material was prepared in sterile conditions and used for further *in vivo* testing. The data on partial purification of *Der p* AE are presented in Table 2. As can be seen, parallel to the purification steps the total protein content of purified material decreased approximately fivefold and relative allergenic activity increased twofold. Combined materials from peak 1 recovered from Sephadex G-100 and Sephacryl S-300 were concentrated and tested in the same manner as the allergenic material from peak 2. The

Table 1. Comparison of properties of *Der p* allergen extract (AE) and partially purified *Der p* extract (PAE) with WHO IS of *Der p* allergen extract

	Volume (ml)	Protein content (mg)	Potency	
			RAST inhibition (IU)	Skin prick test (BU)
<i>Der p</i> allergen extract WHO standard*	1	0.31	100000	13000
<i>Der p</i> allergen extract (AE)	1	0.3	650000	65300
Partially purified <i>Der p</i> allergen extract (PAE)	1	0.3	1500000	166000

* Data referring to WHO standard are cited from the literature (7, 26).

Table 2. Partial purification of *Der p* allergen extract (AE)

	Volume (ml)	Protein content (mg)	Relative allergen potency* (IU/mg)	Total allergen potency (IU)	Recovery of total allergen potency (%)
<i>Der p</i> allergen extract (AE)	60	18	2.16×10^6	38.8×10^6	100
<i>Der p</i> allergen extract (AE) concentrated	10	17.5	2.06×10^6	36×10^6	92.9
Sephadex G-100 column peak 2, concentrated	5.4	7.3	4.38×10^6	32×10^6	82.4
Sephacryl S-300 column peak 2, concentrated	6.4	5.44	4.74×10^6	25.8×10^6	66.5
Partially purified <i>Der p</i> allergen extract (PAE)	12.0	3.6	4.50×10^6	16.2×10^6	41.7

* Relative potency was determined by RAST inhibition using *Der p* allergen extract (AE) as reference standard.

total allergenic activity of this material represented only approximately 1% of original *Der p* AE activity.

CIE analysis of AE and PAE of *Der p* is presented in Fig. 3. It was shown that both analyzed samples contain major allergenic components. A number of precipitating lines were the same, but some lines in the partially purified allergen extract were of higher intensity.

Biologic standardization

The results of analyses of the skin prick test in 20 patients tested with dilutions of freeze-dried AE are shown in Table 3. The mean wheal diameter decreases with the dilution of AE. The lowest two AE concentrations are not presented because the wheal diameter was smaller than 2 mm. Such a reaction was considered to be nonspecific (2, 7, 22).

The concentration of *Der p* AE provoking a wheal of the same size as that of histamine 10 mg/ml was estimated by regression line analysis using a log/log model (Fig. 4). The median C_{h1} for freeze-dried AE is 0.049 mg protein per ml, which represents the potency of the tested allergen. That means that AE in the sample with a concentration of 0.049 mg protein per ml contains 10 000 BU/ml, or that one ampoule of the tested extract contains 65 300 BU.

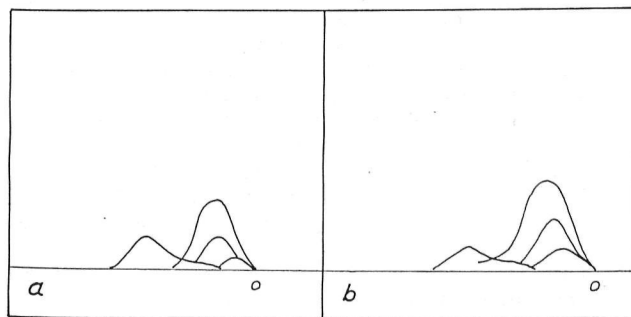


Fig. 3. CIE. Drawings illustrate individually detected arcs from: a) *Der p* allergen extract (AE), and b) partially purified *Der p* allergen extract (PAE). The same rabbit antisera and the same volume of allergens (15 μ l, 45 μ g of proteins) were used for both CIE analyses.

Table 3. Results of statistical evaluation of skin prick testing with histamine 1 (H1), histamine 10 (H10), and fivefold dilutions of *Der p* allergen extract (AE). The lowest two concentrations (*Der p* 1 and *Der p* 2) are not presented because the wheal was smaller than 2 mm

	H1	H10	<i>Der p</i> 3	<i>Der p</i> 4	<i>Der p</i> 5	<i>Der p</i> 6
No. patients	20	20	20	20	20	20
Mean wheal (mm)	6.0	8.4	3.5	5.5	7.2	8.8
SD	0.7	0.8	0.8	0.6	0.5	0.6
SEM	0.14	0.17	0.17	0.14	0.12	0.13
Min. wheal (mm)	5.0	7.0	2.0	4.25	6.0	8.0
Max. wheal (mm)	6.8	9.5	4.6	6.7	7.7	10.5

A stock of freeze-dried ampoules containing *Der p* AE characterized by skin test and RAST inhibition should serve as the national standard for calibration of *Der p* allergen extracts in Croatia.

Analysis of the skin prick test in 12 patients with *Der p* PAE is shown in Table 4. The combined material derived from peak 1 (Sephadex G-100 and Sephacryl S-300 columns) provoked the response comparable to negative control (Table 4, column B). The skin prick test with control solution (50% glycerol) was negative in all patients. The median C_{h2} was 0.018 mg protein per ml for PAE (Fig. 4). One vial of this allergen contains 166 000 BU.

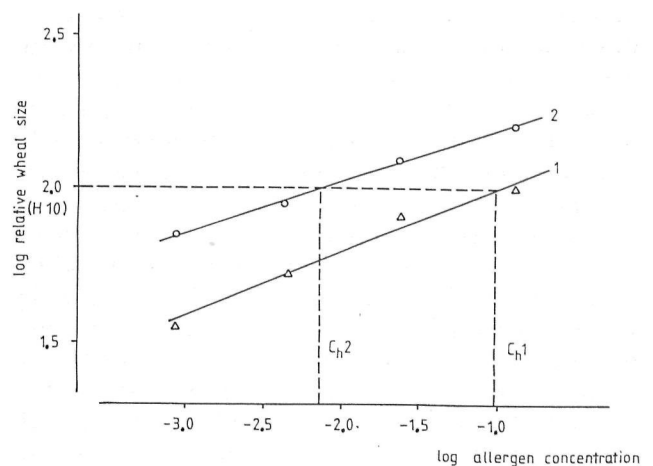


Fig. 4. Estimation of median C_h in 1) 20 patients with AE (C_{h1}); 2) 12 patients with PAE (C_{h2}).

Table 4. Results of statistical evaluation of skin prick testing with histamine 1 (H1), histamine 10 (H10), fivefold dilutions of partially purified *Der p* allergen extract (PAE), and combined materials from peak 1 (B). The lowest concentration (*Der p* 1) is not presented because the wheal was smaller than 2 mm

	H1	H10	<i>Der p</i> 2	<i>Der p</i> 3	<i>Der p</i> 4	<i>Der p</i> 5	<i>Der p</i> 6	B
No. patients	12	12	12	12	12	12	12	12
Mean wheal (mm)	6.9	9.3	3.2	4.9	7.6	10.4	12.3	1.9
SD	0.4	0.4	0.4	0.4	0.4	0.3	0.4	1.3
SEM	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3
Min. wheal (mm)	6.2	8.5	2.1	4.0	6.5	9.8	11.6	0
Max. wheal (mm)	7.5	9.8	3.7	6.1	8.5	11	12.8	3

Discussion

In Croatia, allergen extracts of unknown potency are still used. This fact influences the sensitivity, specificity, and comparability of the diagnostic and therapeutic procedures. We endeavored to establish a national standard for *Der p* allergens. Such a standard, based on WHO IS for *Der p*, should provide a reference preparation for calibration of *Der p* allergens produced in Croatia.

To standardize an allergen, one must select appropriate raw antigenic material, characterize it biochemically (CIE method), and standardize allergenic extracts by biologic standardization and RAST inhibition. RAST inhibition is recommended as an appropriate *in vitro* method to determine total allergen extract activity (25). The criteria for the selection of donors of specific sera were in accordance with the criteria of Dreborg et al. (9, 10). Generally, good correlation exists between the RAST inhibition method and biologic standardization (1). *Der p* IS having 100 000 IU/ampoule exhibits a biologic potency of 13 000 BU/ampoule (7, 26).

Biologic standardization of AE and PAE was performed according to the modified procedure of Dreborg & Grimmer (10). Since, in our hands, no difference in wheal sizes could be observed when testing was performed on the back or arms, the testing was performed on two ulnar and two radial sides of both arms. According to the Nordic Council on Medicines (25), three allergen concentrations (the lowest eliciting the same wheal size as H1) were used to define the biologic activity of tested allergen. Since we did not perform a pretest, we took into consideration concentrations of allergens eliciting wheal sizes both smaller and bigger than those caused by H1. As previously reported (6, 8), we also observed high variability of skin reactions to small concentrations of allergen. In our study, the increasing wheal diameter factor was 1.6 for third and fourth AE concentrations and 1.9 for the fifth and sixth concentration with fivefold dilutions. Some authors observed the increasing factor of 1.5 with 10-fold di-

lutions (6). In preliminary testing we had unsatisfactory results with 10-fold dilutions. Subsequently, we finally adopted fivefold antigen dilutions. The correlation coefficient between the allergen concentration and wheal size was greater than 0.85 in all patients. That is concordant with already published data (1). By regression analysis for each patient's data, the C_h was determined, and the range varied by a coefficient of 6, less than the data from the literature (7). As *Der p* IS is not for human use, we were unable to perform parallel skin prick tests with IS, AE, and PAE.

The purpose of partial purification of AE was to obtain a purified preparation (PAE) containing most of the allergenic active material without inactive ballast material. In this paper we have presented a new combination of purification methods which afforded material of satisfactory quality. As the CIE of the AE and PAE revealed the same number of precipitating lines, all relevant allergenic components were retained in the PAE. Purified preparations are more appropriate for immunotherapy of allergic diseases.

In conclusion, we prepared ampoules of freeze-dried AE standardized biologically and by RAST inhibition. Each ampoule contained 650 000 IU or 65 300 BU of activity. The IU of AE was determined in relation to the WHO international standard for *Der p*. AE could serve as the Croatian national standard for preparation of *Der p* allergenic extracts.

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