

Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen

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A purified allergen, antigen SBP, was obtained from the pollen of the Japanese cedar (Cryptomeria japonica, Sugi in Japanese) by ammonium sulfate precipitation, ion-exchange chromatography on diethylaminoethyl and carboxymethyl cellulose, and gel chromatography on Sephadex G-150. Antigen SBP was a heat-sensitive basic glycoprotein of approximately 40,000 molecular weight. By preparative isoelectric focusing and gel chromatography on Sephadex G-100, antigen SBP could be further separated into four subfractions, differing in both isoelectric point and molecular weight. By immunodiffusion analysis, direct skin testing, and radioallergosorbent test inhibition, it was shown that antigen SBP was the major allergen of Japanese cedar pollen, and the four subfractions were seen to be antigenically and allergenically identical. (J ALLERGY CLIN IMMUNOL 71:77, 1983.)

The allergen extracts used for clinical diagnosis contain nonallergenic materials, occasional irritant components, and active allergens. Purification of the allergenic components and the uses of the purified allergens are important for the standardization of allergen extracts, the immunologic and physicochemical characterization of the individual allergen, and the studies on genetics of the immune response to allergens.

In Europe and North America, allergies to tree pollen are not believed to be as important clinically as allergies to weed or grass pollens because of the shorter pollinating seasons and sporadic abundance of individual species.^{1, 2} However, the Japanese cedar (*Cryptomeria japonica*, Sugi in Japanese) contributes the most important group of pollens causing allergies because of its abundant pollination and widespread distribution in Japan.³ The present study describes the isolation of the major allergen of Japanese cedar pol-

len, which was designated as antigen SBP, its allergenic activity assayed in vivo and in vitro, and some of the distinctive features of the allergen molecule.

MATERIALS AND METHODS

CE

Pollen was obtained from the 1980 crop of the Japanese cedar and 100 gm were defatted with ether and extracted in 2000 ml of 0.125M ammonium bicarbonate (pH 8.0) at 20° C for 48 hr. After extraction, the pollen was separated from the supernatant by centrifugation (10,000 × g) and was extracted in 1200 ml of 0.125M ammonium bicarbonate over a 24 hr period. The combined supernatants were dialyzed against 5 mM ammonium bicarbonate and lyophilized.

Abbreviations used

CE:	Crude extract of Japanese cedar pollen
PAG-IEF:	Polyacrylamide gel isoelectric focusing
PBS:	Phosphate-buffered saline
RAST:	Radioallergosorbent test
SBP:	Sugi basic protein
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
DEAE:	Diethylaminoethyl
cpm:	Counts per minute
CM:	Carboxymethyl

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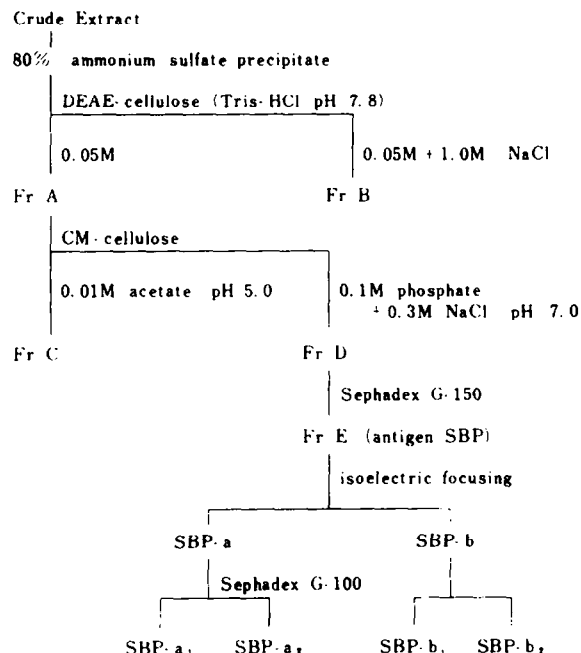


FIG. 1. Schematic diagram of the isolation of antigen SBP and its four subfractions from Japanese cedar pollen.

Fractionation of CE

Ammonium sulfate precipitation. CE obtained from 100 gm of dry pollen was dissolved in 300 ml of 0.05M Tris-HCl (pH 7.8), and solid ammonium sulfate was added with stirring to 80% saturation. After stirring overnight at 4° C, the precipitate was collected by centrifugation. The resulting dark-brown precipitate was dissolved in 100 ml of 0.05M Tris-HCl (pH 7.8) and dialyzed against the same Tris buffer.

DEAE cellulose chromatography. Ammonium sulfate precipitate in 100 ml of 0.05M Tris-HCl (pH 7.8) was passed at 4° C through 10 ml of DEAE cellulose (DE-52; Whatman, Springfield Mill, Kent, England) equilibrated with the same Tris buffer on a glass filter (5 cm diameter), after which 50 ml of the additional same Tris buffer were passed. The combined 150 ml fraction was collected as fraction A. The buffer was then changed to 0.05M Tris-HCl containing 1.0M sodium chloride (pH 7.8), and 100 ml of the fraction were collected as fraction B.

CM cellulose chromatography. Fraction A on DEAE cellulose chromatography was concentrated to 50 ml by negative-pressure filtration,⁴ dialyzed against 0.01M acetate buffer (pH 5.0), and applied at 4° C to 70 ml of CM cellulose (CM-52, Whatman) equilibrated with the same acetate buffer on a glass filter (7 cm diameter). The stepwise elution was then performed with 200 ml of the same acetate buffer and 200 ml of 0.1M phosphate buffer containing 0.3M sodium chloride (pH 7.0). The second eluate, fraction D, was concentrated to 10 ml by negative-pressure filtration⁴ and was subjected to further fractionation by a combi-

nation of gel chromatography and preparative column isoelectric focusing.

Gel chromatography. Chromatography was performed at 20° to 22° C and column effluents were monitored by their ultraviolet absorption at 280 nm with an LKB 2138 Uvicord S spectrophotometer. A Sephadex G-150 column (40 to 120 μ m; Pharmacia, Uppsala, Sweden) (2.6 by 100 cm) and a G-100 column (superfine; Pharmacia) (1.5 by 100 cm) were eluted with a downward flow of 0.05M ammonium bicarbonate (pH 8.0) at a rate of 20 and 5 ml/hr, respectively.

Preparative column isoelectric focusing. Isoelectric focusing in a sucrose density gradient was performed with an LKB 8100-1 column (110 ml) in accordance with the manufacturer's instructions. The sample was focused at 7° C for 65 hr at 600 constant volts with 1.0% Ampholine (LKB Industries, Bromma, Sweden), which had a pH range of 3.5 to 10, 7 to 9, and 9 to 11 in the ratio of 1:2:1. After focusing, column effluents were monitored by their ultraviolet absorption at 280 nm automatically and were collected in 2 ml fractions. The pH was measured in each fraction. Ampholine and sucrose were removed by dialysis.

Human sera and rabbit antisera

Serum was obtained from 15 untreated patients allergic to Japanese cedar pollen. Pooled serum from these individuals was stored at -20° C in small aliquots prior to use.

Antisera to CE were prepared by immunizing rabbits with a mixture of 10 mg of CE in 1 ml of PBS (pH 7.4) and 1 ml of complete Freund's adjuvant (Difco, Detroit, Mich.). The rabbits were injected subcutaneously at weekly intervals for 5 wk and were bled 7 days after the last injection. Antisera to antigen SBP were prepared by injecting rabbits subcutaneously with 1 mg of the antigen in 0.5 ml of PBS emulsified in 0.5 ml of complete Freund's adjuvant. Immunizations were given four times at 4 wk intervals. The rabbits were bled 7 days after the last injection.

Solid-phase antigens and RAST inhibition

Solid-phase antigens were prepared by linking either 2.5 mg of CE or 25 μ g of antigen SBP to 500 pieces of cyanogen bromide-activated paper discs (6 mm diameter) in 25 ml of borate-buffered saline (pH 8.0) for 48 hr at 25° C. The remaining reactive sites on the discs were then blocked with 25 ml of 1M ethanolamine-HCl (pH 8.2) for an additional 48 hr. As mentioned by Paul et al.,⁵ the paper disc linked with excess amounts of antigen required larger quantities of inhibitor to achieve 50% inhibition in RAST inhibition. Therefore, 5 μ g of CE or 50 ng of antigen SBP were coupled to each disc, since in RAST titration a plateau level of binding was achieved with these amounts of antigens.

RAST inhibition was carried out as follows: 50 μ l of diluted (1:5) pooled serum were mixed with 50 μ l of serial dilution of inhibitor and allowed to incubate at 37° C for 30 min and then at 4° C overnight, after which an antigen-coupled paper disc was added and the experiments were completed as described by Ceska et al.⁶ The inhibition was expressed in percent by the relation

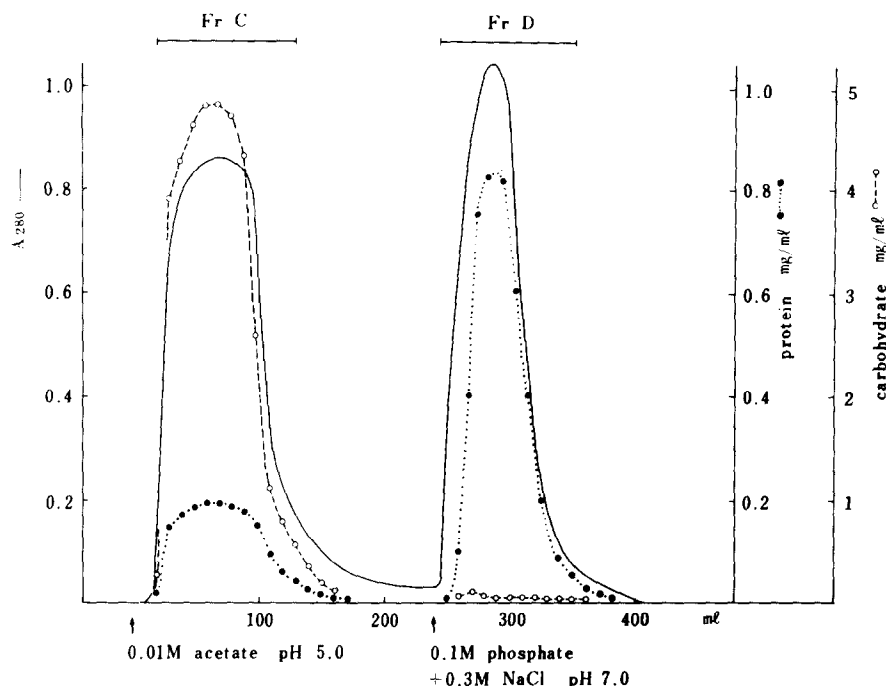


FIG. 2. Chromatography of fraction A on CM cellulose. Seventy milliliters of CM cellulose equilibrated with 0.01M acetate buffer (pH 5.0) on a glass filter (7 cm diameter) were used. Fractions C and D were pooled as indicated.

$$100 \times \frac{\text{cpm of noninhibited serum control} - \text{cpm of inhibited serum}}{\text{cpm of noninhibited serum control}}$$

In all assays, ^{125}I -anti IgE (D ϵ 2) obtained from Pharmacia was used.

Immunodiffusion, radial immunodiffusion, and crossed immunoelectrophoresis

Immunodiffusion was performed in 1.0% agarose (Behringwerke AG, Marburg-Lahn, West Germany) prepared in PBS congruent with the method of Ouchterlony.⁷

Radial immunodiffusion for determination of antigen SBP content in CE was performed with rabbit anti-SBP serum and purified antigen according to the method described for rye grass group I analyses.⁸

Crossed immunoelectrophoresis was performed according to the method of Lowenstein.⁹ In brief, electrophoresis of antigen SBP was carried out in 1.2% agarose gel prepared in Tris-veronal (pH 8.6), followed by a second-dimension electrophoresis into a gel containing appropriate rabbit anti-CE serum.

Skin testing

Intradermal skin tests were carried out by injecting 0.02 ml of serial 10-fold dilutions of each allergen preparation in phenol-saline. The skin reaction was measured after 15 min and a wheal of >10 by 10 mm was regarded as a positive response.

PAG-IEF and SDS-PAGE

Isoelectric focusing was carried out in 5.3% acrylamide gels (5 by 70 mm) containing 2.0% Ampholine (pH 3.5 to 10). The samples were focused at 4° C for 4 hr at 200 constant volts. The gels were stained with Coomassie blue G-250 in compliance with the method used by Diezel et al.¹⁰

SDS-PAGE was performed in 7.5% acrylamide gels (5 by 70 mm), consistent with the method of Weber and Osborn.¹¹ The gels were stained with Coomassie blue R-250 according to the method of Fairbanks et al.¹² Marker proteins used for molecular weight determination were bovine serum albumin (mol. wt. 69,000), ovalbumin (mol. wt. 45,000), chymotrypsinogen A (mol. wt. 25,000), and cytochrome C (mol. wt. 12,400).

Chemical analyses

Total carbohydrate content was determined by the phenol-sulfuric acid method,¹³ using mannose as a standard.

Total protein was determined according to the method of Lowry et al.,¹⁴ using bovine serum albumin as a standard.

The ultraviolet absorption spectrum and the absorption coefficient at 280 nm of antigen SBP were measured with a Hitachi model 200-20 spectrophotometer.

Amino acid analysis was carried out on a Hitachi model 835 automatic amino acid analyzer with a Hitachi model 834 chromatoprocessor. The samples were hydrolyzed in 6N HCl in sealed evacuated tubes for 24 hr at 110° C. The hydrolysates were taken to dryness on a rotary evaporator, then dissolved in sodium citrate buffer (pH 2.2).

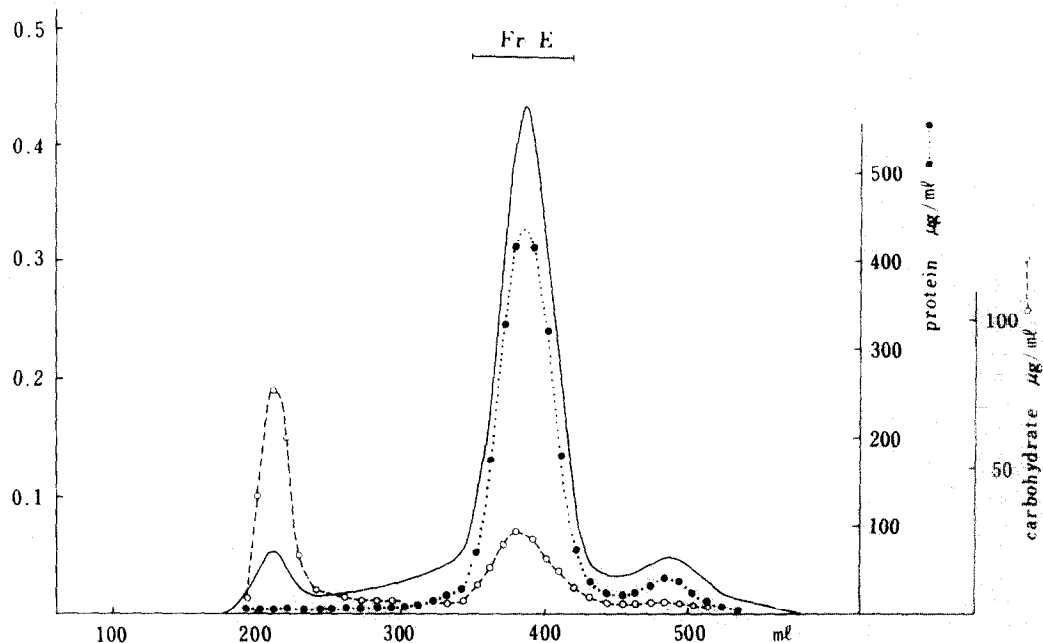


FIG. 3. Chromatography of fraction D on Sephadex G-150 (2.6 by 100 cm) equilibrated with 0.05M ammonium bicarbonate (pH 8.0). Fraction E (antigen SBP) was pooled as indicated.

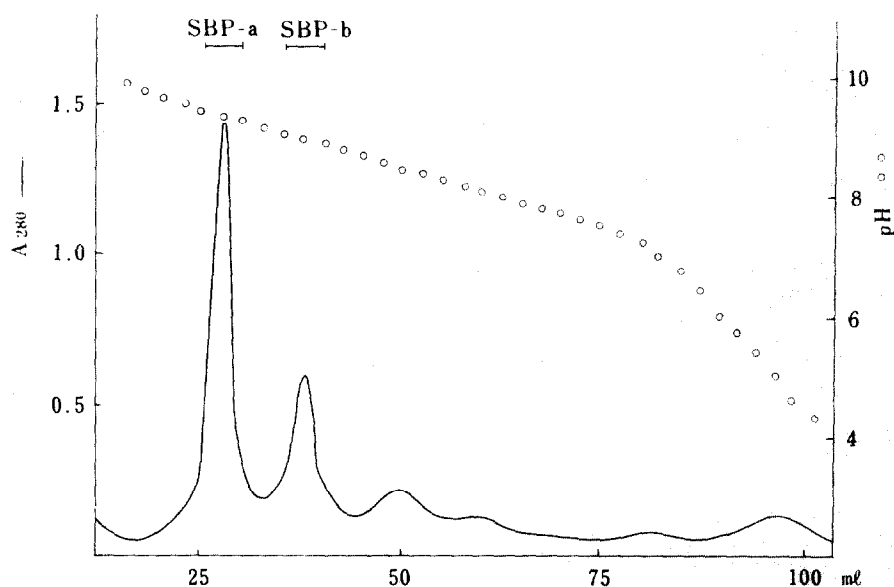


FIG. 4. Isoelectric focusing of antigen SBP in a sucrose density gradient. The sample load was 10 mg. SBP-a and SBP-b were pooled as indicated.

Stability experiments of antigen SBP

A solution of the antigen (1 mg/ml in PBS) was incubated for 30 min at 40, 60, 80, and 100° C. The same concentration of the antigen was incubated in 0.02M buffers at pH 2.0, 4.0, 7.4, 9.0, and 12.0 for 24 hr at 20° C, after which the solution was diluted with 0.15M phosphate buffer (pH 7.4). The allergenic activity of the heat- and pH-treated samples was assessed by RAST inhibition.

RESULTS

Isolation of antigen SBP

A schematic diagram of the method used for purification of the major allergen of Japanese cedar pollen is shown in Fig. 1. It has been previously indicated¹⁵ that the CE contained extremely large amounts of polysaccharides that were allergenically inactive, and the highly allergenic activity was found in the basic

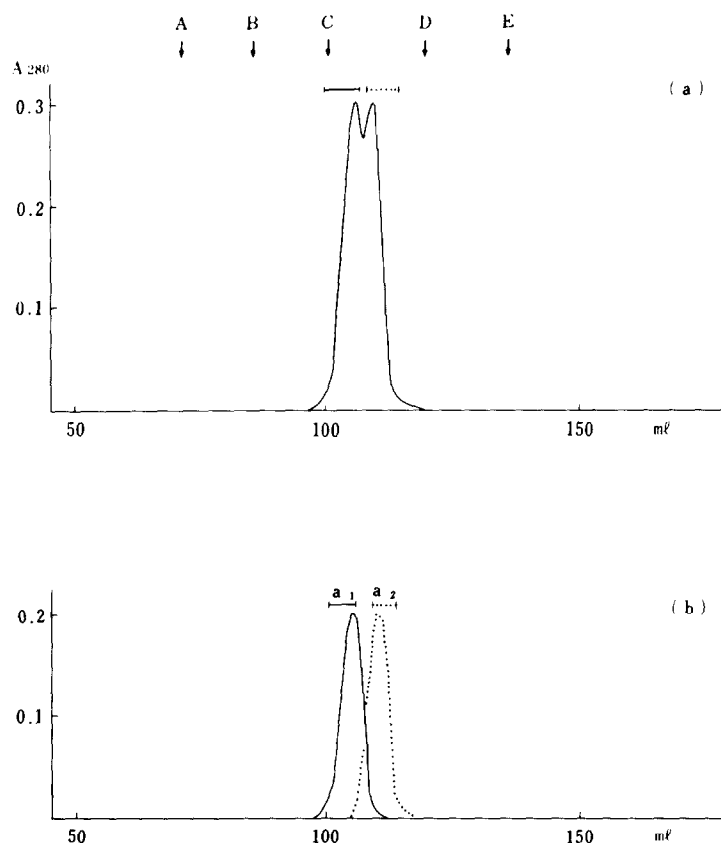


FIG. 5. Chromatography of SBP-a on Sephadex G-100 (1.5 by 100 cm) equilibrated with 0.05M ammonium bicarbonate (pH 8.0). **a**, Initial chromatography of SBP-a. The sample load was 3 mg. **b**, Rechromatography of the first peak (solid line) and the second peak (dotted line) shown in **a**. SBP-a₁ and SBP-a₂ were pooled as indicated. Elution positions of calibration markers are indicated by arrows, including blue dextran (A), bovine serum albumin (B), ovalbumin (C), chymotrypsinogen A (D), cytochrome C (E), and DNP-alanine (F).

protein fraction. Thus a procedure of fractionation in this study was developed for removing the allergenically inactive polysaccharides and isolating the basic protein. From 100 gm of dry pollen, 4.1 gm of CE was obtained, which was found to contain only 10% protein. CE was first precipitated with ammonium sulfate at 80% saturation. By this step, 60% of the polysaccharides in CE was removed. Subsequent ion-exchange chromatography on DEAE and CM cellulose was performed as rapidly as possible at 4°C on a wide glass filter with a small amount of ion-exchange cellulose to prevent loss of protein component by irreversible adsorption to the cellulose matrix.¹ Two fractions, A and B, were obtained by chromatography on DEAE cellulose with a stepwise increase of the ionic strength of the buffer. An acidic protein fraction, fraction B, was not further characterized in this study. Fraction A was then resolved into two fractions, C and D, by chromatography on CM cellulose (Fig. 2). The second eluate, fraction D, which was found to contain most of the proteins in fraction A, was concentrated and applied to a Sephadex G-150

column. As shown in Fig. 3, chromatographic studies on Sephadex G-150 gave one major protein peak, fraction E, which contained a small amount of carbohydrate. Fraction E contained antigen SBP in a yield of 25 mg/100 gm of dry pollen. CE from 100 gm of dry pollen was found to contain 33.4 mg of antigen SBP by radial immunodiffusion analysis, indicating the good overall recovery (74.9%) of the antigen in the fractionation procedures from CE to fraction E.

Separation of antigen SBP into four subfractions

Ten milligrams of antigen SBP was fractionated by isoelectric focusing. As shown in Fig. 4, two major and one minor fraction were obtained. These two major fractions, with a pI of 9.2 and 8.9, were designated as antigens SBP-a and SBP-b, respectively. Since the SDS-PAGE of antigens SBP-a and SBP-b showed an identical pattern of two closely spaced bands, gel chromatography on Sephadex G-100 was next carried out to separate these two bands. When 3

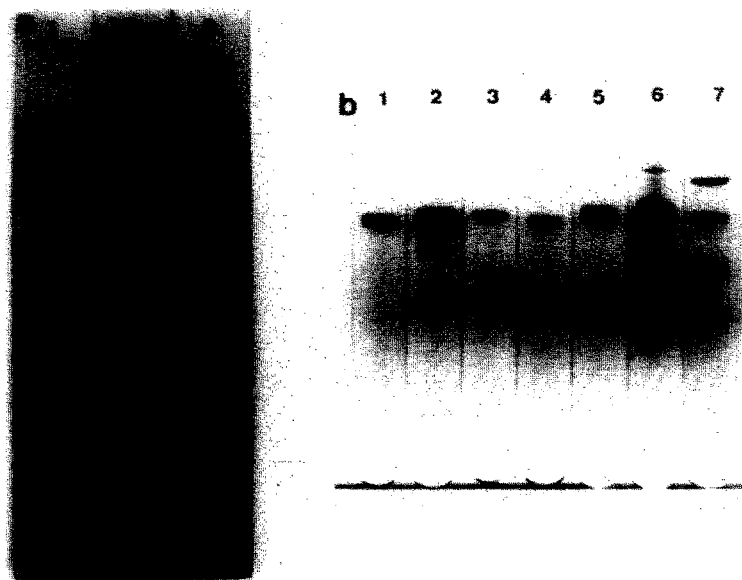


FIG. 6. a, PAG-IEF. The anode is at the top. 1, Antigen SBP (10 μ g); 2, SBP-a₁ (10 μ g); 3, SBP-b₁ (10 μ g). b, SDS-PAGE. 1, Antigen SBP (2 μ g); 2, antigen SBP (20 μ g); 3, SBP-a₁ (2 μ g); 4, SBP-a₂ (2 μ g); 5, an admixture of SBP-a₁ (2 μ g) and SBP-a₂ (2 μ g); 6, CE (500 μ g); 7, an admixture of standard proteins, including bovine serum albumin (mol. wt. 69,000), ovalbumin (mol. wt. 45,000), chymotrypsinogen A (mol. wt. 25,000), and cytochrome C (mol. wt. 12,400).

TABLE I. Isoelectric point (pI) and molecular weight of SBP subfractions

	pI	Molecular weight	
		Gel chromatography*	SDS-PAGE†
SBP-a ₁	9.2	40,000	46,000
SBP-a ₂	9.2	35,000	41,000
SBP-b ₁	8.9	40,000	46,000
SBP-b ₂	8.9	35,000	41,000

*Estimated by gel chromatography on a Sephadex G-100 column calibrated with markers listed in Fig. 5.

†Estimated by SDS-PAGE using standard proteins listed in Fig. 6, b.

mg of antigen SBP-a was chromatographed, there was partial separation of the two fractions (Fig. 5, a). Almost complete separation of the two fractions was achieved by rechromatography on the same column (Fig. 5, b). These two fractions were designated as antigens SBP-a₁ and SBP-a₂, respectively. In a similar procedure, antigens SBP-b₁ and SBP-b₂ were obtained from the antigen SBP-b preparation (chromatographic results not illustrated). The isoelectric points and molecular weights estimated by gel chromatography and SDS-PAGE of the four subfractions are summarized in Table I.

Analyses of homogeneity

The homogeneity of antigen SBP and its four subfractions was assessed by SDS-PAGE and PAG-IEF.

By SDS-PAGE (Fig. 6, b), antigen SBP gave at least four faint bands with two major bands when the large sample load (20 μ g) was employed. No cross-contamination between SBP-a₁ and SBP-a₂, as well as SBP-b₁ and SBP-b₂ (data not shown), was detected. By PAG-IEF (Fig. 6, a), antigen SBP gave two major bands with a pI of 9.2 and 8.9, and one faint band with a pI of 8.6, which corresponded to the minor fraction with a pI of 8.6 in preparative column isoelectric focusing (Fig. 4). SBP-a₁ and SBP-b₁ were slightly cross-contaminated with each other. Furthermore, SBP-b₁ gave one more faint band due to a trace contamination of the minor fraction with a pI of 8.6 (these faint bands are photographically unclear).

Antigenic and allergenic properties of antigen SBP and its four subfractions

Immunodiffusion and crossed immunoelectrophoresis were done to test antigenic purity of antigen SBP and the cross-reactivity between the four subfractions of the antigen. In immunodiffusion, antigen SBP gave one strong line against anti-CE and anti-SBP sera, and no antigenic difference was detected between the four subfractions (Fig. 7). In crossed immunoelectrophoresis, the sample of antigen SBP used was found to contain a trace amount of antigenic impurity, which moved toward the anode (Fig. 8).

The allergenic activity of antigen SBP and its four subfractions was measured by quantitative intradermal skin tests as well as RAST inhibition. Compara-

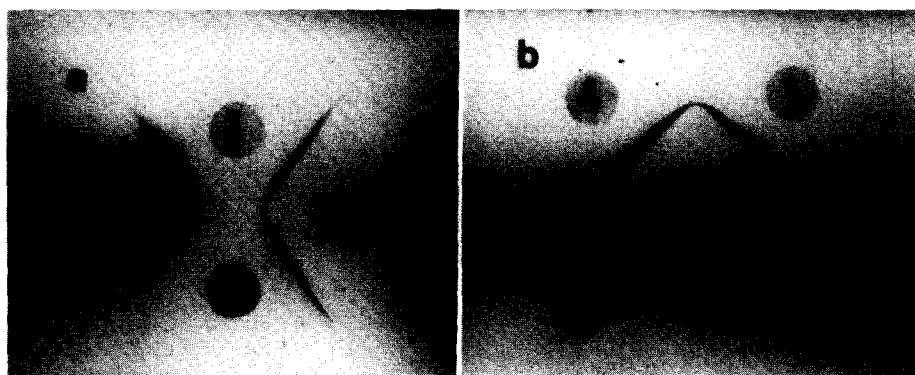


FIG. 7. Immunodiffusion analyses. **a**, CE in well 1 (10 mg/ml); antigen SBP in well 2 (0.1 mg/ml); rabbit anti-CE serum in well 3; rabbit anti-SBP serum in well 4. **b**, SBP-a₁, a₂, b₁, and b₂ in wells 1, 2, 3, and 4, respectively (0.1 mg/ml); rabbit anti-SBP serum in well 5.

TABLE II. Intradermal skin tests with Japanese cedar pollen allergens

Patient	Minimal amount to give a positive reaction (gm/ml)					
	CE	SBP	a ₁	a ₂	b ₁	b ₂
N. S.	10 ⁻⁶	10 ⁻⁹	10 ⁻⁸	10 ⁻⁹	10 ⁻⁸	10 ⁻⁹
R. T.	10 ⁻⁶	10 ⁻⁹	10 ⁻⁸	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹
M. A.	10 ⁻⁷	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹
D. M.	10 ⁻⁶	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸	10 ⁻⁸
E. K.	10 ⁻⁷	10 ⁻⁹	10 ⁻⁸	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹
M. O.	10 ⁻⁷	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹
N. S.	10 ⁻⁷	10 ⁻¹⁰	10 ⁻⁹	10 ⁻¹⁰	10 ⁻⁹	10 ⁻¹⁰
O. S.	10 ⁻⁶	10 ⁻⁸	10 ⁻⁸	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹
M. Y.	10 ⁻⁷	10 ⁻⁹	10 ⁻⁹	10 ⁻¹⁰	10 ⁻⁹	10 ⁻⁹
N. K.	10 ⁻⁷	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹	10 ⁻⁹
Geometric mean	4.6 × 10 ⁻⁷	2.7 × 10 ⁻⁹	5.5 × 10 ⁻⁹	1.7 × 10 ⁻⁹	2.8 × 10 ⁻⁹	1.8 × 10 ⁻⁹

tive skin tests on 10 sensitive patients showed that antigen SBP and its four subfractions were about 100 times more reactive than CE and gave very similar skin responses in all patients (Table II). In RAST inhibition, antigen SBP and CE were coupled to activated paper discs, and the ability of antigen SBP, its four subfractions, and CE to inhibit the binding of IgE antibody in pooled serum to the discs was tested (Fig. 9). Essentially, antigen SBP and its four subfractions showed identical patterns of inhibition toward the binding of IgE antibody to both the SBP and CE discs. Smaller quantities of antigen SBP and its four subfractions (2 ng for the SBP disc and 1 ng for the CE disc) and larger quantities of CE (250 ng for the SBP disc and 200 ng for the CE disc) were required for 50% inhibition of the binding of IgE antibody in pooled serum. More than 70% of the binding of IgE antibody in pooled serum to the CE disc was inhibited with 1 µg of antigen SBP and its four subfractions, indicating that IgE antibody to antigen SBP accounted for a large proportion of IgE antibody to CE. On the basis of these criteria, antigen SBP is

shown to be the major allergen of Japanese cedar pollen and the four subfractions of antigen SBP appear to be antigenically and allergenically identical.

Partial physicochemical characterization of antigen SBP

A solution (0.72 mg/ml in 5 mM ammonium bicarbonate) of antigen SBP showed a typical absorption spectrum of proteins and had a maximal absorbance at 282 nm. An absorption coefficient of $E_{1\%}^{1\text{cm}}$ at 280 nm 12.6 was calculated for antigen SBP. The total carbohydrate content of antigen SBP, determined by the phenol-sulfuric acid method,¹³ was 6.3%. The amino acid analyses of the four subfractions of antigen SBP are shown in Table III. Each of the four subfractions had a very similar amino acid composition.

The stability of antigen SBP was assessed with RAST inhibition. The antigen was stable at temperatures below 40° C and in the pH range 4 to 9. It lost more than 90% of its activity after 30 min at 60° C. When a solution of the antigen was heated above 80° C, the antigen was rapidly aggregated to form precipitates



FIG. 8. Crossed immunoelectrophoresis of antigen SBP (8 μ g) against rabbit anti-CE serum. The anode is to the right in first and to the top in second dimension. Arrow indicates trace impurity.

and completely lost its allergenic activity. The antigen completely lost its allergenic activity by treatment with both an acid (pH 2.0) and an alkali (pH 12.0) solution, although it was still soluble in these conditions (data not shown).

DISCUSSION

The Japanese cedar is a member of the Taxodiaceae family, and those conifers such as the mountain cedar (*Juniperus sabinoides*),¹⁶ which cause allergies in certain areas of the United States, are members of the Cupressaceae family. Yoo et al.¹⁷ reported that the pollen extracts of a large number of species of the Cupressaceae family had a marked cross-reactivity in man and rabbit, yet they also suggested a lack of cross-reactivity between the Cupressaceae family and the redwood (*Sequoia sempervirens*) and the Japanese cedar of the Taxodiaceae family. However, there is evidence suggesting an obvious cross-allergenicity and cross-antigenicity between the Japanese cedar and the mountain cedar or the hinoki white-cedar (*Chamaecyparis obtusa*), a widely pollinating member of the Cupressaceae family in Japan. This was confirmed by skin testing and RAST results in patients sensitive to Japanese cedar pollen and also by immunodiffusion analysis with rabbit antisera (Shimizu T, Yui Y, Yasueda H, and Shida T; unpublished observation). The availability of a purified major allergen from Japanese cedar pollen as well as from mountain cedar pollen^{18, 19} will facilitate further studies on allergenic or antigenic relationships among the species of the order Coniferales.

Direct skin test and RAST inhibition showed that antigen SBP is the major allergen of Japanese cedar

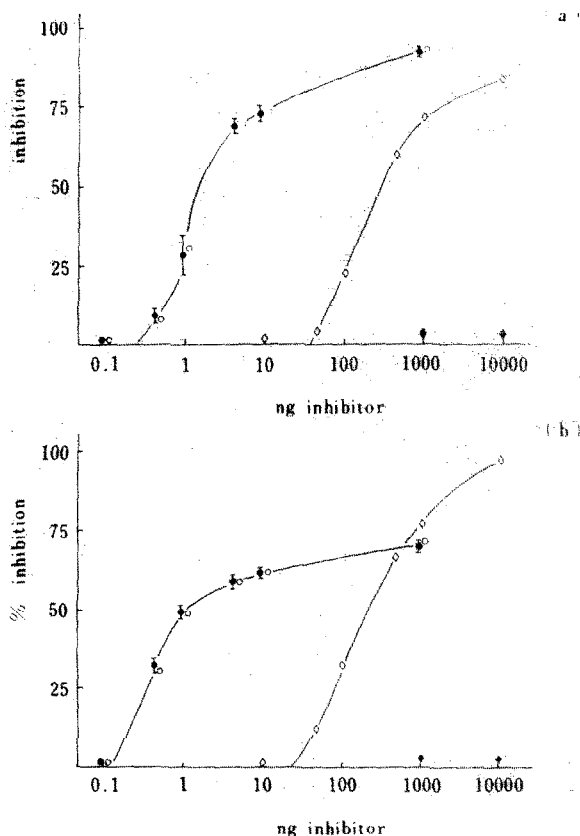


FIG. 9. Inhibition of the binding of anti-Japanese cedar IgE antibody to antigen SBP-coupled disc (a) and CE-coupled disc (b) by CE ($\circ$), antigen SBP ($\triangle$), four subfractions of antigen SBP ($\bullet$), and unrelated antigen, *Dermatophagoides farinae* crude extract ($\blacktriangle$). Inhibition capacity of the four subfractions were shown as single points (mean $\pm$ SD), since undistinguishable results were obtained.

pollen. In addition, SDS-PAGE and immunodiffusion analysis showed that antigen SBP is the most abundant protein in CE. This also supports the fact that antigen SBP is the major allergen of the pollen, since major allergens are usually the most abundant proteins or glycoproteins with a molecular weight range of 20,000 to 40,000 in the extract.²⁰

Antigen SBP was separated into four subfractions by isoelectric focusing and gel chromatography. Several lines of evidence suggested that four subfractions were antigenically and allergenically identical. It would seem unlikely that this identity was due to the cross-contamination of the four subfractions, since each of the four subfractions was only slightly cross-contaminated as judged by SDS-PAGE and PAG-IEF.

There are many allergens on proteins with different isoelectric points, including rye grass group I,¹ short ragweed antigens E and K,²¹ *Dermatophagoides*

TABLE III. Amino acid analysis of SBP subfractions

	Moles amino acid/mole protein*			
	a ₁	a ₂	b ₁	b ₂
Asx	55.3 (15.9)†	48.0 (15.8)	56.3 (16.1)	48.7 (16.0)
Thr	25.8 (7.4)	22.5 (7.4)	25.9 (7.4)	22.5 (7.4)
Ser	31.0 (8.9)	27.3 (9.0)	31.1 (8.9)	27.4 (9.0)
Glx	18.8 (5.4)	16.1 (5.3)	18.2 (5.2)	16.1 (5.3)
Pro	12.5 (3.6)	11.2 (3.7)	13.3 (3.8)	10.7 (3.5)
Gly	37.6 (10.8)	33.1 (10.9)	38.1 (10.9)	33.5 (11.0)
Ala	19.5 (5.6)	17.0 (5.6)	19.2 (5.5)	17.0 (5.6)
half-Cys	8.7 (2.5)	7.6 (2.5)	8.0 (2.3)	7.0 (2.3)
Val	24.4 (7.0)	21.3 (7.0)	24.8 (7.1)	21.9 (7.2)
Met	11.8 (3.4)	9.1 (3.0)	11.9 (3.4)	9.1 (3.0)
Ile	17.7 (5.1)	15.5 (5.1)	18.2 (5.2)	15.8 (5.2)
Leu	20.5 (5.9)	17.9 (5.9)	20.6 (5.9)	18.3 (6.0)
Tyr	14.3 (4.1)	12.8 (4.2)	13.3 (3.8)	12.2 (4.0)
Phe	13.2 (3.8)	11.9 (3.9)	13.6 (3.9)	12.2 (4.0)
Lys	18.1 (5.2)	15.8 (5.2)	17.5 (5.0)	15.5 (5.1)
His	5.9 (1.7)	5.5 (1.8)	6.6 (1.9)	6.1 (2.0)
Arg	12.9 (3.7)	11.9 (3.9)	12.2 (3.5)	11.0 (3.6)

*The molecular weights used for the calculations were 40,000 for SBP-a₁ and b₁ and 35,000 for SBP-a₂ and b₂. The carbohydrate content of each of the four subfractions was assumed as 6.3%.

†Values in the parentheses are the proportion of each residue per 100 amino acid residues in the protein.

pteronysinus antigen P₁,²² and *Cladosporium herbarum* antigen 32.²³ They have been defined as "isoallergens" by Johnson and Marsh.²⁴ Many kinds of proteins purified by a variety of procedures have almost invariably shown heterogeneity when analyzed by isoelectric focusing. This heterogeneity on isoelectric focusing could be explained by several factors, such as deamidation and conformational change or difference in carbohydrate content.²⁵ However, the presence of a common allergen on proteins with a different molecular weight is unusual. Recently, Shafiee et al.²⁶ isolated two glycoproteins (RT₁ and RT₂) from Russian thistle pollen, which were shown to be immunologically identical and to have the same amino acid composition and carbohydrate content but which were different in both molecular weights and isoelectric points. *C. herbarum* antigen 32²³ has also been reported to be heterogeneous with respect to molecular weight, since it was eluted in a relatively wide range of fractions in gel chromatography. This heterogeneity was assumed by the authors to be the result of a variation in carbohydrate content. The amino acid composition of each of the four subfractions of antigen SBP was very similar; thus the heterogeneity of antigen SBP, in relation to molecular weight, may be the result of variation in carbohydrate content, as with *C. herbarum* antigen 32.

However, it is possible that the separation of antigen SBP into subfractions by gel chromatography was

not due to the difference in molecular weight but rather to the difference in protein-gel interaction, since two variants of short ragweed allergen Ra3²⁷ were separated by gel chromatography and were shown to have the identical molecular weight on analytical ultracentrifugation and on the complete amino acid sequence analysis. Whether the separation of antigen SBP into subfractions by gel chromatography was due to the difference in molecular weight or to the difference in protein-gel interaction remains to be determined. Further investigation is needed to determine the accurate molecular weight by analytical ultracentrifugation and the accurate carbohydrate content of each of the four subfractions of antigen SBP.

The allergenic activity of antigen SBP was markedly reduced on denaturation by heating and by acid and alkali treatment. This indicates that the antigenic determinants reacting with specific IgE antibodies are dependent on the native conformation of the molecule.²⁰ The absorption spectrum of antigen SBP showed a single peak at 282 nm, characteristic of proteins. No peak was evident at 305 nm, suggesting the absence of the *N*-glycosidic protein-sugar residues, postulated by Berrens²⁸ to be a common feature of atopic allergens. Antigen SBP is different from the other major allergens from pollens with respect to the net charge of the molecule. Antigen SBP is a basic protein with an isoelectric point of about 9, whereas the other major allergens from pollens, including

short ragweed antigen E,²¹ rye grass group I,¹ and timothy grass antigen B²⁹ are acidic proteins with isoelectric points in the range of 4 to 6. However, allergenicity may not depend on the net charge of the molecule, since it is not the net charge but the charges of and around the limited antigenic determinants that influence binding to the complementary combining site of the IgE antibodies.³⁰

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