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Identification and Partial Purification of Pollen Allergens from Artemisia Princeps

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The pollen of Artemisia has been considered as the main late summer-autumn allergen source in this country. To identify its allergenic components, Artemisia princeps pollen extracts were separated by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane, where IgE binding components were detected by the reaction with sera of twenty Artemisia-allergic patients and ^{125}I-anti-human IgE, sixteen components in the molecular range of 10,000 and 85,000 daltons were detected. Twelve bands bound to IgE from 50% of the sera tested, and two bands (37,000, 23,000 daltons) showed the highest (85%) frequency of IgE-binding in twenty sera tested. When the gel of SDS-PAGE with Artemisia pollen extracts was sliced into 11 allergenic groups (AG) and the protein of each AG was obtained by the gel elution method, the wormwool-RAST inhibition test showed that the AG 10 demonstrated to be the most potent, and the AG 7 was the next. Six AGs showed significant responses (more than 100% of wheal size to histamine, 1mg/ml) on the skin prick test in more than 50% of the patients tested. It is suggested that electrophoretic transfer analysis with SDS-PAGE may be a valuable method for Artemisia allergen identification, and the possibility of partial purification of allergens by employing gel elution is discussed.

Key Words: Allergens, Artemisia princeps, electroblotting, gel elution

Mugwort (Artemisia vulgaris L.) pollen is one of the common causes of allergic reaction during late summer in northern europe (Charpin *et al.* 1974; Holopainen *et al.* 1979). In Korea, the pollen of Artemisia is reported to be abundant in the air of Seoul from the end of August through September (Hong *et al.* 1986), and is known to be one of the main sources of late summer-autumn respiratory allergy in this country.

A few studies have been performed to characterize this pollen extracts with regard to their IgE-binding components and their chemical or biochemical compositions. Recently, Ipsen *et al.* (1985) have identified 10 allergens from Art. vulgaris pollen and Paulsen *et al.* (1985) have observed 7 allergens among 18 antigens by CRIE. Nilsen and Paulsen (1986) have partially purified and defined Ag 7 as the major allergen from Art. vulgaris extracts. Jaggi and Gangal (1987) have purified one important allergen with a molecular weight of 14,300 daltons from Art. scoparia pollen.

The principal aim of this study was to detect the allergenic components in Art. princeps pollen and to purify these allergens, that is important for the further investigation of Art. pollen allergy. To achieve this aim, we have used the procedures recently published for allergen identification (Towbin *et al.* 1979; Sutton *et al.* 1982; Tovey and Baldo 1984), including SDS-PAGE, immunoblotting, and the gel elution method for allergen purification. The results showed the presence of IgE-binding components (and hence possible allergens) in Art. princeps pollens, and

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ABBREVIATIONS

Art.	: Artemisia
SDS-PAGE:	Sodium dodecylsulfate polyacrylamide gel electrophoresis
RAST	: Radioallergosorbent test
NC	: Nitrocellulose membrane
PBS	: Phosphate buffered saline
AG	: Allergenic group
WPE	: Whole pollen extracts
MW	: Molecular weight
CRIE	: Crossed radio-immunoelectrohoresis

provided valuable information on the precise spectra of components recognized by individual patients.

MATERIALS AND METHOD

Preparation of whole pollen extracts

Art. princeps pollens were collected from flowering plants during the season. The dried pollens were defatted with diethylether and extracted with 0.125M NH_4HCO_3 (pH 8.3), 1:50w/v, for 72 hours at 4°C, followed by the centrifugation at 10,000g at 4°C for 1 hour. Sold ammonium sulfate was added in the supernatant to 70% saturation with stirring. After stirring in a cold room for 18 hours, the precipitate was collected by centrifuging at 10,000g at 4°C for 1 hour. The resulting precipitate was dissolved in one tenth of the original volume of 0.125M NH_4HCO_3, dialyzed against distilled water, and lyophilized at −70°C in a sealed vial. The protein content was measured by the Bensadoun method (Bensadoun and Weinstein, 1976), and it was 380 ug per mg of lyophilized extract dissolved in 1 ml of PBS.

Human serum

Sera were collected from 20 untreated patients allergic to Artemisia spp. pollens at the Allergy Clinic, Severance Hospital, Yonsei University College of Medicine (Seoul, Korea). Sera from individuals who showed negative responses to 50 common inhalant allergens on the skin prick test were also obtained and pooled. All sera were tested for Art-specific IgE in a wormwood-RAST assay with the Pharmacia wormwood paper disc (W5). All sera presented RAST class 3 or 4 except one case (class 2).

Ion-exchange chromatography

The lyophilized extracts of Art. princeps pollens were dissolved in 50 mM tris-chloride buffer (pH 8.3), loaded on a 2.5 by 8.5cm sized DEAE Sephadex A_{50} column equilibrated with 50 mM Tris-chloride buffer and eluted with tris-chloride buffer, and the absorbance was monitored at 280nm. When the absorbance dropped to 0.01, the column was finally eluted with 1.0 M NaCl in tris-chloride buffer, as shown in Fig. 1. All fractions were pooled, dialyzed and concentrated by Amicon (Amicon Laboratories, Inc., Nashville, Tenn.). This concentrated eluate (1164 ug/ml of protein measured by the Bensadoun method) was subjected to SDS-PAGE.

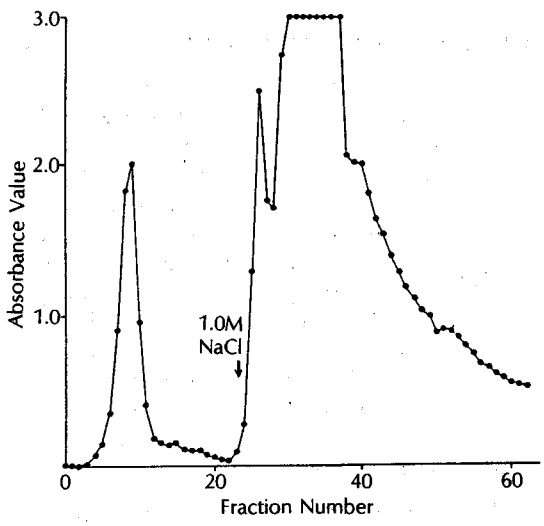

Fig. 1. Elution profile of Artemisia princeps extracts of DEAE Sephadex A_{50}. The column was eluted with 50mM tris-chloride buffer (pH 8.3), monitoring the absorbance at 280nm. When the absorbance dropped to 0.01, the column was eluted with 1.0M NaCl in the buffer.

Gel electrophoresis

SDS-PAGE was carried out by a modified method of Laemmli (1970) and 10% homogeneous gels were used. Art. pollen extracts (200 ug of protein per well) were treated with 2-mercaptoethanol, and heated at 100°C for 2 minutes, then they were subjected to electrophoresis at constant 30 mA. The gels were stained with 0.01% Commassie Brilliant Blue (Sigma Chemical Co., St. Louis., Mo.). To ensure reliability of the molecular weight estimations, low molecular weight markers (Sigma Chemical Co., St. Louis Mo.) were included in each gel. The molecular weight of each protein was allotted after the comparison with the marker proteins.

Electrophoretic transfer of proteins

Proteins were transferred at 0.4 mA for 18 hours to NC membranes (Millipore® HAHY 304 FO Millipore, 0.45um, Bedford, Massachusetts, USA) using a modified method of Towbin et al. (1979). After transfer, the NC membranes were incubated with 3% bovine serum albumin in 10 mM Tris buffer for 4 hours to block unbound membrane sites, then were washed with 0.9% NaCl and air-dried.

Probing of NC membrane strips and autoradiography

The NC strips (5mm width) blotted were incubated with sera (diluted 1 in 4 with 0.05% Tween 20 in PBS) for 18 hours, washed and followed by the incubation with ^{125}I-anti-IgE (Phadebas RAST tracer, about 300,000 cpm per strip) for 18 hours. After washing three times with 0.05% Tween 20 in PBS, the NC strips were air-dried at room temperature and loaded into a Kodak X-omatic cassette (Eastman Kodak Co. Rochester, N.Y) together with photographic films (Fuji Rx-safety, Tokyo, Japan). The cassette was then stored at −70°C for 5 days or 14 days. Two pooled sera from non-allregic individuals were used as controls.

Elution of proteins from the gel

The protein of allergenic fractions was eluted from the 11 sliced gels by shaking with 3.5ml PBS containing 0.05% SDS overnight and the eluates were centrifuged at 15,000 rpm for 1 hour at 4°C. The supernatant was dialyzed against PBS and concentrated into 200μg/ml. The protein content of each AG was measured by the Bensadoun method.

RAST inhibition

A competitive RAST inhibition was used to compare the allergenicity of the 11 separated AGs of proteins; 50ul of 1:10 diluted pooled serum of Art-sensitive patients (wormwood RAST class 4) was incubated with various dilutions of protein (0.5-10.0 ug) for 1 hours at room temperature. The inhibited pooled serum was incubated with a wormwood paper disc (Pharmacia Co., Uppsala, Sweden) for 3 hours at room temperature. After washing three times with washing solution, 50ul of ^{125}I-labelled anti-human IgE was incubated with the disc for 18 hours. After the repeated washing step, disc-bound IgE was determined by r-counter (Packard) and inhibition of the binding of specific IgE to the allergen disc was calculated then compared to control samples. The amount required for 50% inhibition was interpolated on a semi-logarithmic graph by plotting protein concentration versus percent inhibition.

Allergy skin test

The skin prick test with the 11 AGs and crude WPE were performed simultaneously with 10 Art.-sensitive patients and 4 negative responders on skin prick test with 50 common inhalant allergens. The results were read at 15 minutes after the prick. The wheal sizes were estimated as the maximum diameter and vertical length at the mid-portion of maximum length, and it was represented as the percent ratio of mean wheal size to histamine (1mg/ml, Bencard, Great Britain).

RESULTS

Gel electrophoresis

In order to understand the protein components of Art. pollen, the concentrated eluate from second peak on DEAE Sephadex A_{50} chromatography was analysed by 10% SDS-PAGE. As shown in Fig. 2, more than 20 protein bands were observed.

Probing of NC-blotted proteins for IgE binding

Fig. 3 demonstrates the bindings of IgE from atopic sera on blotted NC. Sixteen bands in the molecular weight range of 10,000 to 85,000 daltons bound IgE antibodies in the patients' sera. Protein bands with the highest frequency of IgE binding were number 9 (37,000 daltons) and 12 (23,000 daltons). Table 1 shows the molecular weight of each allergenic frac-

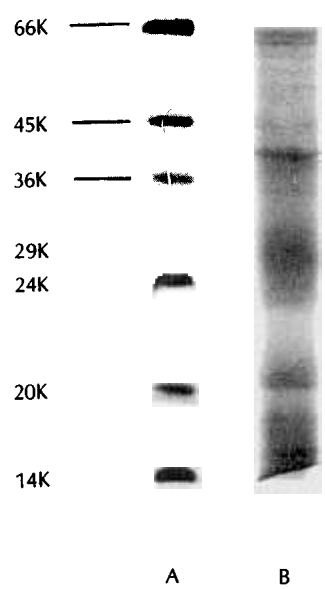

Fig. 2. Separation of Korean Artemisia princeps extracts by 10% SDS-PAGE. (Lane B)
Lane A: Molecular weight markers, bovine serum albumin (66,000), egg albumin (45,000), 3-phospho glycer aldehyde dehydrogenase (36,000), carbonic anhydrase (29,000), L-lactoalbumin (14,200).

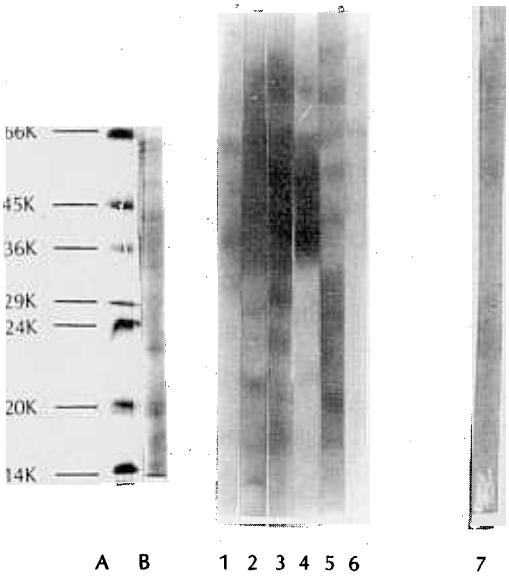

Fig. 3. Identification by autoradiography of IgE-binding components transferred to NC after SDS-PAGE of Artemisia princeps extracts in Artemisia-sensitive patients (Lane 1-6), and negative control (lane 7).
Lane A: Molecular weight markers.
Lane B: Separated Artemisia princeps extaracts.

tion estimated. Of the 16 IgE-binding bands, 12 (number 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15) were bound with at least half of the atopic sera tested. Two pooled sera from subjects not allergic to any common inhalant allergens did not bind to IgE. There was no significant difference in the frequency of allergenic fractions among allergic diseases. There was no relationship between an individual's wormwood RAST reactivity and the total number of Art. princeps allergenic fractions to which he or she reacted.

Protein bands of the allergenic group

10% SDS-PAGE was carried out as previously described. Art. pollen extracts (concentrated eluate; Lane A) and each AG were subjected to electrophoresis as shown in Fig. 4. There were no obvious bands in AG 1 and AG 2. Table 2 reveals one or several protein bands and their molecular weights in each AG. The estimated molecular weight of protein in AG 3 was 72,000 daltons. Ag 7 included three bands and their molecular weights were 42,000, 40,000 and 37,000 daltons, which had the high IgE binding capacity as shown in autoradiography. AG 10 included one band with a molecular weight of 23,000 daltons.

Table 1. The distribution of IgE-binding components in 20 Artemisia-sensitive patients and 2 negative control determined by autoradiography

Fractions	Artemisia-sensitive patients																			Control		Total
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	1	2	(%)
1 (85K)		●				○						○											3 (15)
2 (76-80K)						○		○		○	○					●	●	●	●				8 (40)
3 (69-71K)	●	●		●	○	○	○	●	●	○		●		●	●		●	●	●	○			16 (80)
4 (58-62K)	●	○	○							○	○		●			○							7 (35)
5 (56-58K)	○	○		●	○		○		●		●	●	●	●	●	●	●	●	●				15 (75)
6 (50-52K)	●		○			○		○	●	●		●	○	●	●	●				○			12 (60)
7 (46-48K)					○	○	○		●	●	○			○			●	●	●				10 (50)
8 (39-41K)	●			●	○	○	○	●	●	●	●		●	●		●	●						13 (65)
9 (36-39K)		○	○	●	○	○	○	○	●	●	●	●		●	●	●	●	●	●				17 (85)
10 (31-32K)		●		○			○	○	●		●	●	○		●		●		●	○			12 (60)
11 (26-28K)	○		○	○	○				●	●				●	●	○	●	●	●	○			13 (65)
12 (22-23K)	○	○	○	○	○	○	○		●	●	●	●	○			○	●	●	●	○			17 (85)
13 (18-19K)		○	○	○	○	○				●	●	●	○	●	●		●	●		○			14 (70)
14 (15-16K)		○		○	○	○				●	●	●				●	●	●	●	○			12 (60)
15 (13K)		○				○				●	●	●	○	●	●		●	●	●	○			12 (60)
16 (10K)		○								○				●			●	●					5 (25)
Total	7	11	7	9	9	11	7	6	9	13	11	10	8	10	8	9	13	11	10	8	0	0	

● Developed within 5 days K: Kilodalton
○ Developed within 14 days

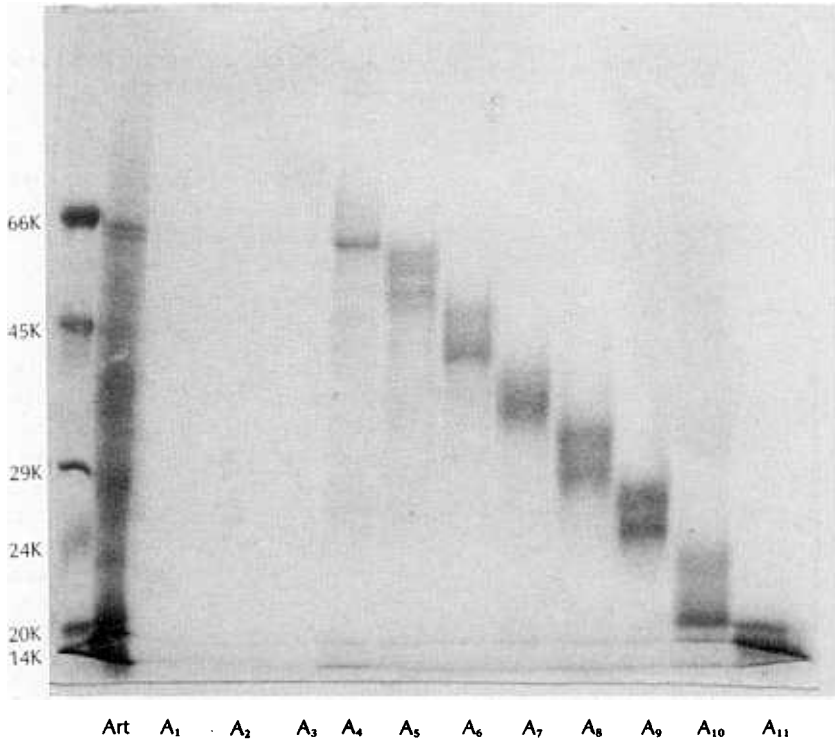

Fig. 4. Separation of allergenic fractions by 10% SDS-PAGE in 11 allergenic groups. Molecular weight markers; bovine serum albumin (66,000), egg albumin (45,000), 3-phospho glyceraldehyde dehydro genase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), L-lactoalbumin (14,200).

Table 2. Molecular weights of allergens determined by 10% SDS-PAGE in 11 allergenic groups

Group		Molecular weight of each fraction		
AG	AG 2	None		
AG		72,000		
AG		59,000		
AG		58,000	54,000	51,000
AG		48,000	46,000	43,000
AG		42,000	40,000	37,000*
AG		34,000	33,000	
AG		29,000	27,000	
AG		23,000*		
AG		22,000	19,000	17,000

*: Fractions containing highest IgE binding components

RAST inhibition by allergenic group

To compare the allergenic potency, various concentrations of proteins from 11 AGs were used as inhibiting antigens. As shown in Fig. 5, the addition of increasing amounts of protein produced an increasing inhibition of the binding of specific IgE antibodies to wormwood disc. Eleven AGs were different in their allergenic potency. The results of RAST inhibition expressed in a dose-related semilogarithmic graph revealed that AG 10, including the allergenic fraction number 12 (23,000 daltons), was found to be the most potent inhibitor, requiring 2.4ug of protein to produce 50% inhibition of specific IgE binding to wormwood paper-disc. The other group, AG 7 including fraction number 9 (37,000 daltons), required 2.6ug of protein, and AG 9, 3.0ug of protein, in order. However, WPE of Art. princeps required only 0.32ug of protein.

Skin prick test using allergenic groups

Table 4 shows that there was considerable variation in the percent ratio of wheal size from one patient to another. These wide discrepancies in wheal size ratio made it impossible to establish any correlation with the 50% RAST inhibition data. There were

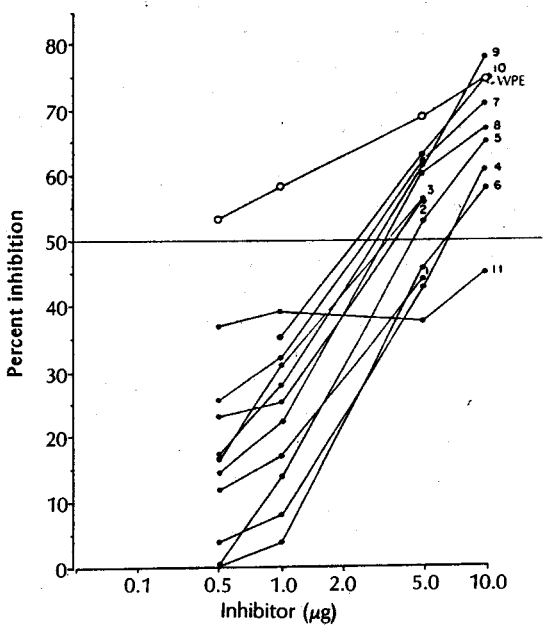

Fig. 5. Percent inhibition of wormwood-RAST following serial addition of 11 different antigens (●; A_1-A_{11}) and whole pollen extracts (0).

six groups (AG 6, AG 7, AG 8, AG 9, AG 10, AG 11) which showed significant responses (more than 100 in percent ratio) in at least half of the atopic patients tested.

Table 3. Protein content that inhibits 50% of specific IgE binding on W5-paper disc

Group	Protein content (ug)
AG 1	–
AG 2	3.4
AG 3	3.6
AG 4	6.6
AG 5	4.5
AG 6	6.2
AG 7*	2.6
AG 8	3.2
AG 9	3.0
AG 10*	2.4
AG 11	–

* : Fractions containing highest IgE binding components
–: No data

Table 4. Results of skin prick tests using each group of proteins separated by SDS-PAGE in Artemisia-sensitive patients

Allergenic group Patients	Percent ratio of wheal size to histmaine											Korean Artemisia pollen extracts
	AG 1	AG 2	AG 3	AG 4	AG 5	AG 6	AG 7	AG 8	AG 9	AG 10	AG 11	
A	–	43	–	79	86	100	107	121	229	221	121	250
B	44	50	44	44	50	111	78	83	106	117	56	94
C	107	164	179	214	ND	214	179	271	236	214	300	164
D	47	47	65	59	ND	159	206	282	229	271	112	24
E	50	50	50	81	ND	144	69	63	150	188	125	131
F	75	73	63	–	ND	50	63	50	69	63	125	81
G	50	50	50	50	ND	63	69	88	131	75	50	50
H	89	56	44	–	ND	83	100	150	217	300	56	50
I	141	–	124	–	ND	305	259	188	329	336	82	200
J	–	–	–	–	ND	–	–	–	–	47	47	47
K*	–	–	–	–	ND	–	–	33	50	67	–	–
L*	–	–	–	–	ND	–	–	–	–	–	–	–
M*	–	–	–	–	ND	–	–	–	32	39	31	–
N*	–	–	–	ND	ND	–	–	–	–	50	–	–

ND : Not done
A-J : Artemisia-sensitive patients
K-N*: Negative responders on allergy skin test
AG : Allergenic group

DISCUSSION

In Korea, there are two discrete pollen seasons, the tree pollen season from March through May, and the weed pollen season from the end of August through September. During the latter season, the pollen of Artemisia with those of Humulus japonicus and Ambrosia are abundant in the air of Seoul (Hong *et al.* 1986). The results of skin prick tests with Korean pollen extracts, commonly found in the air of Seoul, revealed that the incidence of sensitization to Artemisia spp. pollen was 14.2%, and sensitization to ragweed was 10.4% (Kim *et al.* 1987). Park *et al.* (1989) reported 26 cases of atopic bronchial asthma induced by Korean Artemisia spp. pollen extracts. Although 26 species of Artemisia have been found in Korea, Artemisia princeps is the most prevalent species all over the country*. Therefore, this Artemisia spp. pollen is considered to be one of the main respiratory allergens during the late summer-autumn season in this country.

In this study, western blot analysis of Art. princeps pollen proteins for the identification of allergens has successfully been accomplished, as other investigators who have done with other pollens (Ford *et al.* 1986; Ford and Baldo 1987) and house dust mites (Tovey and Baldo 1985). Of the protein bands separated by 10% SDS-PAGE and transferred electrophoretically to nitrocellulose, 16 (in the molecular weight range 10,000 to 85,000 daltons) fractions have bound IgE antibodies from the individual serum of 20 Art.-sensitive patients. This number is greater than that observed with other pollens (Ford *et al.* 1985; Ford *et al.* 1986) studied so far with the electroblotting technique. Further studies are needed to clarify that each band represents a separate allergen, when in fact there may be isoallergens or electrophoretically distinct forms of the same allergen, as are known to exist for Amb a I (antigen E of short ragweed pollen, Paull *et al.* 1979).

Classification of some of the mugwort antigens as allergens was established by means of CRIE. Ipsen *et al.* (1985) identified that Ags 20 may be regarded as only one major allergen among 10 allergens and four (Ags 4, 21, 37, 38) can be regarded as intermediate allergens. Paulsen *et al.* (1985) reported that 18 anodic antigens were found in a crude extract of mugwort pollen, of which 7 were considered to be allergens by CRIE. Nilsen and Paulsen (1986) also revealed by CRIE that Ag 7 (pI value; 4.3) can be regarded as an important allergen in mugwort pollen. In this study, two (numbers 9 <37,000 daltons> and 12 <23,000 daltons>) fractions revealed by far the highest frequency of IgE bindings, accounting for 17 (85%) of 20 sera tested. It seems that there are many allergenic components in each kind of allergen extracts, and different patients may develop different IgE responses to allergenic components (Fort *et al.* 1986; Ford and Baldo 1987; Shen *et al.* 1988). Although sera from 85% of the patients showed IgE binding to the number 9 and 12 fractions on autoradiography, all patients exhibited individual differences in the allergen-recognition spectra. Of the 20 sera, no two sera showed the same patterens of IgE binding. This feature would have to be taken into account in understanding the pathophysiologic mechanisms of individual differences in allergen sensitization.

The WPE of Art. vulgaris were found to contain a substantial quantity of yellow-brown pigments that were too difficult to remove by treatment with various organic solvents (Paulsen *et al.* 1985). It was considered to be important to precipitate the WPE of Art. princeps, as in the case of Art. scoparia (Jaggi and Gangal 1987), with ammonium sulfate before loading on the DEAE sephadex A_{50} column. This was probably due to the high amount of pigmented material in the extracts that might have disturbed the separation of proteins by binding strongly to the gel matrix. Recently, Paulsen *et al.* (1985) fractionated Art. vulgaris pollen extracts on a DEAE Sephadex A_{25} column, and were able to detect the presence of at least three allergenic proteins in fractions eluted with the starting buffers, 0.5M of NaCl and 1M of NaCl, respectively. The fractions probably corresponded to Art. V and VI proteins: the most potent allergenic fractions eluted from DEAE Sephadex A_{50} column in Jaggi's study (1987). In this study, the chromatography of ammonium sulfate (70% saturation) precipitated proteins on DEAE Sephadex A_{50} and elution with 1.0M NaCl in the buffer decreased the pigmented material to some extent, and improved the resoluton on 10% SDS-PAGE.

Meier-Davis *et al.* (1988) recently purified an Alternaria allergen with a molecular weight of 66,000 daltons which is considered to be an important allergen, from the extracts by electroelution. In this study, we used the gel elution method, which is a single step separation of Art. allergens from crude pollen extracts that might be useful for the separation of antigens to raise polyclonal or monoclonal antibodies. This method in turn could be used to investigate the possibility of shared allergen among various species of Art. or to standardize Art. extracts,

* Prof Lee Y.N.; Oral communication

although the obtained proteins have less biologic activity.

The potency of allergenic extracts can be determined by means of skin tests (intradermal and prick), histamine release and RAST inhibition (Lowenstein *et al.* 1987). These responses are dependent on the patient panel used. From this study, 2.4ug of AG 10 protein was required for 50% wormwood RAST inhibition and 2.6ug of AG 7, whereas only 0.32ug (an approximation) of WPE protein produced 50% inhibition. It seems that AG 10 demonstrated to be the most potent, and AG 7, was next. As in the case of ragweed (Gleich *et al.* 1974) and ryegrass (Smart and Knox 1980), purified allergenic components had less biologic activity as compared to the WPEs. The lower biologic activity of the purified fraction, as observed in the RAST-inhibition activity, could be due to the partial denaturation of the protein during extractions or due to the removal of specificity on purification (Cotton *et al.* 1986). We used the wormwood disc as a solid phase in the RAST inhibition test. Lin *et al.* (1989) suggested that there were very strong cross-allergenicities among most Art. species, including wormwood and Art. vulgaris. The skin prick test showed variable responses and no obvious relationships between the wheal size and the RAST inhibition results. Although the obtained results will vary with the patient panel, significant responses (more that 100% of the wheal size ratio) were found in six groups (AG 6–AG 11).

In summary, western blot analysis with SDS-PAGE has revealed a spectrum of Art. princeps pollen IgE-binding components that is greater in number than that which has been previously described. In order to obtain sufficient information on the nature of the allergens from Art. princeps pollen, isolation and biochemical characterization of the major components should be performed, and in addition, comparison of the electroblotting results obtained under both dissociating and non-dissociating gel conditions will reveal the possible modification of allergenic proteins that may occur in vitro.

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