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Intraspecific variations in pollen extracts of *Ricinus communis* (castor bean) prepared from different source materials

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Pollen of *Ricinus communis*, an important aeroallergen was collected from different stages of development of flower, different time intervals, different years and different places in India, to see variability, if any, in the protein content and protein profiles of the extracts. The protein content showed significant variation in the extracts from different sources. IEF and SDS - PAGE protein profiles from pollen antigens of mature buds and flowers showed almost similar pattern. However, appreciable variation was recorded in the protein pattern of pollen extracts from different years and places. It is therefore suggested to have a comprehensive quality control of pollen raw materials before processing for antigenic extracts.

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Of the various bioparticles present in the atmosphere, pollen are offending allergens. Efforts are being made globally for standardization of pollen extracts so that every time the antigen prepared from the pollen of the same species should have the same antigenic and allergenic determinants for effective diagnosis and immunotherapy of respiratory allergy patients (Helm et al. 1984; Gjesing et al. 1985; Stewart et al. 1988). The attention given to the final product of extraction of an allergenic substance often eclipses the importance of quality of the source material. Thus in the overall frame work of allergen standardization, the investigation of pollen raw materials before production of allergenic extract is of paramount importance (Anderson 1985). The quality of the raw material determines the quality of the final extract. Therefore any deficiency in the raw material will lead to the production of poor allergenic extract. Control measures employed for collection of pollen source material include pollen authenticity, method of collection, processing and storage conditions prior to extraction. Aimed at standardization of pollen raw materials, we selected *Ricinus communis* (castor bean), an important pollen allergen of India (Shivpuri & Dua 1963; Singh et al. 1973), to study the protein content and heterogeneity in the protein profiles of the extracts of pollen collected from: (a) different stages of flowering, (b) different time intervals during the same flowering season, (c) different regions of India, and (d) stored for different years after collection.

MATERIALS AND METHODS

Pollen Collection

Polliniferous materials were collected from *Ricinus communis* at regular intervals of about two weeks from wildly growing communities on waste places in Delhi during 1987–1988 season. On each visit polliniferous material from unopened mature buds and fully bloomed flowers were collected. A total of 24 samples, on 12 visits were collected from buds and flowers during the pollination season which starts in September and ends in March/April. After drying, pollen were procured by crush head method and by passing through different grades of sieves. Pollen were also collected from three different stages of flowering on the same day i.e., immature buds (< 4 mm dia.), mature buds (> 4 mm dia.) and blooming flowers. Pollen collected for routine preparation of allergenic extracts in our laboratory during the pollen season of 1984 to 1987, from Delhi region and stored at 4°C were used for studying variations in pollen stored for different years. Pollen from six other places of India were also collected in 1988 from flowers. The places in addition to Delhi were Bangalore, Bhopal, Nagpur, Tiruchchirappalli, Trivandrum and Visakhapatnam.

Microscopic examination

Microscopical analysis of all the pollen raw materials procured from different sources were carried out according to the method of Cour & Loublier (1980). Pollen samples were examined for the pollen, its plant parts, pollen from other species, dust particles and fungal spores. Pollen from buds and flowers, collected at different time intervals, were > 96% pure, whereas pollen collected from different places and years had pollen purity varying between 88 and 95%.

Preparation of antigenic extracts

Pollen collected were defatted with solvent ether and then dried in a vacuum desiccator. Antigens were extracted in 0.05 M phosphate

Table I. *Protein content of extracts (10%, W/V) of Ricinus communis prepared from pollen of buds and flowers collected at different time intervals.* *Pollen samples not available.

Date of collection	Protein mg ml^{-1}	
	Buds	Flowers
02.10.87	4.9	4.2
12.10.87	3.3	*
25.10.87	3.7	4.5
13.11.87	3.9	4.0
26.11.87	3.4	4.5
08.12.87	4.5	5.0
24.12.87	4.7	5.7
11.01.88	6.2	6.8
23.01.88	5.6	7.5
30.01.88	8.0	4.0
09.02.88	9.1	5.5
20.02.88	8.2	9.8
24.03.88	*	9.1
Mean (CV,%)	5.5 (35)	5.9 (32)

buffer (pH 7.8)/containing 0.5% saline by continuous stirring at 4°C for 20 hrs in 10% concentration (w/v). Following extraction, samples were dialysed against distilled water using Visking dialysis tubing with a nominal cut off point of 3000–3500 daltons. After dialysis extracts were lyophilised and stored at -70°C till further use.

Estimation of protein

Protein concentration was estimated, after precipitating the proteins with phosphotungstic acid, by the modified Lowrys method (Lowry et al. 1951). A calibrated solution of bovine serum albumin was used as standard. The mean protein concentration and coefficient of variation (%) were calculated to find the extent of variation among the samples.

Isoelectric focussing (IEF)

IEF was performed on Ampholine PAGE plates (Pharmacia LKB Biotechnology, Sweden, pH 3.5 – 9.5.) according to the manufacturers manual. 10 ul of extract containing 100 ug of protein was loaded. pI markers obtained from Pharmacia were used to determine the pI points of different proteins. The gel was stained with Coomassie Brilliant Blue R 250.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A polyacrylamide gel (10%) containing 0.1% SDS was used. 20 ul of samples containing 80 ug of protein were heated with equal amount of sample buffer (2% SDS, 10% glycerol, 5%, Betamercaptoethanol, 0.01 M Tris pH 8.0, 0.001 M EDTA, 0.1% bromophenol dye) at 100°C for 2 min. and applied to each well. The gel was calibrated with a marker protein obtained from Sigma, Chemical, Co. U.S.A. The gel was stained with Coomassie Brilliant Blue R 250.

Table II. *Protein content of extracts of Ricinus communis pollen collected from different stages of inflorescence.*

Stages of inflorescence	Protein mg ml^{-1}
Buds (<4mm)	1.2
Buds (>4mm)	3.3
Flowers (anthesised)	5.8
Mean (CV,%)	3.4 (55)

RESULTS

Protein concentration

In the extracts from pollen collected at different time intervals from buds, protein concentration varied from 3.3 – 9.1 mg ml^{-1} and in flowers from 4.2 – 9.8 mg ml^{-1}. The mean values were 5.5 mg ml^{-1} ± 35% CV and 5.9 mg ml^{-1} ± 32% CV for buds and flowers respectively (Table I).

Protein contents of the extracts from pollen collected on the same day (12.01.89) from immature buds (< 4 mm dia.), mature buds (> 4 mm dia.) and flowers were found to be 1.2, 3.3, and 5.8 mg ml^{-1} respectively (Table II). The mean value was 3.4 mg ml^{-1} ± 55% CV.

Protein contents from extracts of the pollen samples stored for different years and collected from different places of India are given in Table III. Extracts prepared from pollen stored from 1984 to 1989 pollen seasons showed variation from 1.1 to 5.8 mg ml^{-1} (mean 3.2 ± 57% CV). Antigenic extracts from pollen of different places during the same season, showed variation from 1.3 to 5.8 mg ml^{-1} (mean 3.9 ± 35% CV).

IEF patterns

IEF patterns of the 24 extracts prepared from pollen collected at different time intervals from buds/flowers during the same flowering season did not exhibit marked variation as can be seen from samples 1–19 electrofocussed (Fig. 1A, B). Major bands are detected in the pH range of 4.0–5.5.

Table III. *Protein content of extracts of Ricinus communis.* A. stored for different years (Delhi). B. collected from different places of India, in 1988.

Years	A. Protein mg ml^{-1}	B. Places	Protein mg ml^{-1}
1984	1.1	Bangalore	4.4
1985	1.6	Bhopal	3.7
1986	2.8	Delhi	5.8
1987	2.3	Nagpur	2.7
1988	5.5	Tiruchchirappalli	4.5
1989	5.8	Trivandrum	1.3
		Visakhapatnam	4.8
Mean (CV,%)	3.2 (57)	Mean (CV,%)	3.9 (35)

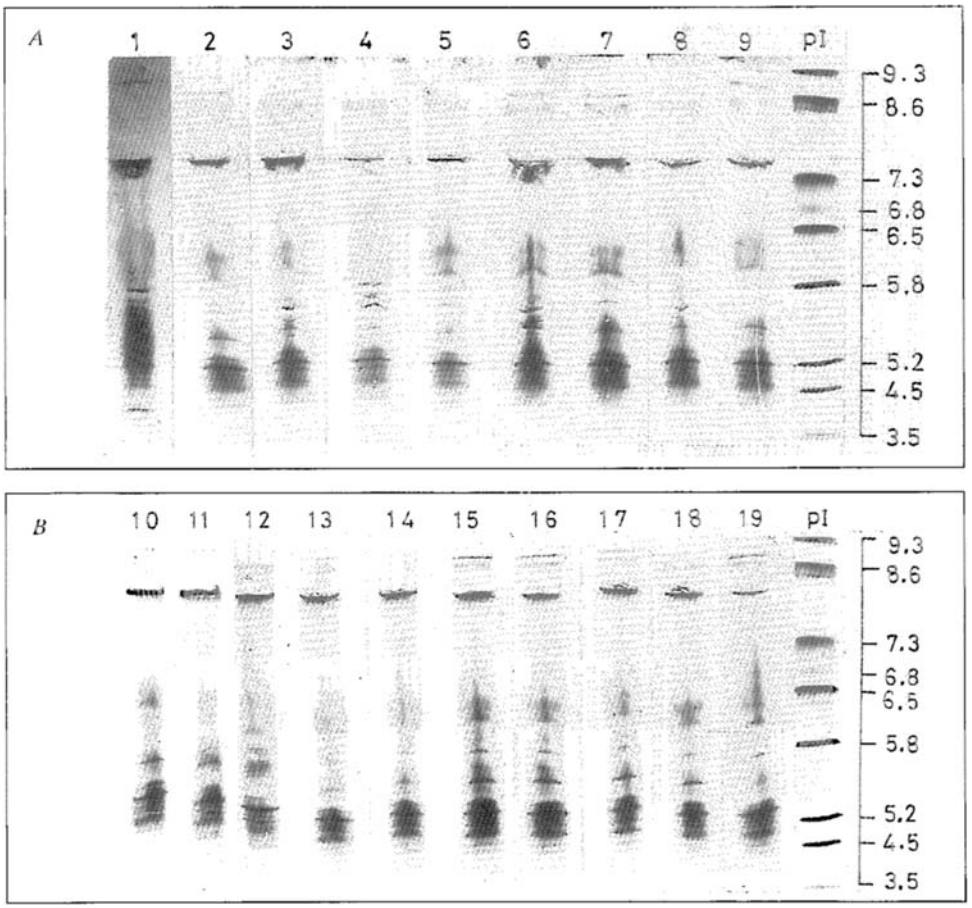

Fig. 1. Isoelectric focussing patterns of extracts of pollen collected from unopened buds and bloomed flowers at different time intervals during the same flowering season.
pI = Marker proteins with their pI points.
A. Samples 1, 3, 4, 6, 8 from buds and rest from flowers.
B. Samples 10, 12, 14, 16, 18, from buds and rest from flowers.

Three bands are present in alkaline region (pH 8.4 – 8.8). The banding pattern is almost similar except for one band around pH 6.8 which starts appearing in the extracts prepared from pollen collected from the month of February onwards. When the extracts from immature, mature buds and flowers collected on the same day were analysed for IEF patterns, it was found that extract from pollen of immature buds had fewer protein bands (Fig. 2). Not much difference was seen in the extracts of mature buds and flowers.

The IEF protein pattern obtained from pollen extracts of different years and places can be seen from Fig. 3. The bands of acidic region (pH 4.0 – 5.5) are generally present in all the extracts prepared from pollen stored from 1984 onwards. In the extracts of 1988 and 1989 bands are present through out the length of the gel. In the extracts from pollen of 1984 to 1987 bands of basic region are absent. Significant variation is seen in the antigenic composition of extracts prepared from pollen collected from different places. The bands of acidic region are present in all the samples but these are not sharp in the extracts of Bangalore and Trivandrum. A band at pH 8.6 is present in the extract of Nagpur, Tiruchchirappalli and Visakhapatnam. Protein bands of the basic region are present in the extracts of Bhopal and Delhi only.

SDS-PAGE

The protein profiles of the extracts of pollen from different time intervals are shown in Fig. 4 and 5. The bands are present in molecular weight range of 14,000 to 70,000. Two bands of 66,000 d and 70,000 d are major protein bands present in all the samples. Bands of low molecular weight 20,000 – 29,000 d are also present in all the samples but they become more sharp and distinct from December onwards. No difference was seen in the banding pattern of extracts prepared from pollen collected from mature buds and flowers except that in extract of flowers a protein band around 20,000 d is more prominent. In immature buds very few bands are present although they are in the molecular weight range of 14,000 – 70,000 d but they are very faint.

SDS-PAGE pattern obtained from pollen extracts of different years are given in Fig. 6. The samples from 1988 and 1989 showed maximum number of bands ranging from 14,000 to 70,000 d. In samples from 1984 to 1987 only four to five bands are present. SDS PAGE pattern of samples obtained from different places (Fig. 7) showed significant variations. Pollen sample from Delhi showed higher number of bands. In Bangalore, Nagpur, Tiruchchirappalli and Visakhapatnam banding pattern is almost similar except for the absence of 70,000 d molecular weight protein in Banga-

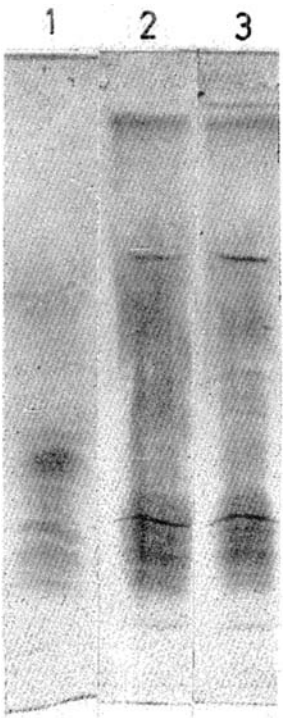

Fig. 2. IEF patterns of extracts of pollen collected from three different stages of inflorescence on the same day.
1. Immature buds (< 4 mm dia.) 2. Mature buds (> 4 mm dia.)
3. Bloomed flowers.

lore and Nagpur samples and two protein bands around 15,000 d in Bangalore samples. In extract of Bhopal bands of low molecular weights are not distinct.

DISCUSSION

Castor bean plants are grown commercially in Brazil, China, Thailand and France for their unsaturated oil which has wide industrial applications (Rubsamen-Waigmann et al. 1985). In India, it is extensively cultivated in Andhra Pradesh, Uttar Pradesh, Bihar and Karnataka (Kulkarni & Ramanamurthy 1977). However, it also grows as weed along road side and waste places in different regions of the country. High concentration of its pollen in the atmosphere, high pollen productivity (c. 400 pollen/anther) in addition to high skin reactivity in respiratory allergy patients suggest that it is an important aeroallergen (Shivpuri 1980; Singh & Babu 1982; Singh 1984). The allergenicity of *Ricinus* pollen have been proved using intradermal skin, Prausnitz Kustner (PK) and bronchial provocation tests (Singh et al. 1973). The study is primarily aimed at standardization of pollen raw material of *Ricinus communis* for preparation of allergenic extracts for diagnosis and immunotherapy.

Protein concentration showed variation in the antigenic extracts of pollen collected at different time intervals during the same flowering season in buds (CV = 35%) and flowers (CV = 32%). In both buds, and flowers, maximum protein content was in the extracts prepared from pollen collected in the month of February and least in the month of November. It is interesting to note that protein concentration of the extract from pollen of immature buds was very low as compared to mature buds and flowers showing high coefficient of variation (54%). Some of the pollen from immature buds were in the tetrad stage of development. Changes in the antigenic composition of short ragweed has been demonstrated by Larson & Gleich (1975). They have reported that AgE is present in very small amounts prior to pollination season, increased with anther and pollen grain maturation and decline gradually with the flowering season. Our results showed similar protein pattern which increased gradually with the flowering season but a trend of decrease was conspicuously absent.

Protein concentration of the extract from pollen stored from different years (1–5 yrs.) showed high variations (CV = 57%). The quality of pollen from different years is also reported to be variable (Anderson 1985; Maasch et al. 1987). Variable patterns in protein concentrations has also been observed from antigens of pollen samples procured from different populations in India (CV 35%). The variation in the protein contents in the extracts from pollen of different years is more than from different places. This could be due to climatic factors, variable soil composition,

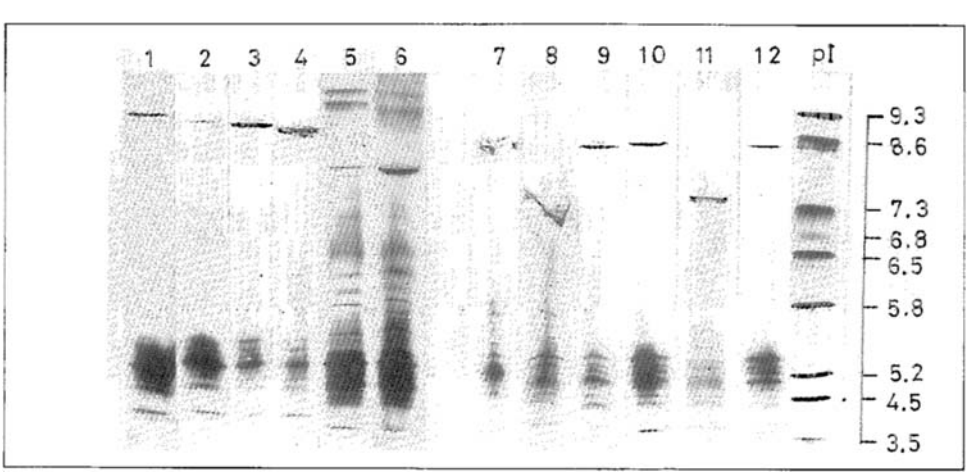

Fig. 3. IEF patterns of extracts prepared from pollen stored for different years collected from Delhi and from different places of India.
1–6. 1984 to 1989 (Delhi)
7. Bangalore 8. Bhopal 9. Nagpur 10. Tiruchchirapalli 11. Trivandrum 12. Visakhapatnam
pI. Marker proteins with their pI points.

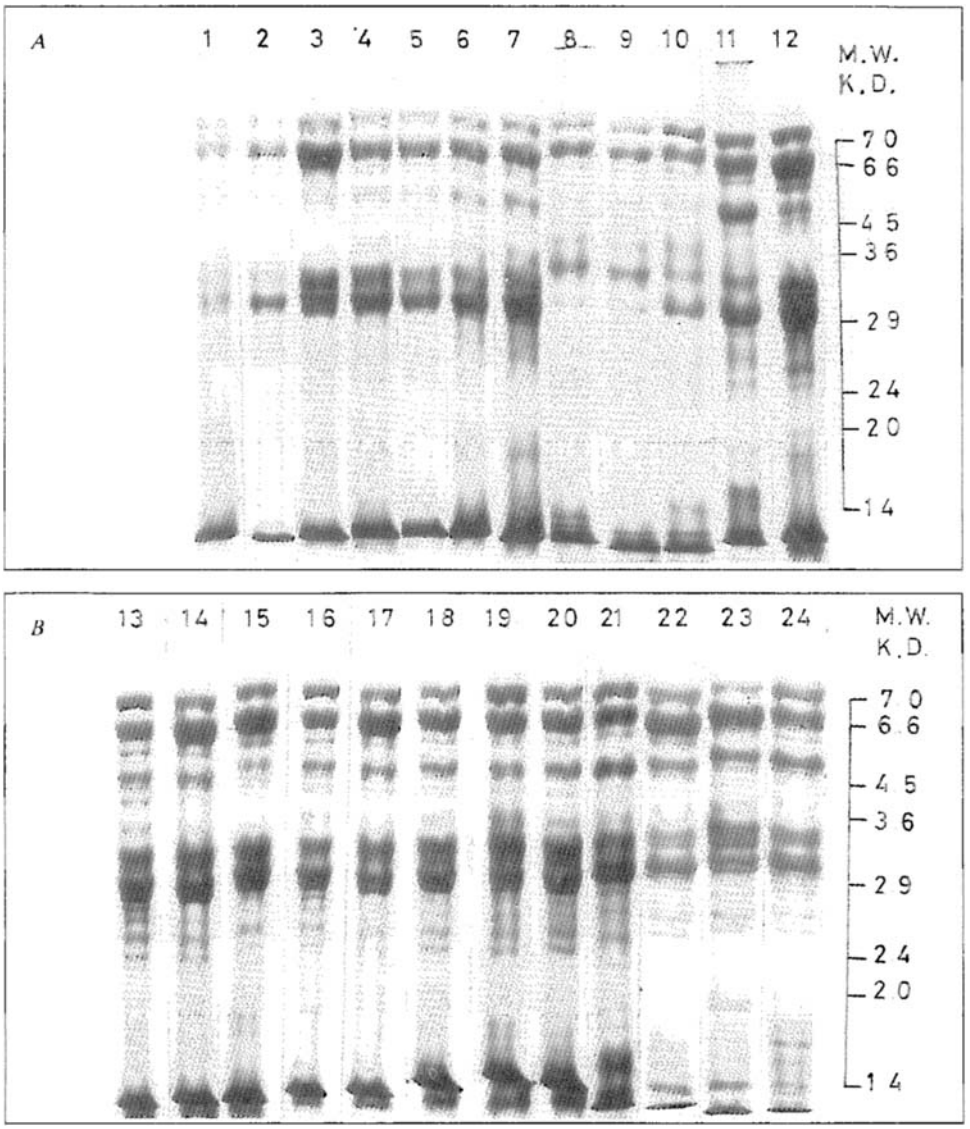

Fig. 4. SDS-PAGE patterns of pollen extracts collected at different time intervals from buds and flowers.
A. Samples 1, 3, 4, 6, 8, 10, 12, from buds and rest from flowers.
B. Samples 14, 16, 18, 20, 22 from buds and rest from flowers.

collection process, time of collection and storage conditions.

The IEF pattern of the antigens prepared from pollen of mature buds and flowers at different time intervals were in good agreement with each other showing only slight differences in staining intensities of some of the bands towards the latter part of flowering season. One band around pH 6.8 starts appearing in the extracts prepared from pollen collected in the month of February onwards. Although protein bands are present through out the length of the gel but higher number of bands were obtained between pH range 4.0 – 5.5. Three bands were also present in the alkaline region (pH 8.4 – 8.8). However, pollen samples from immature buds showed fewer number of bands which were not sharp and no band was seen in the alkaline region. This is significant in view of the bulk collection of pollen from the nature which includes inflorescence of buds and flowers and thus affect the quality of the pollen extract. It is, therefore, suggested that small buds should be discarded while collecting pollen for antigenic preparation for diagnosis and treatment.

IEF pattern of the pollen samples of different years from the same geographical region, and from places in South and Central India showed variations. Most of the protein bands in acidic region were present in all the samples. In the stored samples from different years (1984–1987) the absence of certain bands and low intensity was recorded. As the protein profile of the stored samples at the time of their collection is not available, it is difficult to attribute the variability due to storage effect or the absence of the bands from the beginning. The variability among pollen samples from different places could be due to geographical effect with extremely variable climatic conditions. Geographical variation in the antigenic components of *Olea* pollen samples has also been reported from Spain by Barber et al. (1990).

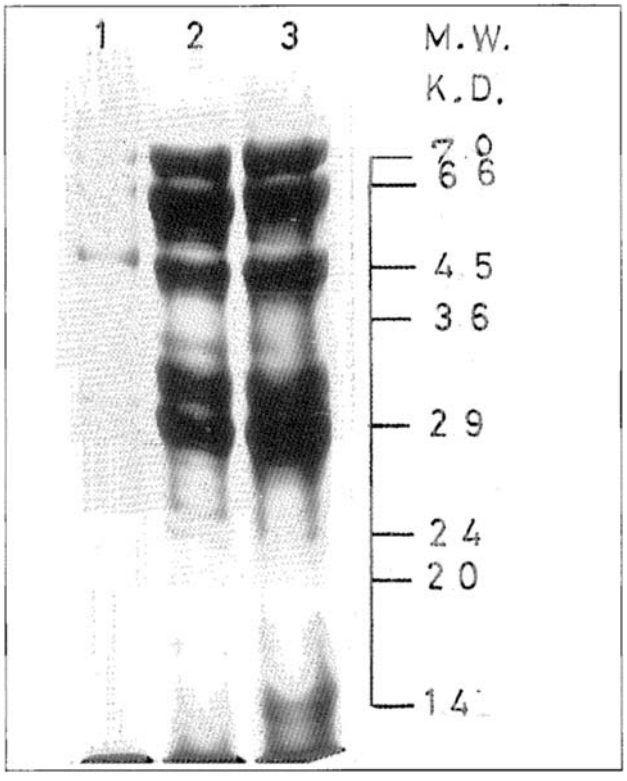

Fig. 5. SDS-PAGE pattern of extracts of pollen collected on the same day from different stages of inflorescence.
1. Immature buds (< 4 mm dia.) 2. Mature buds (> 4 mm dia.) 3. Bloomed flowers.

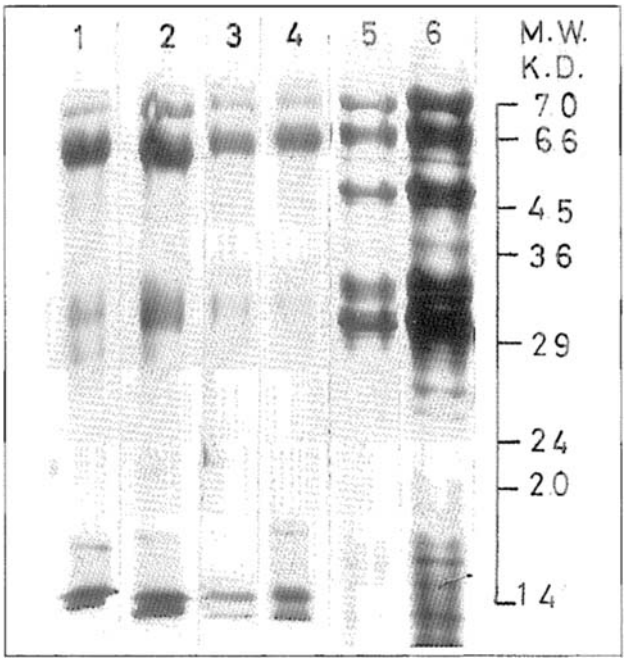

Fig. 6. SDS-PAGE pattern of extract prepared from pollen stored for different years.
1–6. 1984 to 1989.

Protein profiles of various samples from buds and flowers collected at different time intervals, when subjected to SDS-PAGE showed almost the same pattern as that in IEF. Samples from different years and places confirmed remarkable variations as that in IEF.

Large variations in the antigenic components of ragweed pollen from different suppliers have also been reported by Maasch et al. (1987). However, similar variations with respect to *Phleum pratense* was not observed (Maasch et al. 1986). We observed variations in the pattern of IgE binding proteins in the pollen extracts of different source materials, with the sera of hypersensitive patients (unpublished).

On the basis of the observations, it is concluded that there exists an intraspecific variations in the protein components of the pollen procured through different sources. For antigenic preparation, time of collection, maturation of inflorescence, place of collection and period of storage should be taken into account to minimize variability in the antigenic/allergenic components.

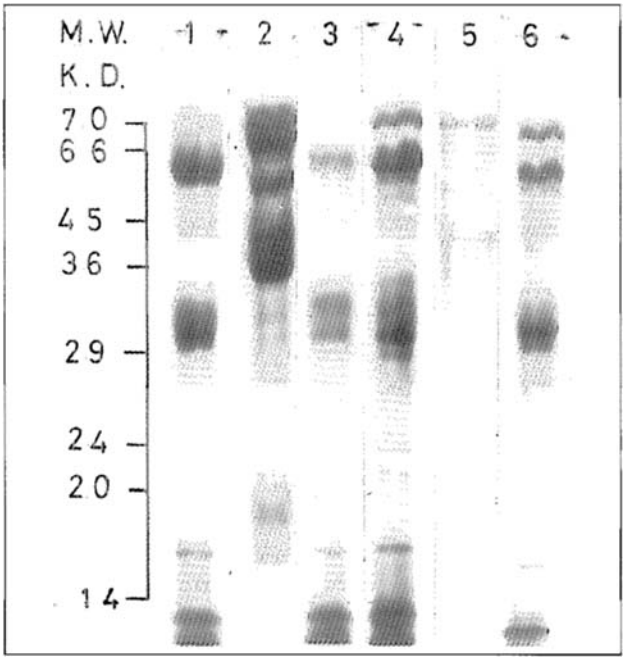

Fig. 7. SDS-PAGE Pattern of extracts prepared from pollen collected from different places of India.
1. Bangalore 2. Bhopal 3. Nagpur 4. Tiruchchirappalli 5. Trivandrum 6. Visakhapatnam.

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