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A Study on Antigenic and Allergenic Changes During Storage in Three Different Biological Extracts

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SUMMARY **The stability of three allergens common in tropical countries was evaluated under different storage conditions.** *Prosopis juliflora* **(PJ),** *Rhizopus nigricans* **(RN), and wheat dust (WD), were taken as representatives of various groups of allergens viz, pollen, fungi and dust. The extracts were stored in buffer containing phenol (0.4%) or glycerol (50%) at temperatures ranging from 4-55° C for 15 to 60 days. Protein content of PJ extract was reduced remarkably when it was stored at 40° C for 45 days. Thin layer isoelectric focusing and rocket immunoelectrophoresis of PJ showed that certain antigenic proteins degrade rapidly even at 25° C as early as day 15. However, two to three proteins of PJ remain stable at a higher temperature (40° C) for two months. Relative radioallergosorbent test (RAST) inhibition showed substantial loss of allergenic activity in all the three extracts, when stored at higher temperatures (25-55°C) even for short durations, i.e., 15 days. Extracts (PJ and RN) containing 50% glycerol were found to be stable, retaining more than 50% activity, even when stored at 55° C for 40 days, while extracts without glycerol lost more than 75% of their allergenic activity. However, addition of glycerol did not change the stability of wheat dust allergenic extract. The present findings indicate that allergenic extracts behave differently when stored. Hence, the stability of each extract should be determined individually.**

Allergenic extracts are heterogenous mixture of biological substances and have been observed to degrade rapidly during storage.[1,2] Several changes occur in extracts on storage depending upon temperature, dilution, the diluent used and the type of allergen.[2-6] Studies on various antigenic extracts revealed that the major components responsible for allergenic activity are proteins and glycoproteins.[7] Some of the proteins present in the extract may be heat labile, whereas others may be quite stable at higher temperatures. Therefore, the changes that occur in extracts under storage are quite complex and need to be investigated in detail. The alteration in the quality of extracts may affect diagnosis as well as their therapeutic efficacy. Hence, the present study was undertaken to determine various changes in *Prosopis juliflora* (PJ), *Rhizopus nigricans* (RN) and wheat dust (WD) allergen extracts under different storage conditions.

MATERIALS AND METHODS

Preparation of extracts

Prosopis juliflora pollen was collected in bulk from wild plants in and around Delhi. A pollen sample of more than 95% purity was subjected to defatting with diethyl ether. Antigen extraction was carried out in ammonium bicarbonate buffer (0.05 M, pH 8.8) by continuous stirring for 18 hours at 4°C. *Rhizopus nigricans* was mass cultured on Sabouraud's broth at 28 ± 2° C for 15 days. The surface growth (myceliaspore mass) was dried, milled to a fine powder, defatted using solvent ether and extracted in ammonium bicarbonate buffer as described above.

Wheat dust was collected from different flour mills using a small vacuum cleaner. Different samples were pooled together and passed through a 60 mesh sieve to remove large woody particles. The wheat

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dust used was a mixture of fragments from the outer coat of the grains, particles resulting from breaking up of some grains, together with debris from associated organisms, such as insects and fungi. The method of preparation of antigen was the same as followed for PJ and RN materials.

The extracts thus obtained were passed through a Millipore filter (0.22 μm), dispensed in 2 ml sterile vials and lyophilized. The moisture content of the lyophilized materials was < 2% and they were stored at -20° C until used. The study was performed using antigenic extracts from the same batch prepared in a single lot.

Storage conditions

Extracts were rehydrated with phosphate buffered saline (0.1 M, pH 7.8) containing 0.4% phenol and/or 50% glycerol for two sets of experiments. Vials of freeze dried extracts were rehydrated (1:20 w/v) on days 0, 15, 30, 45 and stored at specified temperatures (Figs. 1, 2, 3). The vial reconstituted on day 0 was stored for 60 days; day 15 for 45 days; day 30 for 30 days and day 45 for 15 days. In this way, all the analyses were carried out simultaneously on day 60. Similarly, vials were rehydrated (1:50 w/v) on day 0, 25 and kept at different temperatures up to day 40, enabling storage for 40 and 15 days, respectively (Table 1). In all the experiments a freshly rehydrated sample was used as a control.

Protein estimation

The method of Lowry *et al*[8] with a slight modification was followed for protein estimation of extracts using phosphotungstic acid reagent (15% PTA in 10% HCl) to precipitate proteins from the samples. Bovine serum albumin (Sigma Chemical Co., USA) was taken as a standard for calibration.

Thin layer isoelectric focusing (TLIEF)

The extracts were focussed on commercially available polyacryla-

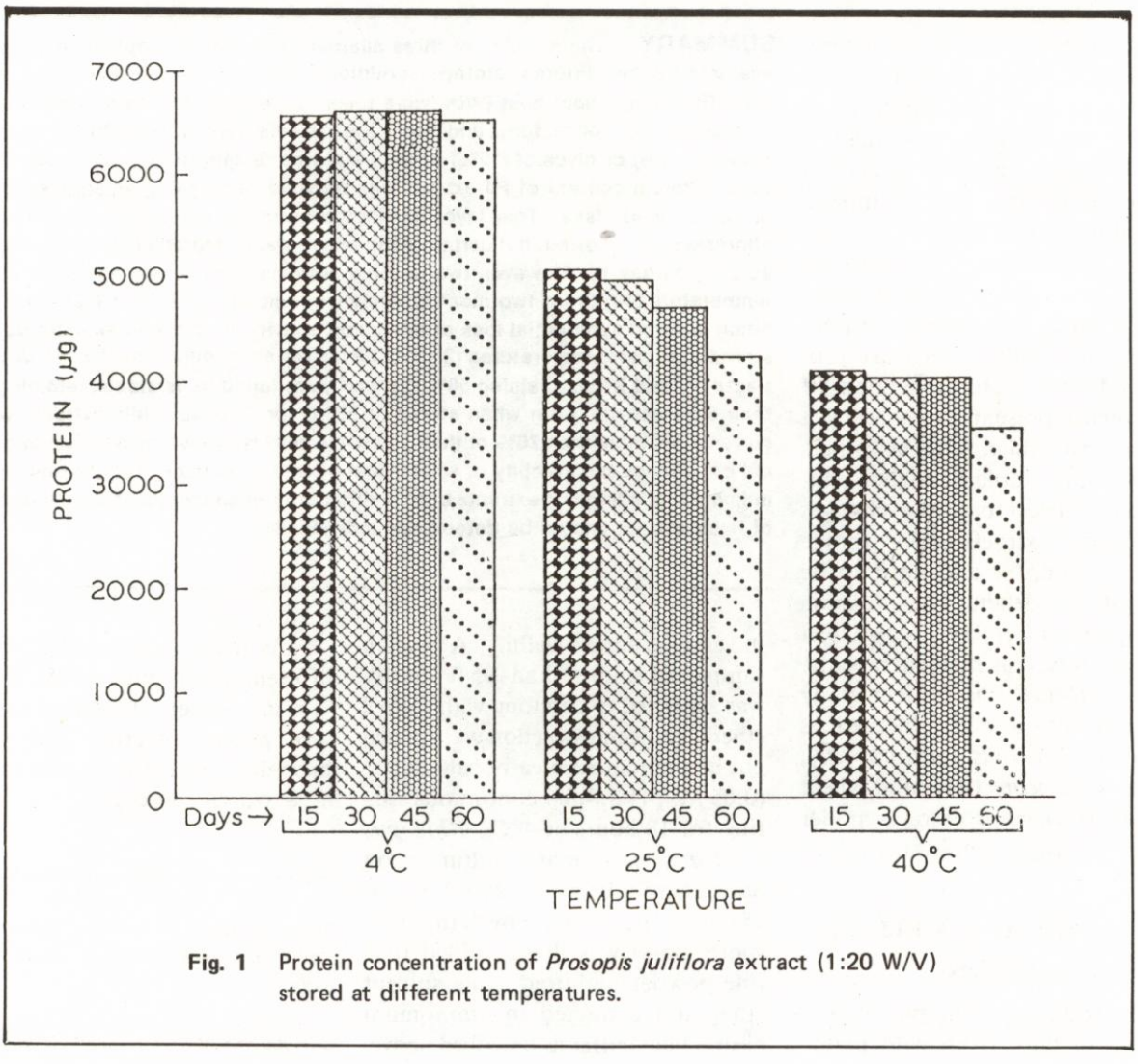

Fig. 1 Protein concentration of *Prosopis juliflora* extract (1:20 W/V) stored at different temperatures.

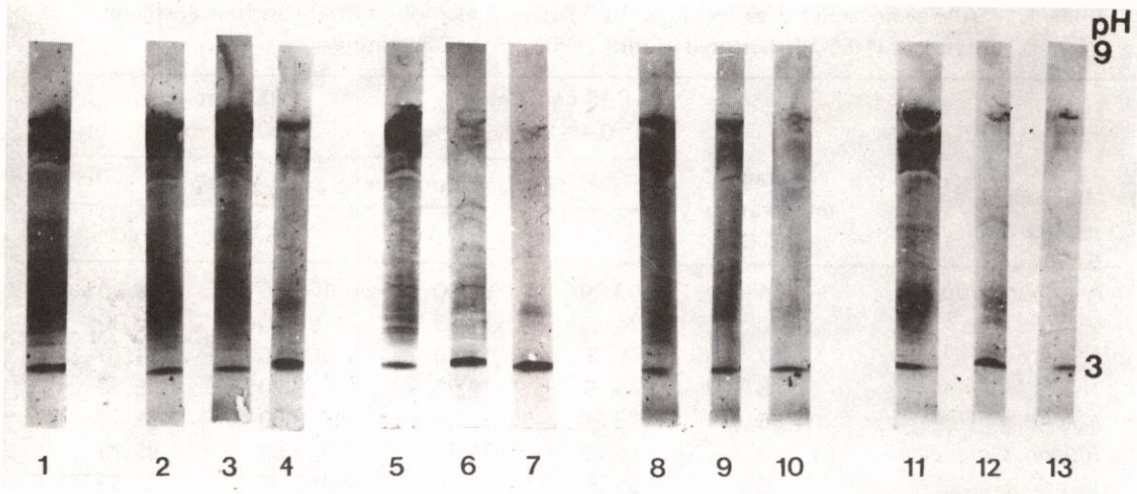

Fig. 2 TLIEF pattern of *Prosopis juliflora*—(Left to right) Lane 1: freshly reconstituted extract from lyophilized material; 2, 3, 4; extract stored for 15 days at 4°, 25° and 40°C; 5, 6, 7; extract stored for 30 days at 4°, 25° and 40°C; 8, 9, 10; extract stored for 45 days at 4°, 25° and 40°C; 11, 12, 13; extract stored for 60 days at 4°, 25° and 40°C. Anode is towards the bottom.

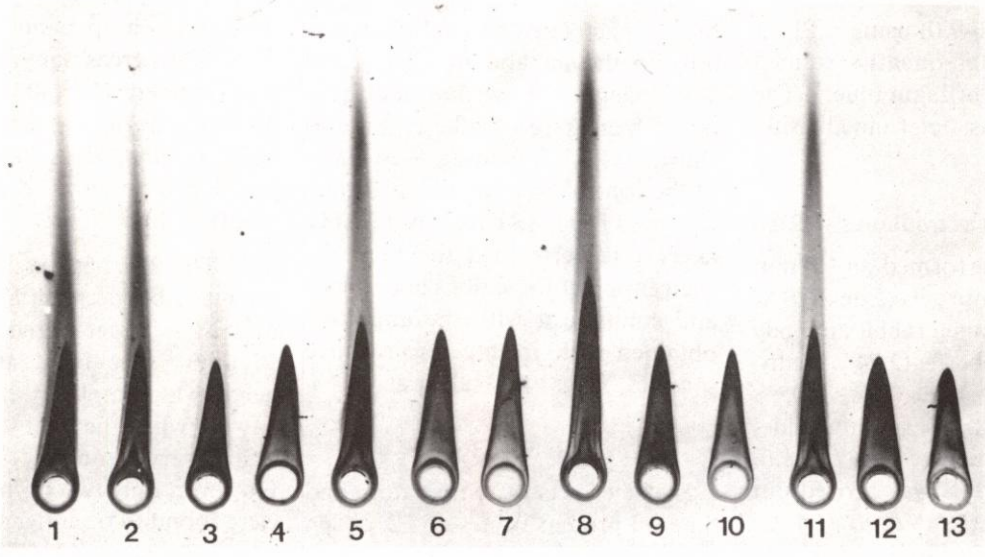

Fig. 3 RIE analysis of *Prosopis juliflora*—(Left to right) Lane 1: freshly reconstituted extract from lyophilized material; 2, 3, 4; extract stored for 15 days at 4°, 25° and 40°C; 5, 6, 7; extract stored for 30 days at 4°, 25° and 40°C; 8, 9, 10; extract stored for 45 days at 4°, 25° and 40°C; 11, 12, 13; extracts stored for 60 days at 4°, 25° and 40°C.

Table 1. Allergenic activity as measured by relative RAST inhibition of various allergenic extracts (1:50 W/V) stored in PBS at different temperatures.

Allergenic extract	Storage temperature in °C	PBS containing 0.4% phenol		PBS containing 50% glycerol	
		Duration in days			
		15	40	15	40
Prosopis juliflora	4	100.00	89.90	100.00	100.00
	30	74.20	19.03	98.00	100.00
	37	60.50	19.14	86.80	81.70
	45	44.80	19.70	79.70	65.60
	55	26.20	19.14	78.50	62.40
Rhizopus nigricans	4	93.50	89.70	100.00	92.15
	30	65.30	27.40	93.00	88.60
	37	49.80	27.20	85.00	74.90
	45	38.10	25.20	77.00	64.50
	55	25.00	24.00	80.00	54.80
Wheat dust	4	91.10	85.30	97.30	80.30
	30	80.90	70.10	86.90	70.10
	37	65.10	69.60	86.20	72.10
	45	59.90	64.10	79.40	72.20
	55	55.50	50.70	60.80	58.60

mide gel (pH 3.0-9.0) using a Phast system and subsequently stained with Coomassie brilliant blue.[9] The pH gradient was determined using standard pI markers.

Rocket immunoelectrophoresis (RIE)

RIE was performed on 1.8 mm thick (1%) agarose gels (Litex HSA, Denmark) containing rabbit antibody using Svendsen's buffer (2.24% diethyl barbituric acid, 4.4% Tris, 0.05% calcium lactate, 0.1% sodium azide, diluted 1:5 v/v before use) pH 8.6. The electrophoresis was carried out for 18-20 hours (1.5 V/cm) at 4° C. The gels were pressed dried and stained using Coommassie blue solution.

Radioallergosorbent test (RAST) inhibition

The extracts (PJ, RN and WD) were coupled separately to cyanogen bromide activated paper discs according to the method of Gleich and Yunginger.[10] A serum pool prepared from sera of allergy patients showing RAST binding > 10 times of the control (normal human serum) was used for RAST inhibition. The assay was performed to check the allergenicity of the various test samples and compare it with the inhibition obtained with freshly reconstituted samples, which was taken as 100%.

RESULTS

The effects of temperature and time on protein values of PJ pollen extracts are given in Fig. 1.

It is evident that the amount of protein decreased substantially, when antigen samples were stored at high temperatures for longer duration. When these samples were analysed by TLIEF, it was found that the samples stored at 4° C, even upto 60 days gave sharp bands between pI 3.5-7.0, whereas samples stored at high temperature (40° C) exhibited no bands, as early as day 15. Storage at 25° C for 45 days showed a significant deterioration in protein content (Fig. 2).

The RIE pattern demonstrated a remarkable decrease in antigenicity of the PJ extract stored at 25° C for 15 days. The band at maximum height was completely eliminated by day 60, while the light shadow band was observed on days 45 and 30. However, only two or three proteins were found to be antigenically stable when stored at 40° C for 60 days (Fig. 3).

The allergenic activity of PJ, RN and WD extracts as measured by relative RAST inhibition are presented in Table 1. The PJ extract lost more than 80% of its activity when stored in buffer containing

phenol at 30° C for 40 days. Almost 40% of the activity was lost within 15 days, when PJ was stored at 37° C. The glycerinated PJ extract was found to be comparatively more stable, retaining more than 60% of its activity, at 55°C for 40 days. There was hardly any loss of activity, when PJ pollen extract containing glycerol was preserved at 30° C for 40 days.

Rhizopus nigricans extract also demonstrated deterioration, when stored in buffer containing phenol at different temperatures. Almost 50% of its allergenic activity was lost within 15 days when stored at 37° C. Glycerinated extract was relatively more stable, retaining more than 50% of its activity when stored at 55° C for 40 days.

Wheat dust extract in buffered saline containing phenol retained more than 50% of its allergenic activity, even when stored at 55° C for 40 days and was not inferior to glycerinated extract (Table 1).

DISCUSSION

The changes that occur in allergenic extracts during storage affect both the results of diagnosis by skin testing and the administration of immunotherapy.[11] India, being a tropical country, has a wide variation in day temperatures, which reach 45° C at several places during summer months. The allergenic extracts have to be transported over long distances before being used. Hence, it was very important to know the changes which occur in allergenic extracts at high temperatures. Thus, determining the potency of the extract by standardized techniques is meaningless if the antigenic activity of the same extract is not maintained thereafter. Different allergenic extracts may vary in their behaviour on storage, therefore, representatives of pollen, fungi and dust were selected for the present study. Those chosen are predominant aeroallergens for patients with nasobronchial allergy in India.[12-14]

The nature of changes which occur in PJ, RN and WD extracts during storage at different temperatures for specified periods have been identified in the present investigation. The results obtained here with PJ extract were similar to those with other pollen extracts reported earlier.[4,15] There was a significant loss in protein content of PJ extract, with no distinct bands appearing on TLIEF when it was stored at 40° C for 15 days (Fig. 2). The RIE pattern also clearly demonstrated the complex nature of the antigens present and the loss of potency was probably not due to the loss of a single substance (Fig. 3).

Radioallergosorbent test (RAST) inhibition, when carried out properly, can be a reliable method for measuring the relative potency of the allergenic extract.[16] The results obtained here confirm the previous findings,[3,15] that storage at 4° C results in minimal loss of allergenicity in all the three (PJ, RN and WD) extracts during the 60 days storage period. Some workers[4,5] have recommended the use of human serum albumin (HSA 0.03%) and/or 50% glycerol, as stabilizing agent for allergenic extracts. However, an appreciable fall in allergenic activity has been reported in extracts containing HSA and/or glycerol.[11] The preserving effect of glycerol is mainly attributed to its capacity to inhibit sugar clearing enzymes. The pollen extracts are known to contain several such enzymes in large amounts.[4] The stabilizing effect of glycerol was also studied in the case of PJ, RN and WD extracts. In general, addition of 50% glycerol resulted in better retention of allergenic activity than did the addition of phenol (0.4%) in PJ and RN extracts, while it had no affect on WD allergen. Wheat dust extract was found to retain more than 50% of allergenic activity in buffer containing phenol, even at high temperatures. This is probably due to the presence of thermostable antigens in the extract, since the source material (WD) in its native environment is subjected to considerable heat over a period of time.

The results of this study are in agreement with those of Bosquet *et al*[4] and indicate that different extracts behave differently when stored under similar conditions. Hence, their shelf life should be determined individually. Preferably, allergenic extracts should be stored in lyophilized form at -20° C and they should not be exposed to high temperatures for long duration.

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