

Effect of various stabilizing agents on *Imperata cylindrica* grass pollen allergen extract

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Summary

Background Allergen extracts are unstable, heat labile or susceptible to proteases. Stability of allergen extracts is important for proper diagnosis and therapy of allergic disorders.

Objective The present study was undertaken to determine the preservation and stabilization conditions of *Imperata cylindrica* (Ic) grass pollen extract.

Methods The Ic extract was kept with 0.1 M ϵ -aminocaproic acid (EACA), 0.75 M sucrose, 5% glycerol, 0.03% human serum albumin (HSA) or 0.4% phenol for different time periods. The extracts were stored for 3, 6 and 12 months each at 4 °C, 4 °C with daily exposure to room temperature (RT) for 1 h, and RT. The quality of extracts was analysed by SDS-PAGE, Western blot, ELISA, ELISA inhibition and skin test.

Results Extracts kept with EACA and sucrose retained most of the protein bands followed by glycerol as determined by SDS-PAGE and Western blot during all storage periods and conditions in comparison with standard extracts. The extracts kept with HSA, phenol and without preservative (WP) showed protein degradation below 33 kDa after 3 months storage at all conditions. However, a 67-kDa allergen was stable in these extracts. EACA extract required 75 to 120 ng of protein for 50% inhibition in IgE binding under different conditions, whereas standard extract required 70 ng for the same. ELISA also demonstrated high allergenic reactivity of EACA extract. ID test on allergy patients with EACA extract demonstrated same allergenic potency as that of standard extract.

Conclusion EACA is the best preservative/stabilizing agent of Ic pollen extract, followed by sucrose and glycerol. Ic extract kept with phenol, HSA and without preservative showed degradation within 3 months. EACA preserved extract is equally potent as that of standard extract up to 1 year's storage.

Keywords ϵ -aminocaproic acid, pollen extract, preservative, protease inhibitor, stability, storage
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Introduction

Allergen extracts are prone to degradation by proteases when stored alone or mixed with other extracts. Grass pollen allergens are susceptible to deleterious effects of proteases derived from certain fungal and insect allergen preparations. Potency of timothy grass, Bermuda grass, white oak, box alder, Russian thistle and cat extracts reduced considerably on combination with one or more extracts containing proteases, i.e. *Alternaria tenuis*, *Cladosporium herbarum* and *Periplaneta americana* [1]. A recent study demonstrated degradation of major birch and timothy grass pollen allergens when mixed with the mold *Fusarium culmorum* [2]. In mixed allergen extract preparations, it is impossible to know the extent of proteolytic activity present in a specific extract. This suggests that allergens/extracts should not be mixed with other extracts containing proteolytic enzymes [2].

Nevertheless, extracts containing proteases may cause self-degradation.

Allergen extracts may contain unstable allergens that are characteristic of an allergenic source. Various preservatives and stabilizers, such as ϵ -aminocaproic acid, sucrose, glycerol, human serum albumin, phenol, etc., have been reported either alone or in combination as maintaining the potency of allergen extracts [3–8]. The extracts are subjected to harsh conditions during transportation over long distances to hospitals and clinics. Apart from this, the method of handling of the extracts by clinicians also affects their allergenic potency.

Imperata cylindrica is an allergenically important wild growing grass and is prevalent in warm and temperate regions of Asia, South Africa and Australia. The pollen extract of this grass shows allergenic and antigenic cross-reactivity with other important tropical grasses, e.g. *Cenchrus ciliaris*, *Cynodon dactylon*, *Pennisetum typhoides* and *Sorghum vulgare* [9]. It shares group 4 and 5 allergen homologues of *Phleum pratense* [10]. A 67-kDa allergenic protein purified from *Imperata cylindrica* demonstrated presence of at least three allergenic determinants. These purified peptides of the 67 kDa protein also cross-reacted with important tropical grasses [11]. We recently

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demonstrated that *Imperata cylindrica* extracts prepared from freeze dried pollen are allergenically more potent and retained major and minor allergenic proteins [12]. The present study was undertaken to see the effect of five different preservatives on *Imperata cylindrica* extract prepared from freeze dried pollens.

Materials and methods

Collection of pollen and preparation of extract

The inflorescence of *Imperata cylindrica* (Ic) were collected during peak flowering season and the pollen was obtained as described [12]. The pollen was defatted in diethyl ether, dried, and the extracts were prepared in phosphate buffered saline (PBS, 1/20 w/v).

Protein estimation

The protein content in Ic extract was estimated by modified Lowry's method as described earlier [13].

Preservation and storage of extracts

To the Ic extracts, different preservatives were added separately to attain a final concentration as follows: (i) 0.1 M ϵ -amino caproic acid (EACA); (ii) 0.75 M sucrose (SUC); (iii) 0.03% (w/v) HSA; (iv) 5% (v/v) glycerol (GL); (v) 0.4% (w/v) phenol (PH); and (vi) without preservatives (WP). The extracts were filtered through a 0.22 micron filter for sterility and stored in airtight sealed vials.

Initially, three sets of each extract were reconstituted for storage at 4 °C, 4 °C with daily exposure for 1 h at room temperature (RT) (to mimic the handling of extracts by allergologists), and at RT. These sets were stored for 12 months. After 6 months, the sets were prepared again and stored for 6 months. After 9 months, the sets were prepared again and stored for 3 months. This scheme of preparing and storing the extracts enabled analysis of all the extracts simultaneously.

In-vitro analysis of extracts

After the completion of storage periods, the extracts were dialysed against distilled water (MW cut-off 1500), lyophilized and used for analysis. A standard extract (lyophilized extract stored at -70 °C) was also reconstituted and used as a positive control for comparison in ELISA, ELISA inhibition and immunoblot.

SDS-PAGE

The lyophilized extracts were electrophoresed on a 10% reducing polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue (CBB) and the protein bands were identified after destaining the gel as described earlier [12].

Immunoblot

The allergenic profile of Ic extracts was determined as described earlier [12]. Briefly, the electrophoresed proteins were transferred onto a nitrocellulose membrane. The non-specific sites on NC were blocked with 3% defatted milk (Bio-Rad) and incubated with Ic hypersensitive pooled patient's sera (1/10 v/v). After washing the membrane with PBST, the allergenic proteins were identified by incubating with anti-human IgE antibody-HRP (1/1000) at 37 °C for 3 h. The colour was developed with di-aminobenzidine.

ELISA

The ELISA for Ic extracts was carried out as described earlier [12]. Briefly, microtiter wells were coated with the extracts in carbonate buffer pH 9.6 (1 µg/100 µL/well, Nunc Maxisorp, Roskilde, Denmark). After blocking the non-specific sites with 3% defatted milk for 1.5 h, the protein was incubated with Ic hypersensitive pooled sera (1/10) overnight at 4 °C. After washing the wells with PBST, the bound IgE was determined by incubating with anti-human IgE-HRP for 3 h at 37 °C (1/1000, Sigma, St Louis, MO, USA). The colour was developed using o-phenylene diamine.

ELISA inhibition

As described in ELISA, 1 µg of standard Ic extract (protein) was coated per well of microtiter plate. After blocking, the solid phase antigen was incubated with a pre-incubated mixture of 1/10 diluted *Imperata* hypersensitive pooled sera and graded amounts of *Imperata* extract, i.e. 5, 10, 50, 100, 500 and 1000 ng, as inhibitor. The remaining steps were the same as followed for ELISA.

In-vivo analysis of preserved and stored Ic extracts

ID test was performed on 52 respiratory allergy patients with standard Ic extract (SE, i.e. freshly reconstituted -70 °C preserved lyophilized powder) as a positive control and with EACA, SUC and GL extracts stored for 12 months at 4 °C with daily exposure to RT for 1 h. PBS containing different preservatives were taken individually as negative controls.

Collection of patient's sera

Intradermal tests were carried out with Ic extract on allergy patients visiting the Clinical Research Centre of Vallabhbbhai Patel Chest Institute, Delhi, as described [9]. The sera showing marked ID reactions (2+ to 4+) were collected and pooled for *in-vitro* analysis of Ic extracts. Guidelines of the Human Ethical Committee of the Institute (Vallabhbbhai Patel Chest Institute, Delhi) were followed whilst doing skin test and sera collection.

Results

SDS-PAGE

The standard Ic extract (SE) showed 37 protein bands between 12 and 110 kDa on SDS-PAGE (Fig. 1). The quality of EACA and SUC extracts was comparable with standard extract under all storage conditions. However, both these extracts showed slightly diffused protein bands after 12 months of storage at RT. The GL extract also showed a similar protein profile for short period at 4 °C with daily exposure to RT but the protein bands were slightly diffused. Extracts with HSA revealed loss of protein bands below 33 kDa after 3 months storage at all three temperatures. The degradation was more prominent in extracts stored at RT. The PH extract showed maximum degradation compared with other preserved extracts. The profile of WP extract was similar to that of PH extracts during all storage conditions. The degradation was quite prominent after 12 months storage at RT. However, complete degradation of proteins was not observed at 12 months storage at RT (Fig. 1).

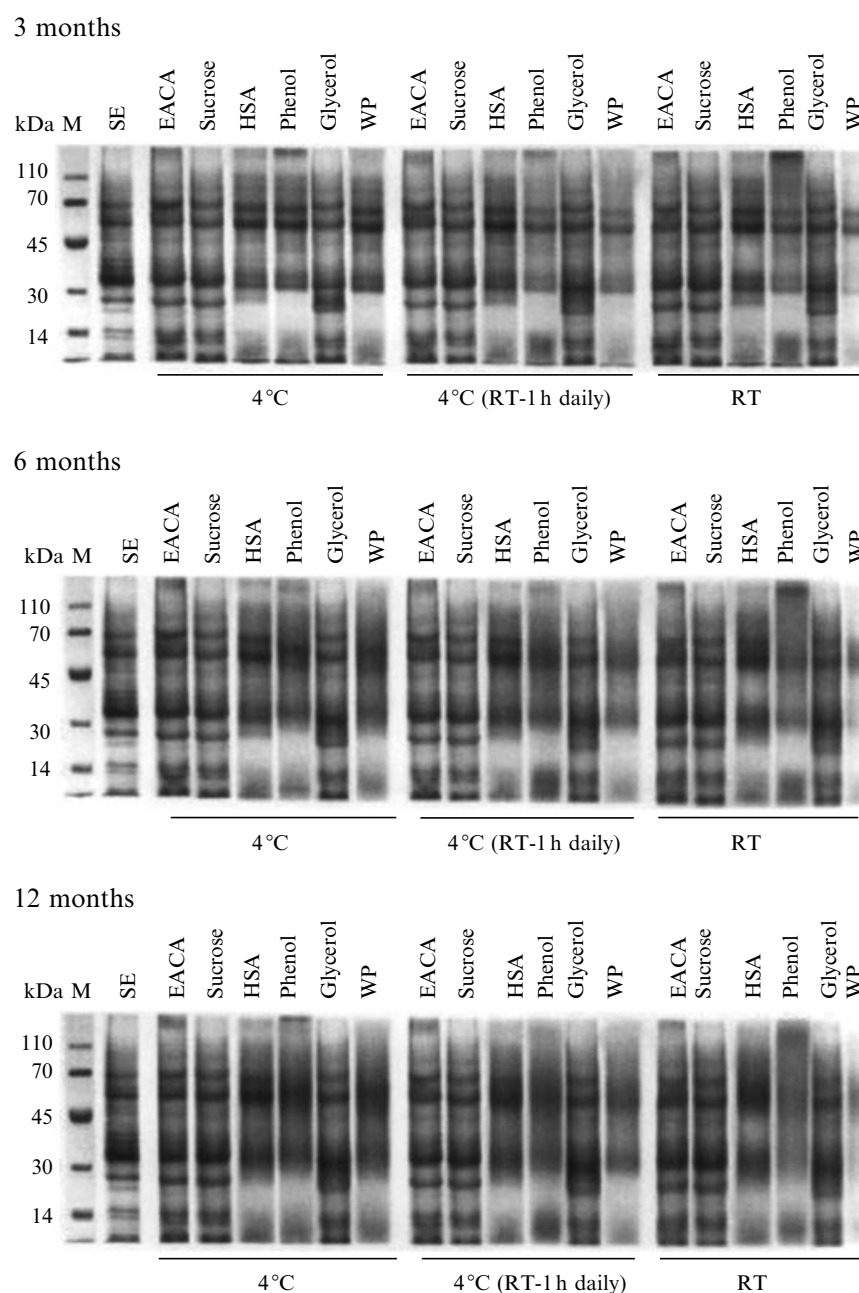


Fig. 1. SDS-PAGE (10% reducing) of Ic extracts with preservatives, stored at different temperatures for 3, 6 and 12 months. The protein bands were identified after staining with Coomassie Brilliant blue. M: molecular weight marker; SE (standard extract): freshly reconstituted extract from -70°C lyophilized powder. EACA: 0.1 M, Sucrose: 0.75 M, HSA: 0.03%, Phenol: 0.4%, Glycerol: 5%, WP: extract without preservative.

Immunoblot

To identify qualitative differences in different Ic extracts, immunoblot was carried out. Immunoblotting identified 30 IgE binding proteins in SE, EACA and SUC extracts under all storage conditions. The bands were diffused at RT after 12 months storage in EACA and SUC extracts (Fig. 2). The allergenic profile of GL extract was almost similar to EACA and SUC extracts under all storage conditions. The immunoblot of HSA extracts revealed prominent bands from 50 to 70 kDa and 33 kDa. The allergenic proteins below 33 kDa were almost absent after 6 months storage at 4°C and RT. The allergenic profiles of PH extracts were similar to HSA extracts. HSA, PH and WP extracts revealed reduced IgE

binding on immunoblot. However, a 67-kDa allergenic protein was stable in extracts after 12 months storage at RT.

ELISA

To determine quantitative difference, ELISA with different Ic extracts was carried out using *Imperata* hypersensitive pooled sera. The allergenic activity of EACA and SUC extracts was similar to SE extract under all storage conditions (Fig. 3). The GL extract showed reduced activity as compared with EACA and SUC extracts. However, PH, HSA and WP extracts showed low allergenic activity under all conditions that was negligible after 12 months storage at RT.

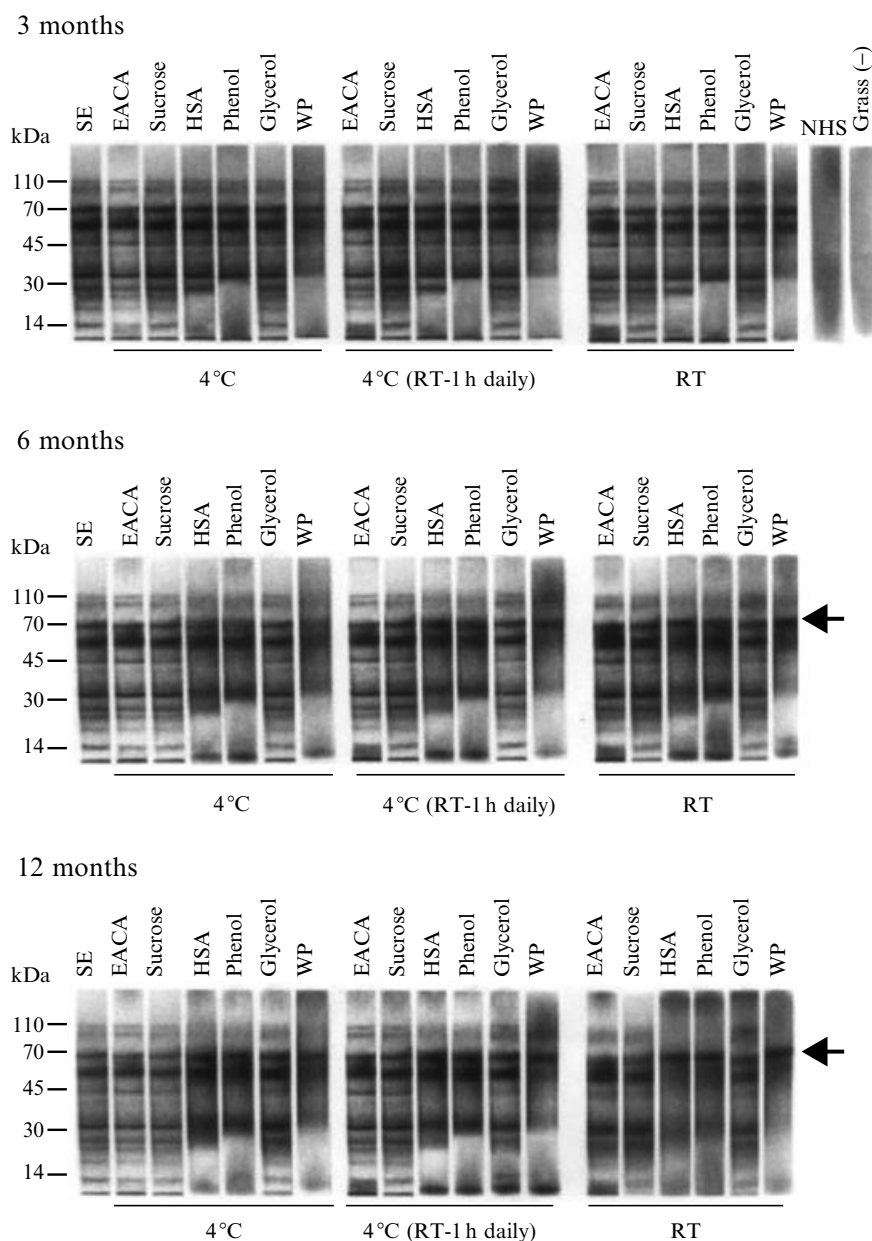


Fig. 2. IgE immunoblot of Ic extracts containing different preservatives, stored at different temperatures at various time periods. The electrophoresed extracts were electrotransferred onto NC sheets and incubated with Ic hypersensitive pooled patient's sera (1/10 v/v dilution) and the bound IgE was probed with anti-human IgE-HRP (1/1000 v/v dilution). The colour was developed using DAB. The preservatives indicated are the same as referred to in the legend for Fig. 1. NHS and grass (-): blots with normal human serum (non-allergic, non-atopic) and grass-negative serum, respectively. The arrows indicate the 67 kDa stable allergen.

ELISA inhibition

The allergenic potency of the preserved Ic extracts was studied by ELISA inhibition. Under different storage conditions, EACA extract required 75 to 120 ng of self-protein for 50% inhibition in IgE binding to solid phase SE (Fig. 4, Table 1). This was closely followed by SUC and GL extracts. The PH, HSA and WP extracts demonstrated comparatively lower potency at various storage conditions. The potency of HSA, PH and WP extracts was 10 to 15 times lower (> 1000 ng for 50% inhibition) after 12 months storage at RT.

ID test

The ID test was performed with EACA, sucrose and glycerol preserved Ic extracts that were stored for 12 months at 4 °C with

daily exposure to RT for 1 h. Out of 52 patients, five showed positive reactions (2 + to 4 +) each with SE and EACA extracts. Of these five patients, three and two patients showed reactions with SUC and GL extracts, respectively. No adverse reactions were observed with preservatives alone.

Discussion

Allergen extracts are vital for proper diagnosis and effective immunotherapy of allergic disorders and should contain both major and minor allergenic proteins [12]. Allergenic proteins may be heat labile, thermostable or unstable, or proteases that can contribute to either stabilize or deteriorate the extract. Stability is an essential requirement for an allergen extract.

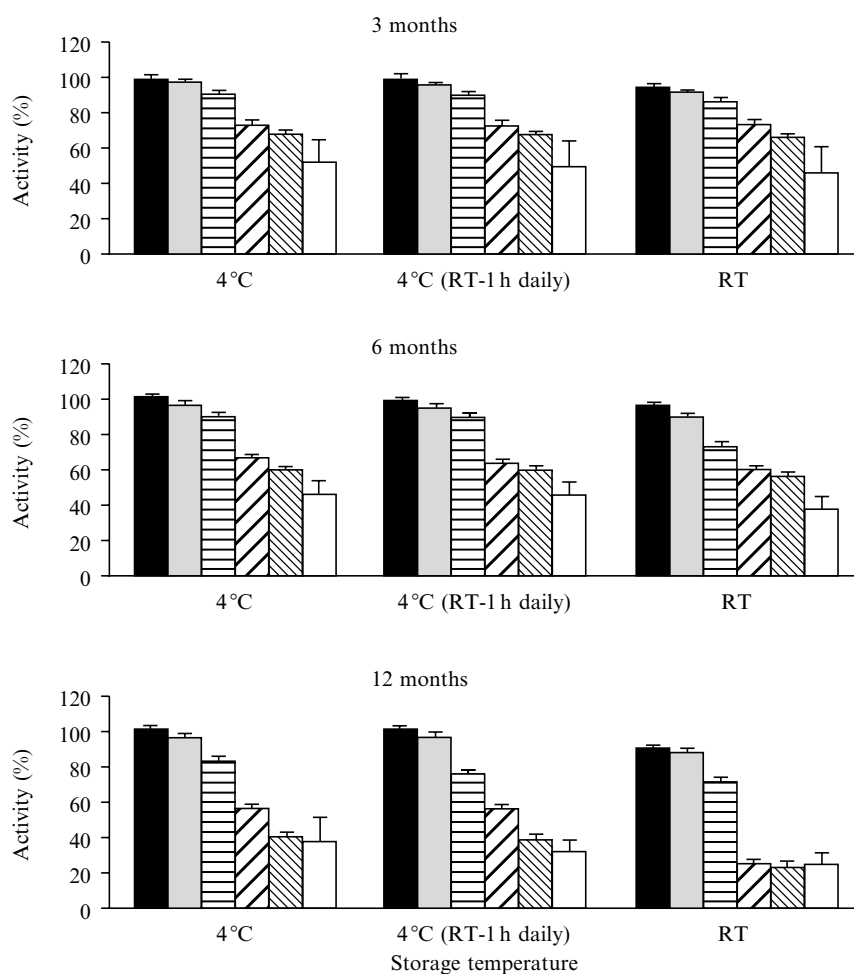


Fig. 3. Percentage allergenic activity as calculated from IgE ELISA (absorbance at 490 nm) of different IgE extracts compared with standard extract (100%). The bars indicate standard deviation from the mean of three individual values. Absorbance at 490 nm: standard IgE extract: 1.38; normal human serum: 0.087.

During storage, loss of activity should be minimal so that no significant change of potency occurs over a specified period. Variations in temperature and humidity can deteriorate extracts on exposure to extreme conditions. The present study was therefore, aimed to determine the stability of *Imperata cylindrica* pollen extract with different preservatives and storage conditions.

In the present study, EACA is the best preservative, followed by sucrose and glycerol. EACA could prevent enzymatic degradation of proteins in IgE extract. This is in agreement with previous studies on the stabilizing effect of EACA on *Lolium perenne* grass pollen extract [3]. EACA is known to be a potent inhibitor of trypsin-like enzymes [14–17]. However, the exact mechanism of inhibition is still not clear. As it is a zwitterion, it can stabilize proteins by preferential hydration [18]. It also prevents protein degradation due to freeze thawing and has beneficial effect over HSA on *Lolium perenne* pollen extract [3]. SUC extracts demonstrated high allergenic potency that was comparable with that of EACA extract. The stability of

GL extract was less compared with SUC extract. This supports a previous report in which 0.5 M–1.0 M sucrose had a more stabilizing effect compared with equimolar (0.5 M) concentration of glycerol in phenolated and non-phenolated extract of *Lolium perenne* [4]. Several studies on allergen stability include analysis with 50% glycerol extract [5, 6]. In the present study, only 5% glycerol was used considering the irritant effects of high glycerol concentration while testing. Both sucrose and glycerol may act in part as a competitive inhibiting substrate for sugar cleaving enzymes. However, sucrose may also change protein conformation and render it less susceptible to proteolytic degradation [19].

Phenol is added to prevent bacterial contamination in extracts. However, initiation of protein degradation in PH and HSA extracts started within 3 months storage at 4°C. Previous reports also suggest that phenol accelerates protein degradation [4, 5, 7]. Phenol, being acidic in nature, is a good protein denaturant. It thereby unfolds proteins and exposes certain sites for proteolytic cleavage. HSA might have prevented the

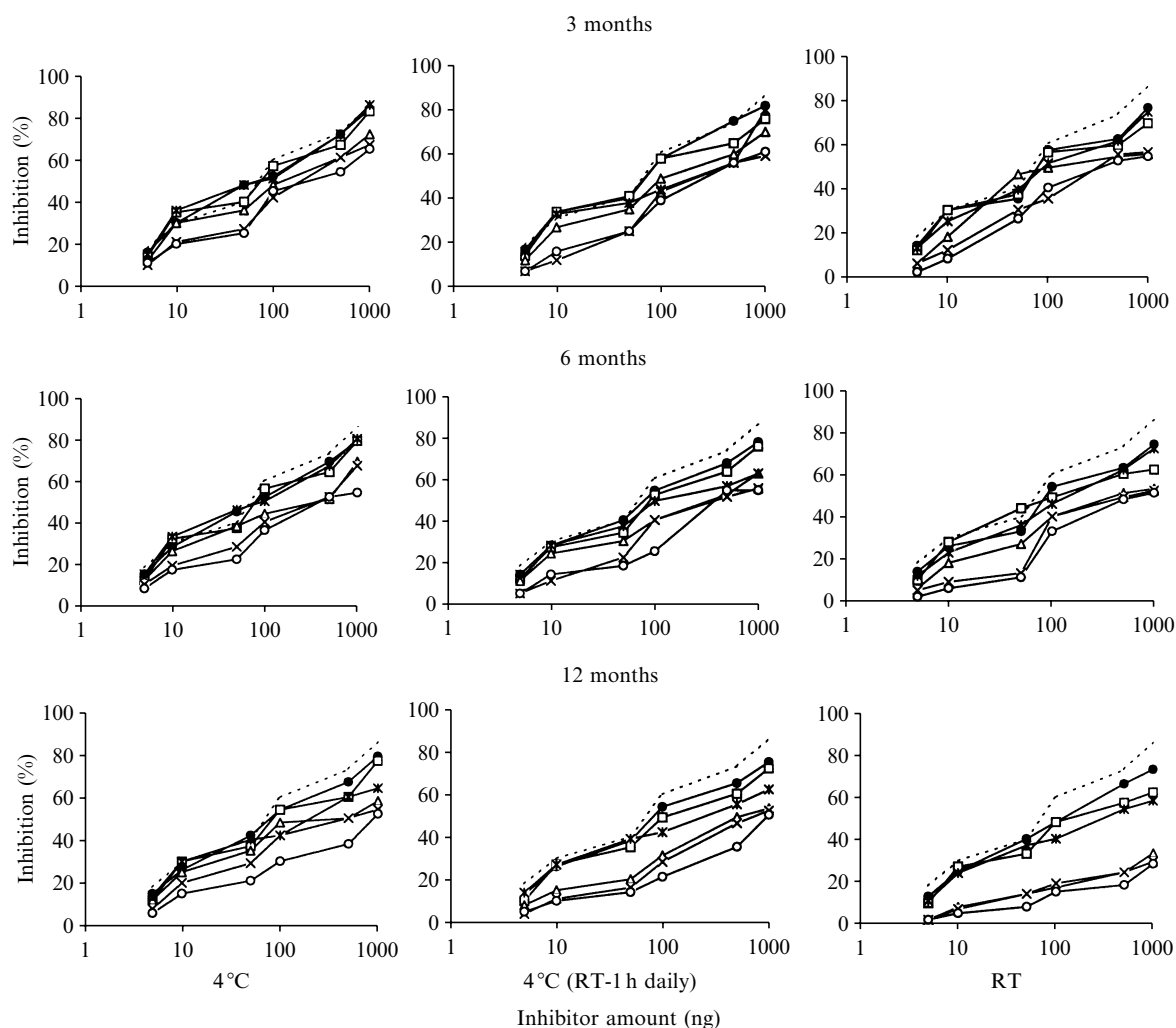


Fig. 4. IgE ELISA inhibition of Ic extracts containing preservatives, stored at varied temperatures for different time periods. Ic extracts 1 µg/well, were coated in microtitre wells and incubated with a mixture containing Ic hypersensitive pooled patient's sera (1/10 v/v dilution) and 5 to 1000 ng of preserved Ic extracts as inhibitors. The bound IgE was determined using anti-human IgE-HRP. (● 0.1 M ϵ -aminocaproic acid, □ 0.75 M sucrose, Δ 0.03% HSA, × 0.4% phenol, ★ 5% glycerol, ○ without preservative, ... standard extract). The values represent the mean of three independent experiments.

adsorption of proteins to the container walls but proteases in the Ic extract may have neutralized its beneficial effect. The degradation of proteins in WP extract shows protease action in the extract. SDS-PAGE, immunoblot, ELISA and ELISA inhibition suggested that HSA and phenol could not retain the potency of Ic extract. However, immunoblotting demonstrated that all proteins are not degraded completely. A 67-kDa allergenic protein of Ic extract was stable after storage in HSA, phenol and WP extracts, suggesting that this protein is protease resistant and thermostable. The remaining activity of Ic extract (Figs 3 and 4) after 12 months storage at RT in HSA, PH and WP may be attributed to the presence of this 67 kDa allergen along with other degraded proteins (Fig. 2). In our previous study, 11 major allergenic proteins, including a 67-kDa protein, were identified in the standard Ic extract [12]. In the present study, all these proteins were preserved in SUC and EACA extract.

ID test suggested that EACA was more effective than sucrose and glycerol in maintaining the potency of Ic extract. Also,

EACA, sucrose and glycerol did not cause any adverse reactions on ID testing. This suggests that EACA is a safe preservative [3].

In conclusion, the study suggests that ϵ -aminocaproic acid is a good stabilizing agent, followed by sucrose, for *Imperata cylindrica* pollen extract and can be safely used on allergy patients.

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Table 1. ELISA inhibition by lc extracts preserved at different storage conditions

Time period	Preservative	4 °C (ng)	4 °C (RT 1 h daily) (ng)	RT (ng)
3 months	0.1 M EACA	75	75	80
	0.75 M sucrose	75	77	80
	5% glycerol	85	85	95
	0.03% HSA	130	130	140
	0.4% phenol	200	250	310
	Without preservative	270	300	380
6 months	0.1 M EACA	80	80	85
	0.75 M sucrose	80	95	120
	5% glycerol	100	125	150
	0.03% HSA	380	380	400
	0.4% phenol	400	425	600
	Without preservative	400	475	800
12 months	0.1 M EACA	80	85	120
	0.75 M sucrose	85	110	150
	5% glycerol	200	280	320
	0.03% HSA	500	600	> 1000
	0.4% phenol	500	800	> 1000
	Without preservative	900	1000	> 1000

The values represent the amount of preserved *Imperata cylindrica* extracts (protein) for 50% inhibition in IgE binding to solid phase SE (freshly reconstituted – 70 °C stored lc extract). Control: SE was used as inhibitor against IgE binding (amount for 50% inhibition = 70 ng).

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