

Stability of Protease-Rich *Periplaneta americana* Allergen Extract During Storage: Formulating Preservatives to Enhance Shelf Life

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Allergenic proteins in extracts degrade rapidly and lose potency on storage. Hence, formulation of optimum conditions is required to enhance shelf life of extracts for proper allergy diagnosis and immunotherapy. In the present study, allergenic potency of *P. americana* proteins was evaluated after storage with ϵ -aminocaproic acid (EACA), sucrose, glycerol, pepstatin A, and aprotinin, individually for 1, 3, 6, and 12 months at 4, 25, and 37°C. *P. americana* extract stored with EACA and sucrose individually retained potency comparable to proteins in standard extract (freeze-dried extract, stored at -70°C) upto 6 months at 4°C. The extracts without preservatives or with glycerol, pepstatin A, aprotinin, or stored at 37/25°C were severely degraded and lost potency by 3 months. A formulation containing a combination of EACA and sucrose enhanced the shelf life of *P. americana* proteins upto 12 months at 4°C. Hence, EACA and sucrose together show better potential for stabilization of protease-rich extracts.

KEY WORDS: *Periplaneta americana*; cockroach extract; stability; ϵ -aminocaproic acid; immunoblot; ELISA inhibition.

INTRODUCTION

Extracts used for allergy diagnosis and therapy are heterogeneous mixture of proteins. Allergen extract(s) are required to contain all the defined major, minor allergens and be biologically potent (1). The extracts show maximum potency when they are freshly made but deteriorate on storage. The loss in potency affects the results of allergy diagnosis and immunotherapy (2). Therefore, stability is

an important parameter for an allergenic product under various conditions of storage such as temperature, light, solvent, dilution, etc. (1).

Aqueous allergen extracts are transported long distances under variable temperatures and kept at room temperature while testing. These extracts contain proteases that can cleave self-proteins and other extracts in vaccines. Proteases are present maximally in the extracts of fungi, insects, and house dust mites (3–5). *P. americana* extract is rich in proteases and reduces the potency of timothy grass, Bermuda grass, white oak, box alder, Russian thistle, and cat extracts when mixed for immunotherapy prescription (6). Similarly, there was considerable loss of potency in rye grass extract when mixed with *Helminthosporium* and cockroach extract (7). Various preservatives and stabilizers such as ϵ -aminocaproic acid (EACA), sucrose, glycerol, human serum albumin (HSA), phenol, Tween 80, etc., were used to maintain the potency of allergenic extracts (8–11).

Cockroach allergens play an important role in the etiology of allergic bronchial asthma (12, 13). Commercially available cockroach extracts (aqueous/glycerinated) are highly variable in protein content and allergenic potency (14). Whole body cockroach extracts are widely used but studies defining shelf life based on real-time stability studies are lacking. Each indigenous preparation should be checked for degradation because extracts from different sources behave differently under storage. In the present study, the effect of various preservatives was investigated on *Periplaneta americana* extract under different storage conditions.

MATERIALS AND METHODS

Preparation of Cockroach Extract

Mixed culture of male and female cockroaches (*Periplaneta americana*) were freeze-dried and ground to a fine

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powder. The material was defatted with 1:10 diethyl ether i.e., 1 g of cockroach powder in 10 mL of diethyl ether by repeated changes with fresh ether at 4°C. The antigens were extracted from cockroach powder 1:50 (w/v) in 0.1 M phosphate buffered saline (PBS), pH 7.2, containing 5 mM EDTA and 1 mM phenyl methyl sulfonyl fluoride, by continuous stirring for 8 h at 4°C. The supernatant was collected after centrifugation at $14,170 \times g$ for 1 h and dialyzed extensively against distilled water using 3.5 kDa cutoff membrane at 4°C (15). The extract was lyophilized, stored at -20°C, and protein concentration estimated by modified Lowry's method (16).

Storage of Extracts with Individual Preservatives

P. americana extract was stored with different concentrations of preservatives. Lyophilized extracts were reconstituted to obtain a final concentration of: 1) 0.025, 0.05, 0.1 M EACA (ϵ -aminocaproic acid); 2) 0.25, 0.50, 0.75 M Sucrose; 3) 5, 10% Glycerol; 4) 3 μ M Pepstatin A, and 5) 0.6 μ M Aprotinin. The extracts were filtered through 0.22 μ m filters and stored for 1, 3, 6, and 12 months at 4, 25, and 37°C. After completion of the storage period, the extracts were analyzed.

Storage of Extracts with Combination of Preservatives

P. americana extracts were also stored with a combination of preservatives (0.05 M EACA + 0.25 M sucrose) showing better stability when used separately. The extracts were filtered through 0.22 μ m filters, stored for 1, 3, 6, and 12 months at 4 and 25°C.

In vitro Analysis of Extracts

After the completion of storage periods, the extracts were dialyzed against distilled water (MW cutoff 3.5 kDa) at 4°C, lyophilized, and analyzed. Standard extract (lyophilized extract stored at -70°C) was also reconstituted and used as positive control for comparison in SDS-PAGE, immunoblot, ELISA, and ELISA-inhibition.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). *P. americana* extracts were subjected to SDS-PAGE on 10% resolving gel (15). Sixty micrograms protein of each sample was loaded per lane and electrophoresed at 120 V. The gel was stained with Coomassie brilliant blue.

Immunoblotting. SDS-PAGE separated proteins were electrophoretically transferred onto nitrocellulose membrane and probed with cockroach hypersensitive pooled

patients' sera (15). Briefly, the membrane was blocked with 3% defatted milk for 3 h at 37°C, washed with TBS (tris buffered saline), and incubated overnight with a pool of 20 cockroach-positive patients' sera containing 75–235 ng/mL of specific IgE with an average of 149 ng/mL (1:10 v/v) at 4°C. Membrane was then washed with TBST (TBS containing 0.2% Tween 20) followed by TBS and incubated with antihuman-IgE peroxidase (Sigma) (1:1000) for 3 h at 37°C. Following washing, IgE reactive bands were visualized with diaminobenzidine and hydrogen peroxide in acetate buffer.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was carried out with *P. americana* extracts as described earlier (15). Briefly, microtiter plate was coated with cockroach protein (1 μ g/100 μ L/well) in carbonate buffer, pH 9.6 and incubated overnight at 4°C. The free sites were blocked with 3% defatted milk in PBS for 3 h at 37°C and incubated with a pool of 20 cockroach-positive patients' sera (1:10 v/v) at 4°C overnight. After washing with PBST (PBS containing 0.2% Tween 20), the plate was incubated for 3 h at 37°C with antihuman-IgE peroxidase (Sigma) (1:1000) and color developed with *o*-phenylene diamine. The reaction was stopped by adding 5N H₂SO₄ and read at 492 nm in an ELISA reader. ELISA was performed in triplicates and the average of three readings was taken for analysis.

ELISA—Inhibition. Specific IgE inhibition was carried out to determine the potency of the various treated extracts. Microtiter plate was coated with standard *P. americana* extract (1 μ g/100 μ L/well) and blocked with 3% defatted milk in PBS. The plate was incubated with a preincubated mixture of 1:10 v/v diluted cockroach hypersensitive pooled patients' sera and graded amounts of *P. americana* extracts (0.1, 1, 10, 100, 1000 ng) as inhibitor in a 100 μ L volume. The remaining steps were the same as followed for ELISA.

In Vivo Analysis of Extracts

In vivo analysis of extracts was done by skin tests on 53 respiratory allergy patients referred to the Outpatient Department, V.P. Chest Institute, Delhi. Informed consent of each patient was obtained for skin tests, blood collection, and use of sera samples for this study. The protocol was approved by the Human Ethics Committee of the institute for the testing of *P. americana* extracts stored under different conditions.

For intradermal (ID) tests, 0.02 mL of each extract (1:500 w/v) was injected into the forearm of the patient raising a bleb of 2–3 mm and reactions were graded after 20 min on the basis of wheal size of positive control,

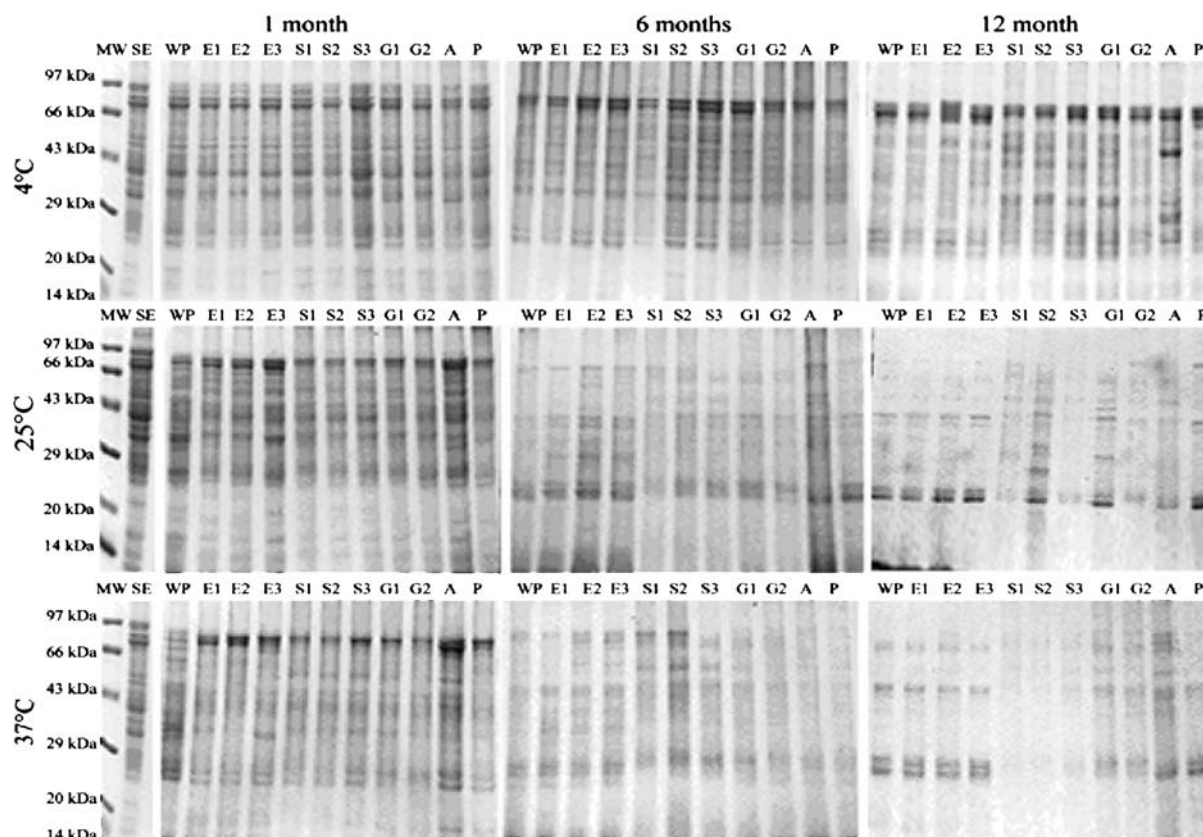


Fig. 1. SDS-PAGE profiles of *P. americana* extracts stored at 4, 25, and 37°C for 1, 6, and 12 months. MW: molecular weight marker, SE: standard extract, WP: without preservative, E1: 0.025 M EACA, E2: 0.05 M EACA, E3: 0.1 M EACA, S1: 0.25 M sucrose, S2: 0.5 M sucrose, S3: 0.75 M sucrose, G1: 5% glycerol, G2: 10% glycerol, A: 0.6 μ M Aprotinin, and P: 3 μ M Pepstatin A.

i.e., histamine diphosphate (100 μ g/mL) (17). Blood was collected from patients' showing marked positive skin reactions (wheal size equal to positive control and or more), sera separated and stored at -70°C . Normal human serum (NHS) was collected from 10 volunteers with negative skin reactivity to cockroach and other allergens.

ID testing was performed with standard *P. americana* extract and extracts stored individually with 0.05 MEACA and 0.75 M sucrose or with a combination of these preservatives. PBS containing EACA, sucrose, or both were also tested as negative controls.

Statistical Analysis

Statistical analysis was performed to compare specific IgE values and skin test reactivity of the various stored *P. americana* extracts with the standard extract using student's *t* test. $p < 0.05$ were considered statistically significant in all cases.

RESULTS

Analysis of the Extracts Stored with Individual Preservatives

SDS-PAGE and Immunoblot Profiles. Standard extract resolved into 45 bands on SDS-PAGE and demonstrated 22 allergenic bands on immunoblot with pooled cockroach hypersensitive patients' sera. Extracts stored at 4°C for 1 month with any of the preservatives were comparable to standard extract in terms of protein profile (Fig. 1) and IgE reactive bands (Fig. 2). Extracts with EACA and sucrose at 25°C were comparable to standard but those with glycerol and pepstatin A showed substantial loss of allergenic proteins. EACA and sucrose-treated extracts for 3 months at 4 and 25°C were comparable to standard, whereas the extracts stored with glycerol, aprotinin, and pepstatin A showed protein degradation at 25°C (data not shown). The extracts without preservative exhibited considerable degradation at 4, 25, and 37°C after 3 months (data not shown).

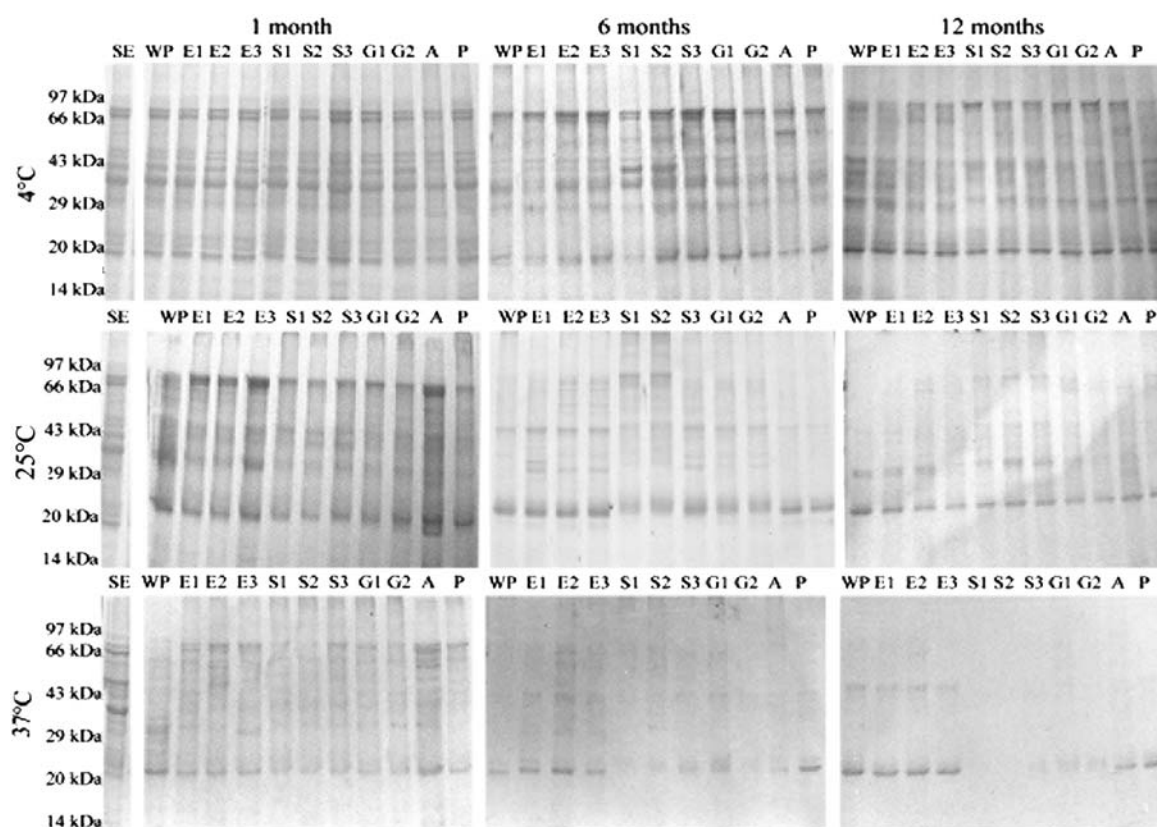


Fig. 2. IgE reactive bands of *P. americana* extracts stored at 4, 25, and 37°C for 1, 6, and 12 months. MW: molecular weight marker, SE: standard extract, WP: without preservative, E1: 0.025 M EACA, E2: 0.05 M EACA, E3: 0.1 M EACA, S1: 0.25 M sucrose, S2: 0.5 M sucrose, S3: 0.75 M sucrose, G1: 5% glycerol, G2: 10% glycerol, A: 0.6 μ M Aprotinin, and P: 3 μ M Pepstatin A.

After 6 months of storage at 4°C, the extracts with glycerol, aprotinin, pepstatin A, and without preservatives showed substantial protein degradation with loss of allergenic proteins as compared to the standard extract (Figs. 1 and 2). Storage at 25°C for 6 or 12 months resulted in moderate to extensive degradation of allergenic proteins in most of the extracts with or without preservatives (Figs. 1 and 2). After 12 months storage at 4°C, degradation was evident in all the extracts and more so in extracts containing glycerol (5–10%) and pepstatin A (Figs. 1 and 2). The extracts with EACA and sucrose showed presence of a majority of the allergenic proteins but the bands were slightly diffused. Extracts stored at 37°C showed extensive degradation as evident in SDS-PAGE and immunoblot and hence, were not analyzed further by ELISA and ELISA inhibition.

The SDS-PAGE and immunoblot profiles just show whether there is degradation in the various extracts in comparison to the standard, but they do not describe the subtle differences between the stored extracts. To substan-

tiate the differences, analysis of the IgE binding activity and potency of the extracts was carried out by ELISA, ELISA inhibition, and skin testing.

Specific IgE Reactivity and Relative Potency

Specific IgE reactivity of the various stored extracts as determined by ELISA using pooled cockroach hypersensitive patients' sera is given in Table I. The specific IgE values of the extracts stored with 0.05 M EACA and 0.25 M sucrose for 1 (152 and 122 ng/mL) and 6 months (119 and 100 ng/mL), respectively, at 4°C were not significantly different ($p > 0.05$). Also, the specific IgE reactivity of these extracts stored upto 6 months at 4°C were not significantly different from the standard ($p > 0.05$). However, the IgE binding was significantly reduced in extracts stored with any other preservative as compared to standard.

EC₅₀ of the extracts stored under different conditions was evaluated by ELISA inhibition wherein standard

Table I. Specific IgE Values of Extracts Stored with Individual Preservatives

Preservatives used	Specific IgE in ng/mL					
	1 month		6 months		12 months	
	4°C	25°C	4°C	25°C	4°C	25°C
WP	72	58	30	12	7	2
EACA 0.025 M	82	72	75	24	18	17
0.050 M	152	105	122	34	29	24
0.10 M	154	108	123	36	29	23
Sucrose 0.25 M	119	84	100	34	16	12
0.50 M	120	85	100	34	17	13
0.75 M	122	87	103	36	18	13
Glycerol 5%	58	20	43	13	10	7
10%	60	22	48	15	14	10
Aprotinin 6 µM	58	22	20	10	7	5
Pepstatin A 3 µM	62	23	18	11	6	4

Note. WP: without preservative; EACA: ϵ -aminocaproic acid. Specific IgE value of standard extract (SE) is 156 ng/mL. Specific IgE value of normal human sera (NHS) is 4 ng/mL.

extract was coated on solid phase and the stored extracts were used as inhibitors. The relative potency (RP) of the stored extracts was determined in comparison to the standard extract, wherein RP value for standard was taken as 1. RP of the extracts stored at 4°C for 6 months with EACA and sucrose individually was 0.55 and 0.46, while RP of extracts stored without preservatives at 4°C for 6 and 12 months was substantially lower (0.06 and 0.02) (Table II).

Analysis of the Extracts Stored with Combination of Preservatives

SDS-PAGE and Immunoblot Profiles. Protein profiles of the *P. americana* extracts stored with a combination of 0.05 M EACA + 0.25 M sucrose, at 4°C for 6 and 12 months were similar to the standard extract, with

the 12-month extract showing mild degradation (mainly of high molecular weight proteins) and slightly diffused bands (Fig. 3A). However, the extracts stored with these preservatives individually at 4°C showed degradation by 12 months (Fig. 1). The extracts stored at 25°C with the combination of these preservatives were comparable to standard extract only upto 3 months (data not shown).

The extracts with the combination of preservatives (EACA + sucrose) at 4°C demonstrated allergenic profile (20 allergenic bands with two minor allergenic bands of high molecular weights missing) similar to the standard extract upto a period of 12 months (Fig. 3B). But extracts stored at 25°C were comparable to the standard only upto a period of 3 months (data not shown).

Specific IgE Reactivity and Relative Potency. Specific IgE reactivity of the extracts stored with a combination of preservatives (0.05 M EACA + 0.25 M sucrose) for 6 and 12 months at 4°C were not significantly different from the standard extract ($p > 0.05$) (Table III). A fall in RP (0.5) was observed in the extract stored upto 12 months with the formulation. However, the extract without preservative exhibited substantially lower IgE reactivity with a RP value of 0.02 after a year at 4°C (Table III).

In Vivo Analysis of Extracts

Of 53 respiratory allergy patients tested, 24 showed marked positive skin reactions with standard *P. americana* extract. The extracts stored individually with EACA (0.05 M) and sucrose (0.25 M) at 4°C for 6 months showed marked positive skin response in all these 24 cockroach-positive patients. Extracts without preservative and those stored individually with EACA (0.05 M) and sucrose (0.25 M) for 1 year showed positive skin reactions in only one, six, and four patients, respectively. No adverse

Table II. EC₅₀ and Relative Potency of the Extracts Stored with Individual Preservatives

Storage duration (months)	Preservatives	EC ₅₀ (ng)			Relative potency (RP)		
		4°C	25°C	37°C	4°C	25°C	37°C
1	WP	25	55	300	0.48	0.22	0.04
	EACA 0.05 M	10	16	80	1.20	0.75	0.15
	Sucrose 0.25 M	14	24	95	0.86	0.50	0.13
6	WP	200	800	>1000	0.06	0.02	<0.01
	EACA 0.05 M	22	110	400	0.55	0.11	0.03
	Sucrose 0.25 M	26	150	550	0.46	0.08	0.02
12	WP	550	>1000	>1000	0.02	<0.01	<0.01
	EACA 0.05 M	55	200	980	0.22	0.06	0.01
	Sucrose 0.25 M	75	300	>1000	0.16	0.04	<0.01

Note. WP: without preservative; EACA: ϵ -aminocaproic acid. EC₅₀ of standard extract (SE) is 12 ng. RP of the stored extracts was determined in comparison to the standard extract assigned RP value of 1.

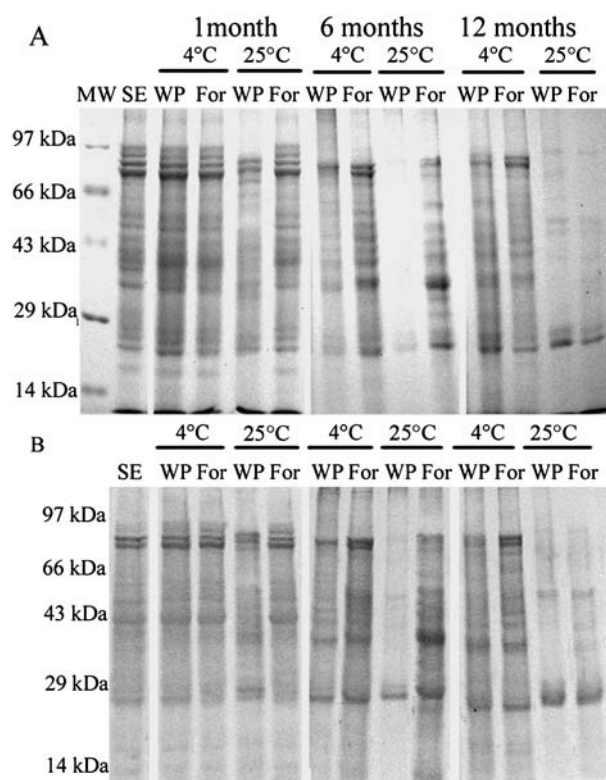


Fig. 3. SDS-PAGE profiles (A) and IgE reactive bands (B) of the *P. americana* extracts stored at 4 and 25°C for 1, 6, and 12 months (A). MW: molecular weight marker, SE: standard extract, WP: without preservative, For: formulation containing 0.05 M EACA + 0.25 M sucrose.

reactions were observed during testing of EACA and sucrose in concentration(s) used as preservatives. ID testing of extracts stored with a combination of EACA (0.05 M) and sucrose (0.25 M) at 4°C for 6 or 12 months demonstrated positive skin reactions in all the 24 patients. The skin test grading of the 24 cockroach-hypersensitive patients tested with the standard and stored extracts is given in Table IV.

DISCUSSION

Allergen extract stability is utmost important for proper allergy diagnosis and effective immunotherapy. Stability and potency of allergen extracts depend upon source material, extraction procedure, storage conditions, etc. (4, 11). Allergenic extracts are known to contain proteins which may be heat labile/thermostable structural proteins or enzymes (proteases) (11). Proteases degrade self and other proteins thereby reducing potency and specific activity of proteins in the extract (4). Storage conditions and preservatives should prevent protein degradation and maintain the shelf life of the allergen extract. Studies revealed that pollen, fungi, and mite extracts behave differently on storage (10, 11, 18) and, therefore, stability of individual extracts need to be evaluated separately. The commercial cockroach extracts show variable potency, electrophoretic banding pattern, major allergens, and protein content (14). In view of these, the present study was undertaken to determine a formulation for storage of *P. americana* extract.

Previous studies with phenol and Tween 80 showed deleterious effects on proteins in extracts (11). Human serum albumin used to prevent adsorption of proteins to vial surfaces was not an effective stabilizer (11). It is also not preferred due to fear of HIV and diseases associated with blood products. Glycerol (50%) offers stabilizing properties to proteins in diagnostic allergen extracts (10, 19). But glycerol has irritant effect at this concentration and cannot be used in therapeutic vaccines. *P. americana* extract stored with lower glycerol concentrations (5 and 10%) showed reduced IgE binding (Fig. 2), low specific IgE, and potency in the present study (Tables I and II). Sucrose has been used earlier for stabilizing many biological allergen extracts (9, 11). It acts as a competitive inhibiting substrate for sugar cleaving enzymes, stabilizing the folded state of proteins, or may change protein conformation rendering it less susceptible to proteolytic degradation (11). In present study, 0.25 M sucrose maintained IgE binding pattern, specific IgE reactivity, and potency

Table III. Specific IgE, EC₅₀, and Relative Potency of the Extracts Stored with the Formulation

Storage duration (months)	Preservatives used	Specific IgE (ng/mL)		EC ₅₀ (ng)		Relative potency (RP)	
		4°C	25°C	4°C	25°C	4°C	25°C
1	WP	68	52	22	50	0.55	0.24
	Formulation	158	135	9	12	1.33	1.00
6	WP	32	15	210	750	0.06	0.02
	Formulation	132	42	16	90	0.75	0.13
12	WP	8	4	525	900	0.02	0.01
	Formulation	110	10	24	600	0.5	0.02

Note. WP: without preservative; EACA: ϵ -aminocaproic acid. Specific IgE value of standard extract (SE) is 156 ng/mL. Specific IgE value of normal human sera (NHS) is 4 ng/mL. EC₅₀ of standard extract (SE) is 12 ng. RP assigned to SE is 1.

Table IV. Intradermal Test Grading of the 24 Cockroach-Hypersensitive Patients

Extracts tested	Number of patients showing 2+ to 4+ skin reaction			
	2+	3+	4+	Total
Standard extract	10	9	5	24
0.05 M EACA (6 months, 4°C)	12	8	4	24
0.25 M sucrose (6 months, 4°C)	13	7	4	24
0.05 M EACA (12 months, 4°C)	5	1	—	6
0.25 M sucrose (12 months, 4°C)	4	—	—	4
Formulation (6 months, 4°C)	9	10	5	24
Formulation (12 months, 4°C)	13	9	2	24
WP (12 months, 4°C)	1	—	—	1

Note. 2+ wheal size equivalent to histamine control. 3+ wheal size greater than histamine control with two pseudopodia. 4+ wheal size greater than histamine control with multiple pseudopodia.

of *P. americana* allergens similar to standard extract for 6 months at 4°C.

Several reports show the self-degradation of allergens by enzymes in the extracts (20). Both mite and cockroach extracts are composed of proteases (cysteine, serine—mainly trypsin-like type, and aspartic proteases) (3). Therefore, in present study, serine and aspartate protease inhibitors, namely aprotinin and pepstatin A, were tested individually for stabilization. These protease inhibitors stabilized *P. americana* extract for 3 months at 4°C (data not shown). The stabilizing effect depends on the specificity of these inhibitors, classes of proteases, and their stability in solution. EACA, a trypsin-like serine protease inhibitor has maintained the stability of grass pollen extracts and protected against thermal degradation (8, 11). EACA has enzyme inhibiting properties and zwitterions effect that stabilizes proteins by preferential hydration and prevents degradation due to freeze thaw (8, 11). In the present study, 0.05 M EACA maintained potency and quality of the cockroach extract similar to standard extract for 6 months at 4°C.

Allergen extract should contain all major and minor allergens and be biologically potent (1). Previously, Per a 1 (26, 31, 45, 51 kDa—group 1 allergen), Per a 3 (72, 78 kDa), and Per a 7 (38 kDa) were detected as major allergens in *P. americana* (21–23). Standard *P. americana* allergen extract used in the present study demonstrated the presence of 22 IgE binding components using pooled sera of patients, this includes nine major allergenic components (23, 28, 35, 38, 40, 49, 72, 78, and 97 kDa) based on reactivity with $\geq 80\%$ of the individual patients' sera and 13 minor allergenic components (15). The formulation (patent filed) containing 0.05 M EACA + 0.25 M sucrose maintained all major and most of the minor allergens and potency of *P. americana* extract upto 12 months at 4°C. Individually, these preservatives were able to give

protection upto 6 months at 4°C. The individual allergens (Per a 1, Per a 3, and Per a 7) could not be measured in the stored extracts due to unavailability of the specific antibodies. Previously, a formulation of phenol and HSA used with *Alternaria* allergens could maintain allergenic activity for 3 months only (24).

Extracts stored individually with EACA or sucrose for 6 months and EACA + sucrose for 12 months could diagnose all the cockroach-hypersensitive patients tested. Although the extract stored at 4°C for 12 months with a combination of preservative showed mild degradation and a drop in relative potency, its specific IgE binding capacity and skin test reactivity was similar to standard extract as it was able to elicit marked positive skin reactivity in all the 24 cockroach-hypersensitive patients. Thus, the potency of the extracts should be assessed based on a combination of both *in vitro* and *in vivo* parameters. The extracts stored with EACA or sucrose individually for 12 months could detect sensitization in only 25% (6/24) and 17% (4/24) cases, respectively. However, extract without preservative identified allergy in just 4% (1/24) of the cockroach-allergic cases ($p > 0.05$). It shows that cockroach extracts without preservative or stored individually with EACA/sucrose for 12 months missed many of the cockroach-hypersensitive patients. This is also substantiated by 25 times more potency of the extract stored with formulation (RP 0.5) as compared to extract without preservative (RP 0.02).

In conclusion, EACA and sucrose stabilized *P. americana* extract individually for 6 months whereas the formulation containing both increases the shelf life upto 1 year and can be used safely for stabilization and storage of protease-rich allergen extracts for diagnosis and therapy.

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