

Introducing a Stabilizer Formulation for Allergenic Mold Extracts

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Abstract

Background: Sensitization to common mold allergens is one of the major causes of allergic rhinitis and asthma. Therefore, there is a critical need for standard sensitivity tests including skin prick tests to improve the stability of fungi extracts in traditional allergenic formulations. To address this concern, the present study aimed to develop a formulation to preserve allergenic activity of mold extracts.

Methods: 48 stabilizer formulations were designed and monitored for allergenic activity during a 40-days incubation period at 37 °C using an ELISA. Specifically, the IgE reactivity of allergenic *A. alternata* extracts were examined. After establishing the most effective stabilizer formulation, we evaluated whether it could protect the allergenic activity of Alt a1, *A. fumigatus*, and *C. herbarum* using an IgE inhibition ELISA after 40 days at 37 °C.

Results: We demonstrated that the most effective stabilizer formulation was a glycerol-based extract containing Arg and Glu. This formulation had an equal ratio of sucrose, sorbitol and protein and was able to preserve more than 95% of allergenic *A. alternata* extract activity during a 40-days incubation period at 37 °C.

Conclusions: The present study reveals a novel formulation that is an efficient stabilizer of allergenic mold extract activity and has practical applications in mold skin prick tests, ELISAs, immunotherapies, and RAST.

Keywords: Allergy, Allergic Rhinitis, Mold, Stabilization, Skin Prick test.

Introduction

Allergic rhinitis is a common, chronic, inflammatory disease that is estimated to affect about 30% of the population worldwide (1). Allergic rhinitis places a huge burden on the health care system annually and affects the quality of life of those affected (2). Therefore, there has been increased attention and effort towards improving traditional diagnostic tests for allergens of interest and to minimize the risk of allergy development (3).

Many investigations have shown that molds are one of the main causes of allergic rhinitis (4). Through the inhalation of mold spores, the immune system of sensitive patients triggers the onset of common allergy related symptoms including itchy and watery eyes, a runny nose, nasal congestion, and sneezing (5). In addition to atopy, fungi can cause a plethora of diseases including superficial skin lesions, asthma, fatal systemic mycoses, amongst other

infectious diseases (6, 7). In this regard, mold extracts have critical industrial and clinical applications (8).

In the past decade, numerous studies have been working to develop allergenic mold extracts for skin prick testing, immunotherapies, enzyme-linked immunosorbent assays (ELISA), and radioallergosorbent testing (RAST) (9). Currently, there is a major concern on how to stabilize mold allergens within allergenic mold extracts (10). Since allergenic mold extracts have high protease content, stabilizing their allergenic activity has proven to be more challenging compared to other allergenic extracts. Importantly, industrial guidelines require allergenic extracts to remain active for two years during storage conditions at 4 °C (11).

Often, current formulations being used in clinical practice for skin prick testing and immunotherapy are complex matrices of proteins and glycoproteins.

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However, only a small portion of these extracts are active mold allergens, and the presence of other allergenic ingredients can cause significant hindrances to their reactivity during sensitivity testing (12). Therefore, the present study was conducted to design an effective stabilizer formulation to improve and maintain mold allergenic extract activity, while keeping in line with industrial demands.

Materials and methods

A total of 45 participants, from the allergy clinic of Qaem hospital in Mashhad Iran, who were between the ages of 15 and 55, were enrolled in the study. This study included patients with allergic rhinitis and rhino conjunctivitis. 15 sensitive patients were selected for each common allergenic mold including *Alternaria alternata* (*A. alternata*), *Aspergillus fumigatus* (*A. fumigatus*), and *Cladosporium herbarum* (*C. herbarum*) (13). Sensitivity was proven by a positive skin prick test for the allergenic mold (Stallergerm Greer, USA). This study was granted approval by the Ethics Committee of Mashhad University of Medical Sciences (code: 940043).

Allergenic Extracts

Standard strains of *A. alternata* (PTCC5224), *A. fumigatus* (PTCC 5009), and *C. herbarum* (PTCC5202) were obtained from the Iranian Research Organization for Science and Technology, and grown at 24 °C for 20 days on Sabouraud dextrose broth. The cultures followed a 12 h night and 12 h day schedule. A high allergenic extract yield was determined by the presence of both spores and mycelia using light microscopy.

Crude *A. alternata* was harvested and resuspended at a 1:10 ratio (w/v) in a 50 mM carbonate buffer (pH 7.5). The buffer contained 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM thiourea, and of 2% insoluble polyvinylpyrrolidone (PVP). Following an overnight incubation at 4 °C, the crude mold product was removed by filtration. The filtrate was then placed in 5% cold acetone solution, which resulted in the formation of a precipitate. Through centrifugation, the acetone was removed, and the precipitate subsequently was dissolved in 25 mM Tris-HCl buffer (pH 7.5) containing 1 mg per ml 3-[3-Cholamidopropyl] dimethyl ammonio]-1-

propanesulfonate (CHAPS). The resulting solution was dialyzed for a molecular cutoff of 12 kDa against a potassium phosphate buffer.

Setting the Stabilizer Formula

To evaluate the effect of sugar on *A. alternata* extract stability, sorbitol and sucrose were included in the final formulation proportionally to the protein concentration, which was measured using a Bradford assay. The first group had an equal ratio of protein, sorbitol, and sucrose (1:1:1), whereas, the second group had the same amount of protein and sucrose but reduced amount of sorbitol (1:1:0.25). Further, the control group did not contain any sugar or polyol. Moreover, 150 mM phosphate buffer saline (PBS) was used as the final solvent buffer for all extracts. In addition to sugar, 25 mM glutamate (Glu) and arginine (Arg), and 1% β-Alanine (β-Ala) (14), glycerol (20%), and human serum albumin (h Alb) (0.03%) were also critically evaluated for their ability to stabilize allergenic *A. alternata* extracts. In total, there were 16 reactions between the chosen amino acids and h Alb (Table 1), and 48 stabilizers, which were categorized into three main groups based on the ratio of sugar to protein.

Evaluating allergenic activity via ELISA

We evaluated the efficacy of sugar, amino acids, h Alb, and glycerol on allergenic *A. alternata* extract stability using an ELISA and during an incubation period of 28 days at 37 °C. The incubation time was pre-selected according to the Arrhenius equation, and is equivalent to two years if incubated at 4 °C (15). Plates pre-coated with treated extracts (100 µg/ml) in 200 mM bicarbonate buffer (pH 9.6) were incubated overnight at 37 °C, and subsequently blocked and incubated at 37 °C for 1 h the following day with 2% bovine serum albumin (BSA) in PBS buffer. Then, approximately 100 µl of pooled sera, collected from 15 *Alternaria* sensitive patients, were added to designated wells. Bound IgE in pooled patient sera were detected after consecutive incubations with biotinylated Anti-human IgE (Santa Cruz), streptavidin-horseradish peroxidase (HRP) (Becton Dickinson, USA) and 3,3',5,5'-Tetramethylbenzidine (TMB). Absorbance values obtained from a spectrophotometer at a wavelength of 540 nm were used to quantify concentration.

IgE inhibition ELISA

Due to obtaining ambiguous data from our previous ELISA, we alternatively used a specific IgE inhibition ELISA and increased the incubation period from 28 to 40 days. We first coated plates with fresh *A. alternata* mold extract without further treatment. Then, we incubated pooled patient sera with mold extracts with different stabilizers at RT for 2 h. Within the patient sera, specific IgE was inhibited by the allergenic activity of mold extracts, and a low absorbance value was indicative of high allergenic activity.

Negative control solutions were prepared either without any extract or antibody, or with substrate only. The negative control of inhibition prepared by mixing pooled sera with PBS, PBS replaced by extract to have no inhibition. Positive control solutions were prepared by incubating pooled sera with fresh extract, and represented as a percentage. This percentage reflects the allergenic activity of treated allergenic *A. alternata* extracts with specific IgE.

Evaluating *Alternaria* allergenic activity

The concentrations of Alt a1 (major allergen of *A. alternata*) were determined using a commercial sandwich ELISA kit (Indoor Biotechnologies, UK) that was performed in accordance to the manufacturer's instructions and recommendations.

Results

Stabilizers effect on IgE reactivity of the extract

As previously described, we conducted an ELISA to examine the effect of possible stabilizer candidates on IgE reactivity (see methods). Through the ELISA, we were able to detect *A. alternata* allergen-specific IgE in pooled patient sera after 28 days of incubation at 37 °C. Firstly, we determined the effect of sugar on *A. alternata* extract stability. The group with an equal ratio of sucrose, sorbitol, and protein had a significantly higher reactivity to *A. alternata* allergen-specific IgE in pooled patient sera compared to other groups reaching an average of 73.7 ± 3.5 . The average IgE reactivity in the *A. alternata* extract with 25% sorbitol and no sugar group was 50.7 ± 4.9 and 48.8 ± 4.9 respectively, however, there was no significant difference between these two groups.

Secondly, we evaluated the stabilizing effect of amino acids, glycerol and h Alb. Our data revealed that the addition of Arg or Glu alone significantly improved allergenic *A. alternata* extract activity. However, the

simultaneous addition of Arg and Glu was more effective and improved the activity up to 75 ± 2.8 while non-treated controls reached only 30 ± 12.7 . Further, comparing the effect of β -Ala to Arg or Glu, revealed that there was no significant difference in allergenic activity between these three amino acids. However, comparing Arg/Glu to β -Ala treatment revealed a significantly higher level of IgE reactivity with Arg/Glu treatment. Lastly, we found that both h Alb and glycerol did not preserve *A. alternata* extract allergenic potency.

In addition to using these stabilizers separately (Table 1), they were also used in combination with each other (Fig. 1). We showed that the group with equal sucrose, sorbitol, and protein, combined with Arg/Glu/glycerol (98%), was the best at preserving allergenic activity. There was no significant difference between this treatment Arg/Glu/glycerol and the fresh extract. Further, there was no significant difference in allergenic activity between the reduced sorbitol group with Arg/Glu/ β Ala (91%) and Arg/Glu/glycerol (94%), which was also similar to our positive control. Lastly, we found that the no sugar group, combined with Arg/Glu/glycerol had an allergenic activity (90%) similar to our fresh extract %. Overall, we found that the presence of amino acids, h Alb, and glycerol alone or in combination of each other had no effect on *A. alternata* extract stability, as there was no significant difference in allergenic activity after a 28-day incubation period at 37 °C compared to controls.

Table 1. Stabilizers formulations based on amino acids groups. These groups are the same in main three groups regarding sugars.

Arg (25 mM)	Alb h (0.03%)
	Glycerol (20%)
	β Ala (2%)
Glu (25 mM)	Alb h (0.03%)
	Glycerol (20%)
	β Ala (2%)
Arg/Glu (50 mM)	Alb h (0.03%)
	Glycerol (20%)
	β Ala (2%)
Without amino acids	Alb h (0.03%)
	Glycerol (20%)
	β Ala (1%)

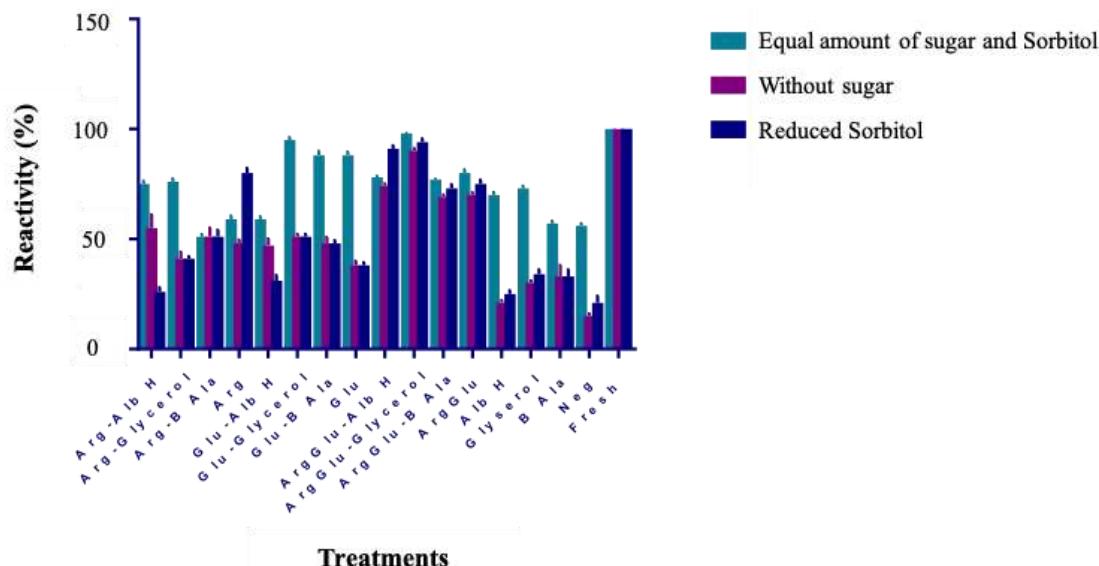


Fig. 1. Allergenic *A. alternata* extract activity after a 28-day incubation period at 37 °C with different stabilizers.

To achieve an effective stabilizer formulation, the allergenic potency of *A. alternata* extracts investigated using a specific IgE inhibition ELISA during an incubation period of 40 days at 37 °C. Our results show that the group with an equal sorbitol, sucrose and protein ratio had a higher percentage of specific IgE inhibition (Fig. 2). The average IgE inhibition among this group was 82 ± 2.4 , which was significantly

higher compared to the no sugar and reduced sorbitol group. The average specific IgE inhibition among *A. alternata* extracts without any sugar was 62 ± 3.2 . In contrast, the group with reduced sorbitol had an average specific IgE inhibition of 64 ± 2.3 . In conclusion, we found that there was no significant difference in specific IgE inhibition between the no sugar and reduced sorbitol groups.

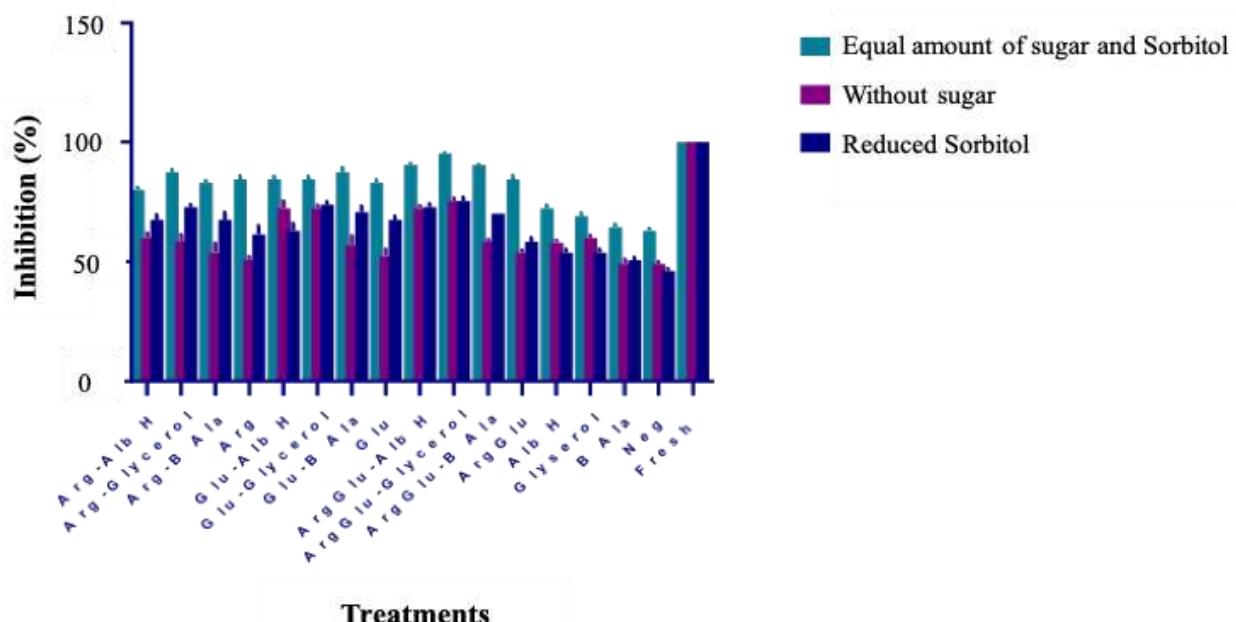


Fig. 2. Inhibition of specific IgE in pooled patient sera with different allergenic *A. alternata* extract formulations after a 40-day incubation period at 37 °C.

Further, specific IgE inhibition in the presence of Arg, Glu, Arg/Glu, β -Ala, h Alb, and glycerol was evaluated. We found that the addition of these stabilizers had no profound effect on allergenic *A. alternata* extract activity compared to untreated controls.

We then evaluated the combinatorial effect of these stabilizers, since there was no significant impact on allergenic *A. alternata* extract activity on their own.

The formulation containing an equal sorbitol, sucrose and protein ratio and Arg/Glu/Glycerol reached a specific IgE inhibition of $95\% \pm 0.05$. In the same sugar group, combining Arg/Glu/ β -Ala or Arg/Glu/ h Alb reached a specific IgE inhibition of 90.7%. To conclude, we used the most effective stabilizer formulation, that protected more than 95% of *A. alternata* extract activity, to evaluate the activities of Alt a1 and other allergenic mold extracts (Table 2).

Table 2. Selected formulation ingredient.

Protein content (mg/ml)	Solvent buffer (mM)	Sugar (μ g/ml)	Glycerol (%)	h Albumin	Amino acids (mM)
100	PBS (150)	Sorbitol (100) Sucrose (100)	20	-	Arg (25) Glu (25)

Alt a1 activity

We conducted an Alt a1 ELISA on days 7, 14, 21, 28, and 40 to compare Alt a1 levels in the presence of our selected formulation and untreated extract, or untreated fresh extract. Further, we evaluated Alt a1 levels in the presence of a formulation similar to our selected formulae. Our data showed that during a 40-day incubation period, there was decreased Alt a1 activity (43%) in untreated extracts. In comparison to the

untreated extract, our selected stabilizer formulation had a significant impact on Alt a1 activity. While the activity of the fresh extract was normalized to 100%, our selected stabilizer formulation showed minimal decline in Alt a1 activity and reached 97.5% after 40 days. Moreover, replacing Arg with Lys demonstrated similar results (93.9%), and there was no significant difference compared to our selected formulation (Fig. 3).

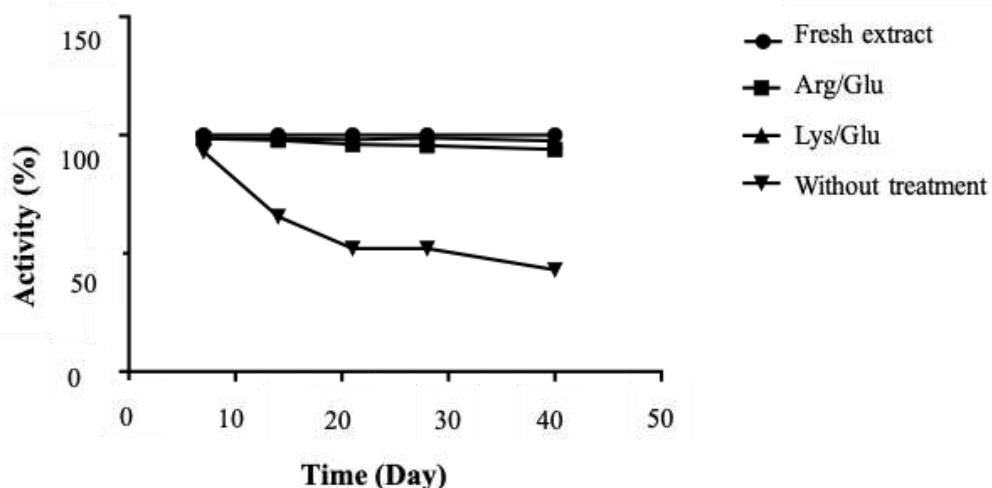


Fig. 3. Alt a1 activity during 40 days of incubation at 37 °C while treated with different stabilizers.

Other molds

The potency of allergenic *A. fumigatus* extracts to inhibit specific IgE during a 40-day incubation period at 37 °C was evaluated using a specific IgE inhibition ELISA (Fig. 4). We clearly demonstrate that specific IgE inhibition in fresh extract was dramatically higher

compared to non-treated extracts which had decreased to 27%. Treating allergenic *A. fumigatus* extracts with a stabilizer that contained Arg/Glu or Lys/Glu led to a higher specific IgE inhibition compared to the non-treated extract, but similar to fresh extract. Furthermore, we found that the specific IgE inhibition for Arg/Glu

and Lys/Glu treatments were 96% and 92% respectively. Although there was no significant difference between these two treatments, Arg/Glu treatment was more favorable. Interestingly, we found similar results with allergenic *C. herbarum* extracts (Fig. 5). Overall, our results show that there is a significant difference between fresh extract and non-

treated extracts. While non-treated extracts experienced 28% allergenic reactivity and inhibition, Arg/Glu treatment, consequently, protected 97% of allergenic mold activity. Lastly, extracts treated with the stabilizer formulation containing Lys/Glu preserved 88% of allergenic mold activity after 40 days, which is significantly different from using Arg/Glu.

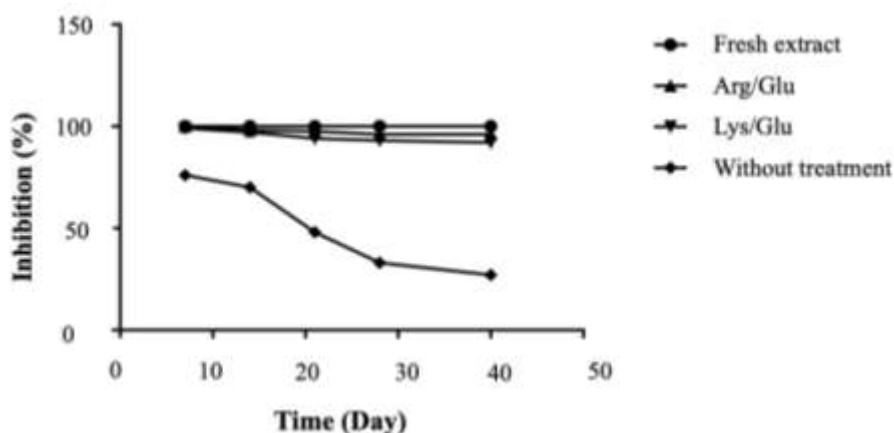


Fig. 4. Specific IgE inhibition with Aspergillus allergenic extract, which treated with different stabilizers during 40 days of incubation at 37 °C.

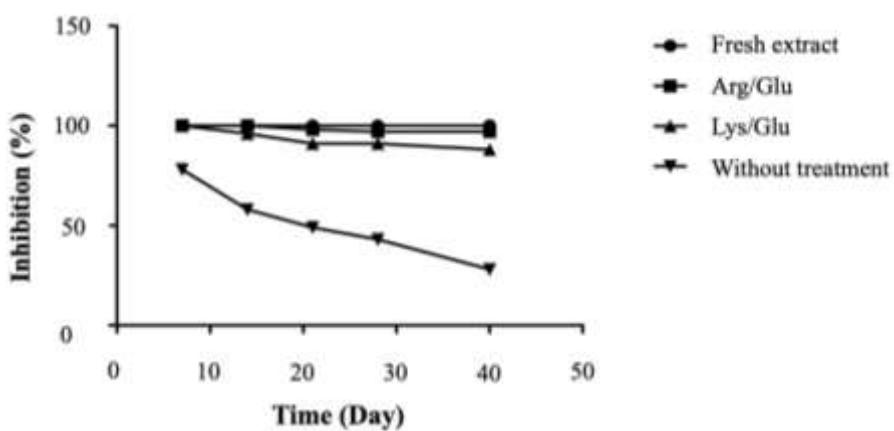


Fig. 5. Specific IgE inhibition with Cladosporium allergenic extract with different stabilizers during 40 days of incubation at 37 °C.

Discussion

With the rise in allergic diseases, allergenic extracts must remain stable and therefore, active during long-term storage conditions for their continued use in clinical and research settings. Allergenic extract stability greatly depends on the number and type of allergen present. Indeed, the presence of stabilizers and another chemical material may also play a critical role in preserving stability (12). Accordingly, any given extract may lose allergenic capacity due to the degradation of specific allergens rather than a general

reduction in the potency of all allergens over time (16). Therefore, specific factors affecting stability must be clearly identified to ensure the functional longevity of allergenic extracts. Furthermore, a universal stabilizer formulation cannot prolong the potency of an entire extract containing allergens of diverse origin such as house dust mites, animal-related allergens, molds or pollens (12). Hence, this study was an attempt to introduce a specific stabilizing formulation for allergenic molds.

Different extraction methods can profoundly affect the potency of allergenic mold extracts. However, to gain a critical understanding of the factors affecting the stability and potency of mold extracts, high-quality extracts are required. In this study, we used a high yield extraction protocol that included an anti-protease supplement to increase the quantity and potency of our allergen of interest. Further, since molds are heavily pigmented with melanin, we included PVP during the extraction process to prevent protein precipitates. Moreover, PVP also binds to polyphenols, which can greatly improve yield. Additionally, EDTA, PMSF, and sodium chloride (NaCl) were also added to inhibit protease activity (17, 18).

Most pharmaceutical companies suggest an expiry date of two years for skin prick tests(19). According to the Arrhenius equation, we increased the temperature to 37 °C and achieved an expiration date of 28 days, which is equivalent to two years at 4 °C (15). Eventually, a 40-day incubation period at 37 °C was used to determine the most effective stabilizer formulation after 28 days. Similar to our experimental system, others have also evaluated extract stability at incubations different from two years at 4 °C, such as 3 days of incubation at 56 °C (20). Moreover, additional days including day 7, 14, 21, and 28 were evaluated to follow an IgE-mediated allergenic activity trend and to reveal any possible points of degradation. Overall, we extended the incubation period to reveal the stabilizing potency of all stabilizers used in this study.

Most articles recommend using 50% glycerol, which has been suggested to be a good stabilizer as it is a common a preservative found in various skin prick tests (10). According to our results, 50% glycerol on its own was unable to stabilize allergenic activity during our specified incubation period and temperature. Since glycerol is a strong irritant, we used 20% glycerol. At this concentration, we aimed to maintain a similar stability as claimed by 50% glycerol users, while preventing bacterial growth (11). Though 20% glycerol was not sufficient to maintain allergenic activity alone, formulations containing 20% glycerol with other components demonstrated better stability. According to recent evidence, allergenic pollen extracts without glycerol, but had included amino acids, had higher stability compared to glycerol-based extracts that contained 50% glycerol (20). In addition to glycerol, others have shown that the addition of β -Ala or h Alb to the final formulation can also stabilize

allergenic potency (21). However, we showed that β -Ala or h Alb individually was unable to maintain allergenic activity during a 28-day incubation period at 37 °C.

Some studies have reported that sugars and polyols can improve stability of protein-based media (22). In our study, we found that sucrose and sorbitol were only effective at an equal concentration to proteins. Further, there was a dramatic decline in allergenic activity if sorbitol was reduced to 25%. Therefore, reducing sorbitol would not be applicable to our purpose, because we found stability to be less than or similar to extracts containing no sugar.

Though there is an overwhelming body of evidence to illustrate the importance of amino acids in stabilizing protein-based media, but their role in the allergenic extracts is much less characterized in the literature (23, 24) Therefore, both Arg and Glu were evaluated in our extract separately and in combination with each other. Separately, Arg and Glu was able to preserve allergenic activity until day 28 but not after 40 days at 37 °C. Thus, the individual use of Arg or Glu is not sufficient to protecting thermostability and allergenic activity of mold extracts during our desired incubation conditions.

When used together, Arg and Glu improved stability remarkably and maintained allergenic activity until day 40. Such a drastic observation could have occurred because Arg or Glu are both charged amino acids, and using them individually could have a profound or permanent effect on the net charge of proteins which could, therefore, promote degradation processes (14).

In addition, based on our observations of the allergenic potency of Alt a1 and other molds with our selected and altered formulation, we concluded that replacing Arg with Lys is not necessary to improve potency of mold extracts. It is unlike previous study on pollen allergenic extracts(23).

As this particular area of study is not well characterized, there are many potential opportunities for future investigations and immunotherapies, as there a plethora of proteins and glycoproteins within mold extracts that also require our attention (25, 26). There is conflicting consensus within the literature on what exactly determines the best possible outcome for allergenic formulations, in terms of stability, for instance, some studies suggest using other amino acids (27), while others recommend other sugars such as

mannitol and trehalose to stabilize extracts (28). In addition, ions used in buffers may also play a pivotal role in stabilizing solutions, and preventing protein degradation (10).

Though there is a critical need for examining all fungi and other chemical stabilizers, this paper provides a simple formulation to improve allergenic fungi extract stability, since many industrial formulations are strictly for commercial use, and thus, confidential property.

In addition to using sorbitol, sucrose, and glycerol, which are among the most traditionally used preservatives to date, we found that amino acids play a larger role in stabilizing allergenic activity in mold

extracts. Though we proved that Arg/Glu was the best candidate during our study, this simple formulation should not be generalized for all allergenic extracts since their allergen content may vary in type and number. In this regard, future research should be conducted for other extracts and amino acids.

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