

Determining the Effect of Amino Acids on the Allergenic Activity of Pollen Extracts

Nazila Ariaee¹, Mojtaba Sankian², Abdolreza Varasteh¹,
Mitra Hosseinpour³, Farahzad Jabbari^{*1}

Abstract

Background: The diagnosis and treatment of allergic diseases require high quality pollen allergen extracts for reliable test results and effective treatments. The quality of the pollen allergen extracts is influenced by pharmacologically inert ingredients, such as stabilizers which are added to prevent the degradation of the allergenic activity. This study was conducted to develop a stabilizer formulation in order to protect the allergenic activity of the pollen's extracts.

Methods: Pine and orchard grass pollen allergen extracts were incubated for 40 days at 37 °C. The effects of chemicals were examined via inhibition ELISA on days 7, 14, 21, 28, and 40 to evaluate the ability of the pollen allergen extracts to inhibit specific IgE in the sera of sensitized patients.

Results: Our findings showed that the pine pollen and orchard grass allergen extracts treated with Lys/Glu had the best stabilizing effect resulting in a 97% IgE inhibition following the 40 days of incubation. In the non-treatment group, the IgE inhibition decreased to 23% at the end of the 40 days. The orchard grass pollen allergen extracts receiving no treatment decreased to 12% IgE inhibition following the 40-day incubation.

Conclusions: Amino acids are able to act as an effective stabilizer for pollen allergen extracts and prevent the degradation of their activity over time. Particularly applying Lys/ Glu in pollen allergenic extracts can protect allergenic activity and potency of the pollen extracts to inhibit specific IgE in human sera.

Keywords: Amino acids, Pollen, Skin Prick Test, Stabilizing.

Introduction

Allergic rhinitis is one of the most common allergic disorders, affecting roughly 30% of the population worldwide (1). Pollens currently rank as the leading aeroallergens in the world and are the main cause of allergic disease, particularly in developed countries (2). Pollen allergens are biologically active substances that are natural products of most plant species (3). These pollen allergens are dispersed throughout the environment and are considered a significant risk factor for seasonal allergic rhinitis and asthma (4). For the diagnosis and treatment of allergic disease, a variety of different pollens are used as source material for the production of allergen extracts. The allergenic content of pollen varies based on the plant species and region in which the plants are grown (3).

Skin prick test (SPT), Radioallergosorbent Test (RAST), and enzyme-linked immunosorbent assay (Pollen allergen extracts are commonly used in many different diagnostic tests for allergic disease including, the skin ELISA). Furthermore, pollen allergen extracts are used in the treatment of allergic diseases in allergen immunotherapy process(5). Pollens are considered an active pharmaceutical ingredient. Therefore, the production and quality of pollen allergen extracts is highly regulated by administrative agencies (3). A significant challenge regarding pollen is the fact that different pollen grains have various allergenic contents. Therefore, a standardized method for the production of pollen allergen extracts cannot be applied to all types of

1: Allergy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran.

2: Immunology Research Center, Medical School, Mashhad University of Medical Sciences, Mashhad, Iran.

3: Shahid Beheshti University of Medical Sciences, 15 Khordad Hospital, Tehran, Iran.

*Corresponding author: Farahzad Jabbari; Tel: +98 51 38012770, Fax: +98 51 384280 14; E-mail: Jabbarif@mums.ac.ir.

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pollen(6). An additional challenge is in stabilizing the pollen allergen extracts for a prolonged period of time (7). According to many related guidelines, allergenic extracts should remain active for two years of storage at 4 °C (8). All extracts used in the clinics for skin prick tests and immunotherapy are complex mixtures of proteins and glycoproteins. In this regard, only a small amount of these extracts are allergens. Therefore, a uniform formula for the entire allergenic extracts with different structure, origin, and chemistry cannot be much efficient (9). In the present study, we aimed to identify the ability of amino acids to stabilize and prevent the degradation of pollen allergen extracts over a prolonged length of time.

Materials and methods

Pollen allergen extracts

Both *Pinus eldarica* and *Dactylis glomerata*, also known as pine and orchard grass, respectively, are common allergens in Mashhad, Iran and were therefore selected as the allergens for this study. Pollen collection was performed by trained pollen collectors from the Research Center for Plant Sciences, Ferdowsi University, Mashhad, Iran. Pollen collection took place throughout the pollination season over three consecutive years. The collected pollen grains were dried, then separated by passing the dried materials through different sieves. The resulting fine powder was examined for purity and pollen content using light microscopy. Pollen grains were defatted using cold acetone. Roughly 2 g of pollen was extracted in 10 ml of phosphate-buffered saline (PBS) 150 mM (pH 7.4) by continuous stirring at 4 °C for 18 h. The supernatants were separated by centrifugation for 20 min 255 g and collected after filtering. To avoid any contamination, the extracts were filtered through a 0.22 µm membrane under sterile conditions. The isolated pollen grain extracts were dialyzed against potassium phosphate buffer (10). The protein content of the pollen allergen extracts was measured using Bradford's method. The allergen extracts were freeze-dried and stored at -20 °C until further analysis.

Patient's sera

A total of 30 participants were enrolled in our study ranging from ages 15-55 years old. All participants enrolled in the study were patients from the allergy clinic at the Qaem Hospital, Mashhad, Iran. Among

this investigation, allergic rhinitis and rhino conjunctivitis were the most prevalent allergic diseases. 3 ml peripheral blood was obtained from subjects and their sera were isolated. Fifteen of the patients were sensitive to pine and the other 15 showed sensitivity to orchard grass. These allergens are among the most common aeroallergens in Mashhad, Iran (11). Allergen sensitivity was determined via skin prick testing (Stallergren Greer, USA). Our study was approved by the Ethics Committee of Mashhad University of Medical Sciences (code: 940043). Prior to participating in the study all patients signed written informed consent agreements.

Pollen allergen extract stabilization

Phosphate Buffer Saline (PBS) with 20% glycerol was used as a final solvent buffer for all extracts (12). Equal parts of sorbitol and sucrose were added based on the measured protein content of the pollen allergen extracts (5). The total protein concentration of the pollen allergen extracts was determined to be 200 µg per ml. Therefore, the final stabilizer contained both sorbitol and sucrose at a concentration of 200 µg per ml.

For stabilizing the pollen allergen extracts, Glutamate (Glu) and Arginine (Arg) were used at a concentration of 25mM (12). Mannitol (Man) and Lysine (Lys) were also used at a concentration of 2% (13). The effect of these chemicals on the stability of pollen allergen extracts and their ability to inhibit specific IgE was evaluated throughout a 40-day incubation period maintained at a temperature of 37°C. An inhibition ELISA was performed on days 7, 14, 21, 28, and 40. According to the Arrhenius equation (14), the Inhibition ELISA on 28 days was selected to have a precise assessment of the stability trend. The length of time required for sufficient stabilization of the pollen allergen extracts was determined to be 2 years if incubated at 4 °C, as most of the factories declare an expiry date of the allergen extracts after 2 years for skin prick tests. Accordingly, we increased the temperature to 37 °C and incubated the extracts for at least 28 days.

Inhibition ELISA

To evaluate the best stabilizing method for pollen allergen extracts, an inhibition ELISA was used. The ELISA plate was coated with 200 mmol bicarbonate

buffer (pH 9.6) solution containing 200 ng per ml of the fresh pollen extracts. The plate was blocked with 2% Bovine Serum Albumin (BSA) and incubated for one hour at 37 °C. In a separate plate, the serum of sensitized patients was incubated with the pollen allergen extracts treated with different stabilizers for 2 h at room temperature. The specific IgE in the pooled sera were inhibited by the allergenic potency of pollen extracts. 100 µl of allergen-treated sera, either for pine or orchard grass, were added to the first plate following the coating and blocking process. Finally, biotinylated Anti-IgE (Santa Cruz) was added to plate and incubated for 2 h. Consequently, streptavidin-horseradish peroxidase (HRP) (Becton Dickinson, USA) was added and 1 h incubated at room temperature. For the substrate solution, 3,3',5,5'-Tetramethylbenzidine (TMB) was used.

For the negative controls, two wells were incubated without any pollen allergen extract and blocked with the block buffer solution, a separate two wells were incubated with nothing except substrate, and two separate wells were incubated with no antibody. For without inhibition control two wells that were incubated with patient serum, and the pollen allergen extract was replaced with PBS. For the positive control, patient serum was incubated with fresh pollen allergen extract.

Statistical analysis

Data analysis was performed using GraphPad Prism software version 6 (California corporation, USA). To analyze the normal distribution of the two groups, the treatment and control groups, the Kolmogorov-Smirnov test was applied. Considering the normal distribution of data in the groups, we used T-test and one-way ANOVA to determine the differences and compared the means of different days in the treated and control groups. Differences were considered statistically significant at a $p < 0.05$.

Results

Different amino acids were observed to significantly affect the IgE reactivity and stability of pine pollen allergen extracts. Our findings revealed that treatments containing Lys/Glu or Arg/Glu had the highest IgE inhibition (Fig. 1). The percentage of IgE-specific inhibition in the allergen extracts containing Lys/Glu was 97%, while the extracts treated with Arg/Glu had 93% IgE inhibition after 40 days

incubation. The control group receiving no amino acid treatment was observed to have 23% IgE inhibition. The stabilizing with Mannitol showed an 83% IgE inhibition following the 40 days of incubation, which was significantly lower than the groups containing Lys/Glu or Arg/Glu.

As seen in Figure 1, our findings show that adding Lys or Glu individually was not as effective as combining them. Additionally, our data indicates that pine pollen allergen extracts treated with Lys had a higher IgE inhibitory effect than extracts treated with Glu. The IgE specific inhibition was 66% in pine pollen allergenic extract treated with Lys, while the extracts treated with Glu showed 38% inhibition. Adding a combination of Mannitol with Glu did not improve the stability of the pollen allergen extracts. In fact, the IgE inhibition of the pine pollen allergen extracts declined to 30%, however, this difference in IgE inhibition with Glu or Glu/Man treatment was not statistically significant. Treatment with a combination of Mannitol and Lys resulted in an IgE inhibitory effect of the pine pollen allergen extracts of 70%, however this improvement was not statistically significant in comparison to the Lys treatment.

The stabilizing effect of amino acids on orchard grass pollen allergen extracts was also examined via inhibition ELISA over a 40-day incubation period. Our findings showed that the addition of amino acids resulted in higher reactivity of specific IgE. As shown in Figure 2, during the 40 days of incubation, the IgE reactivity in all groups decreased. The group with no treatment experienced a sharper decline in IgE inhibition, reaching 30% after the 40 days of incubation. The orchard grass pollen allergen extracts containing Lys/Glu stabilized the reactivity of allergen-specific IgE such that its inhibition was at 97%, while the pollen allergen extracts treated with Arg/Glu showed an IgE inhibition of 80% after 40 days incubation. Similar to the pine pollen allergen extracts, when Lys and Glu were used individually the IgE inhibition reach 65% and 42%, respectively. Adding Mannitol to the treatments with Lys or Glu caused a further decrease in IgE inhibition to 53% and 25%, respectively. With respect to the orchard grass, the IgE inhibition ELISA showed that although Lys and Mannitol reached a fairly similar point on day 40, these two groups had a different trend in stabilization throughout the incubation period, and the difference between them was statistically significant.

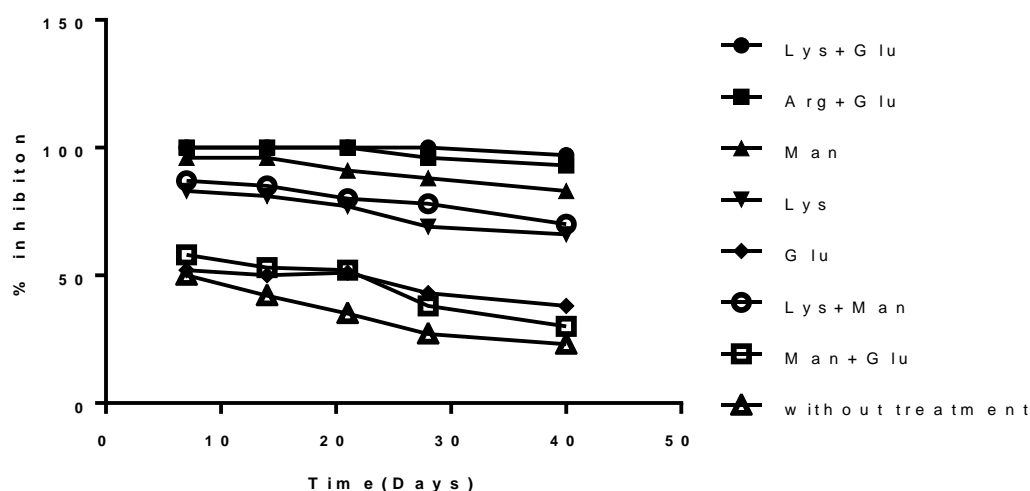


Fig. 1. Comparison of the percentage of IgE inhibition between different treatments in pine pollen allergenic extract throughout 40 days of incubation at 37 °C using inhibition ELISA.

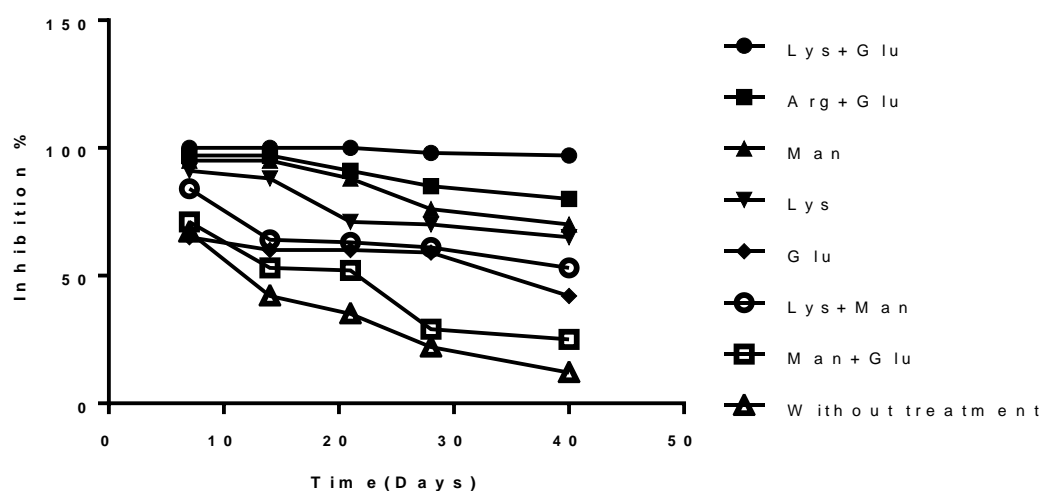


Fig. 2. Comparison of the percentage of IgE inhibition between different treatments in orchard grass pollen allergenic extracts throughout 40 days of incubation at 37 °C using the inhibition ELISA.

Discussion

Allergen extracts are required to remain stable for the proper diagnosis and treatment of allergic disease. The stability of allergen extracts depends on the number of allergens and their activity. The use of stabilizers holds a critical role in preventing the degradation of the allergenic potency of the allergen extract and their ability to inhibit specific IgE (15).

It has been previously suggested that the allergenic activity of the extract may decline due to the degradation of specific allergens rather than a general reduction in the potency of all allergens (16). Therefore, factors affecting stability must be clearly identified for each type of allergen extract to ensure their stability (17). Recent investigation has shown

that a universal method for stability does not work for allergen extracts with different origins such as, house dust mite, animal-related allergens, or pollens (9, 18). Therefore, this study aimed to introduce a specific stabilizing method for pollen allergenic extracts.

Although a large amount of evidence has shown the efficacy of amino acids in stabilizing protein-based media, there are few reports examining the use of amino acids for stabilizing pollen allergen extracts (9, 19). Therefore, we evaluated the effects of different amino acids and amino acid combinations on pollen allergen extract stability. Our findings showed that the use of Arg or Lys did not significantly improve the stability of the pollen allergen extracts, however, when used in combination there was a significant increase in the stability of the extracts. This may occur due to the charge of the amino acids. The use of the charged amino acids individually may affect the net charge of the pollen allergen extract. This change in the net charge is not sufficient for preventing the degradation of the allergen extract (12). The difference between the effect of treatment with Arg/Glu and Lys/Glu may occur due to the differences between Lys and Arg. Although Lys and Arg have the same charge and both are able to neutralize the negative charge of Glu, they have different structures which may account for this variation (20). We also observed that replacing amino acids with an uncharged chemical, such as Mannitol, did not improve the ability of the allergen extract to inhibit specific IgE. Based on our results, treatment with Man/Glu was not effective in stabilizing pine or orchard grass allergen pollen extracts. The effect of this combined treatment was similar to the effects of no treatment. Since the difference between groups including Glu or Man/Glu was statistically significant, this may indicate that a negative charge has less effective on stabilizing and preserving the potency of allergen extracts and preserving their ability to react to specific IgE.

In line with our previous research which enrolled by Jabbari in 2019 (21) our results showed that Mannitol can improve the stability of pollen allergen extracts. Our findings show that the efficacy of Mannitol was less than the combined use of two amino acids with different charges. Furthermore, the combination of Mannitol with charged amino acids

did not improve the stability or maintain the potency of the pollen allergen extracts. Previous research has indicated that amino acids can act as a preservative and have a role in stabilization, in which Lys can inhibit enzymes function (20) and Mannitol can prevent Maillard reactions (22). The findings of our study show that Lys/Man is better than Glu/Man for the stabilization of the pine and orchard grass pollen allergen extracts. It should be noted that Mannitol is unable to maintain the allergenic potency of the extracts beyond day 28, while the combined amino acid treatments of Lys/Glu or Arg/Glu maintained the potency throughout the 40-day incubation period. There is a possibility that other allergen extracts with different origins, such as house dust mites, animal, or even other plant pollens will yield different results for various amino acids. The effect of amino acids on these different allergens should be explored in future studies.

Previous research has shown that glycerol is an effective stabilizer, however it is a strong irritant. In this investigation, we attempted to reduce glycerol contents (23, 24) and selected the minimum glycerol concentration with a bacteriostatic effect. In a previous study, it was shown that pollen allergen extracts without glycerol, that contained amino acids had a higher stability compared with a glycerol-based extract, which typically contains 50% glycerol (21).

In conclusion, although chemicals like human serum albumin and glycerol are the most common and traditional stabilizers, amino acids are a potential candidate for pollen allergen extracts. Despite showing that Lys/Glu is the best stabilizer for pine and orchard grass pollen allergenic extracts, it cannot be generalized for all allergenic extracts because of the different allergenic contents. Future research should examine the effect of amino acids on other allergen extracts to determine the most effective stabilizers for that allergen.

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References

1. Rapiejko P, Jurkiewicz D, Pietruszewska W, Zielnik-Jurkiewicz B, Woron J, Lipiec A. Treatment strategy of allergic rhinitis in the face of modern world threats. *Otolaryngologia polska= The Polish otolaryngology*. 2018;72(2):1-12.
2. Lake I, Colon F, Jones N. Quantifying the health effects of climate change upon pollen allergy: a combined cohort and modelling study. *The Lancet Planetary Health*. 2018;2:S16.
3. Codina R, Lockett RF. Pollen used to produce allergen extracts. *Ann Allergy Asthma Immunol*. 2016;30(1):e6.
4. Asam C, Hofer H, Wolf M, Aglas L, Wallner M. Tree pollen allergens—an update from a molecular perspective. *Allergy*. 2015;70(10):1201-11.
5. Ariaee N, Varasteh A, Jabbari Azad F, Sankian M. Preparation allergenic pollen extracts; the points should be considered to make high-quality products. *Journal of Immunoassay and Immunochemistry*. 2018:1-14.
6. Fernández-Caldas E, Carnés J, Iraola V, Casanovas M. Comparison of the allergenicity and Ole e 1 content of 6 varieties of *Olea europaea* pollen collected during 5 consecutive years. *Annals of Allergy, Asthma & Immunology*. 2007;98(5):464-70.
7. Becker W-M, Vogel L, Vieths S. Standardization of allergen extracts for immunotherapy: where do we stand? Current opinion in allergy and clinical immunology. 2006;6(6):470-5.
8. Plunkett G. Update: stability of allergen extracts to establish expiration dating. Current opinion in otolaryngology & head and neck surgery. 2016;24(3):261-9.
9. Grier TJ, Hall DM, Duncan EA, Gada SM. Allergen stabilities and compatibilities in immunotherapy mixtures that contain cat, dog, dust mite, and cockroach extracts. *Annals of Allergy, Asthma & Immunology*. 2015;115(6):496-502.
10. Tehrani M, Sankian M, Assarehzadegan MA, Falak R, Jabbari F, Varasteh A. Immunochemical characterization of *Amaranthus retroflexus* pollen extract: extensive cross-reactive allergenic components among the four species of *Amaranthaceae/Chenopodiaceae*. *Iranian Journal of Allergy, Asthma and Immunology*. 2010;9(2):87-95.
11. Oskouei YM, Hosseini RF, Ahanchian H, Jarahi L, Ariaee N, Azad FJ. Report of common aeroallergens among allergic patients in northeastern Iran. *Iranian journal of otorhinolaryngology*. 2017;29(91):89.
12. Golovanov AP, Hautbergue GM, Wilson SA, Lian L-Y. A simple method for improving protein solubility and long-term stability. *Journal of the American Chemical Society*. 2004;126(29):8933-9.
13. Bharate SS, Bharate SB, Bajaj AN. Interactions and incompatibilities of pharmaceutical excipients with active pharmaceutical ingredients: a comprehensive review. *Journal of Excipients and Food Chemicals*. 2016;1(3):1131.
14. Pawar S, Dell AI, Savage VM, Knies JL. Real versus artificial variation in the thermal sensitivity of biological traits. *The American Naturalist*. 2016;187(2):E41-E52.
15. Glesner J, Filep S, Schulten VM, Frazier A, Vailes LD, Wünschmann S, et al. Potency Of German Cockroach Extracts For IgE Reactivity Depends On Allergen Content And Allergen-specific IgE Titers Of The Cockroach Allergic Patient. *Journal of Allergy and Clinical Immunology*. 2018;141(2):AB108.
16. Homer W, Helbling A, Salvaggio J, Lehrer S. Fungal allergens. *Clinical microbiology reviews*. 1995;8(2):161-79.
17. Himly M, Nandy A, Kahlert H, Thilker M, Steiner M, Briza P, et al. Standardization of allergen products: 2. Detailed characterization of GMP-produced recombinant Phl p 5.0109 as European Pharmacopoeia reference standard. *Allergy*. 2016.
18. Zimmer J, Bonertz A, Vieths S. Quality requirements for allergen extracts and allergoids for allergen immunotherapy. *Allergologia et immunopathologia*. 2017;45:4-11.
19. Grier TJ, Hall DM, Duncan EA, Coyne TC. Mixing compatibilities of *Aspergillus* and American cockroach allergens with other high-protease fungal and insect extracts. *Annals of Allergy, Asthma & Immunology*. 2015;114(3):233-9.
20. Platts L, Darby SJ, Falconer RJ. Control of Globular Protein Thermal Stability in Aqueous Formulations by the Positively Charged Amino Acid Excipients. *Journal of pharmaceutical sciences*. 2016;105(12):3532-6.
21. Mansouritorghabeh H, Sankian M, Varasteh A-R, Moghadam M, Khameneh B, Farid-Hosseini R, et

- al. Determination of Optimum Excipients for *Platanus orientalis* Pollen Extract by Accelerating Chemical Stability Test and Their Synergistic Effect. *Reports of Biochemistry and Molecular Biology*. 2019;7(2):189-95.
22. Ohrem HL, Schornick E, Kalivoda A, Ognibene R. Why is mannitol becoming more and more popular as a pharmaceutical excipient in solid dosage forms? *Pharmaceutical development and technology*. 2014;19(3):257-62.
23. Jutel M, Agache I, Bonini S, Burks AW, Calderon M, Canonica W, et al. International Consensus on Allergen Immunotherapy II: mechanisms, standardization, and pharmacoeconomics. *Journal of Allergy and Clinical Immunology*. 2016;137(2):358-68.
24. Jutel M, Kosowska A, Smolinska S. Allergen Immunotherapy: Past, Present, and Future. *Allergy, asthma & immunology research*. 2016;8(3):191-7.