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PHD THESIS

**IGE SENSITIZATION PROFILES IN RAGWEED POLLEN ALLERGY
AND CLINICAL RELEVANCE OF THE RECOMBINANT AMB A 1
AND AMB A 8 ALLERGENS**

A B S T R A C T

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ABSTRACT

GENERAL PART

Allergy is an exaggerated reaction of the immune system to harmless exogenous stimuli and involves various types of hypersensitivity reactions, which can lead to respiratory, skin, gastrointestinal, ocular or other symptoms, including anaphylaxis. Type I hypersensitivity reaction, also called immediate immune response, is an abnormal immunological response caused by a harmless substance from the environment, called an allergen, and is mediated by IgE antibodies.

Currently, there is a consistent increase in allergy prevalence, attributed to changes in lifestyle (mainly to western lifestyle), dietary habits and environment, including climate change. Worldwide, 8-10% of people suffer from allergic diseases, ranging from mild symptoms such as rhinitis (10-30% of the population) or conjunctivitis to severe symptoms such as asthma (3-9% of the population) or anaphylaxis.

One significant cause of the increased incidence of allergic diseases is ragweed pollen, a major problem for the healthcare system. *Ambrosia artemisiifolia*, also known as common ragweed, is a weed native to North America that has spread to several continents due to its invasive nature. In Europe, the most ragweed pollen-affected area is the Pannonian Plain, but it has also a significant negative impact in Romania, increasing the number of allergic patients affected during the pollen season, from August to September. Recent data collected by allergists report a high rate of sensitization and severe respiratory symptoms caused by ragweed pollen. It is even predicted that sensitization to ragweed in Europe will increase from approximately 33 million to 77 million individuals (40% of the population) by 2041-2060, due to high concentration of ragweed pollen spreading over a broad geographic area.

Currently, 11 molecular allergens have been identified and described for ragweed pollen, two of which are major allergens: Amb a 1 and Amb a 11, while the rest are considered minor allergens. Amb a 1 belongs to the family of pectate lyases and is considered a major allergen because it has the potential to cause allergic reactions in more than 90% of patients sensitized to ragweed pollen. Additionally, Amb a 1 is an extremely abundant protein, constituting up to 15% of the total protein content of ragweed pollen and representing 54–78%

of the allergenic component. Amb a 1 has 5 documented isoforms, each with different IgE binding capacities, with the first isoform, Amb a 1.01, showing the highest allergenic activity.

Among the minor allergens are two plastocyanins (Amb a 3 and Amb a 7), a defensin (Amb a 4), a basic protein whose function is not yet known (Amb a 5), the pan-allergens Amb a 6 (lipid transfer protein), Amb a 8 (profilin), Amb a 9 and 10 (polcalcins), and enolase Amb a 12. Although they are considered minor allergens based on their IgE binding frequency, this does not mean they are not clinically relevant. However, this relevance needs to be determined for each allergen individually, which requires obtaining them as purified allergens. Among the ragweed allergens with potential clinical importance is also Amb a 8, a profilin structurally similar to homologous proteins from other allergenic sources such as mugwort, birch, and timothy grass pollen. Producing and including clinically relevant allergens in the molecular diagnosis of ragweed allergy will improve the clinical management of this disease.

SPECIAL PART

Among the main aspects that outline the motivation for choosing this study topic is the negative impact of ragweed pollen allergy on public health caused by the constant increase in prevalence and the severe symptoms that affect the quality of life of allergic patients. Furthermore, it is important to have a clear overview of the molecules involved in ragweed allergy and, based on the IgE reactivity profile, to identify which allergens are clinically relevant. Additionally, producing them as recombinant allergens will facilitate molecular allergy diagnosis and help overcome the limitations of allergenic extracts. Identifying the IgE epitopes of a clinically important allergen can also contribute to the development of novel molecular immunotherapies.

The main scientific objectives of this research study were

1. Identification of sensitization profile of patients allergic to ragweed pollen and allergenic potential assessment of other ragweed allergens besides Amb a 1;
2. Identification of potential associations between the IgE sensitization profile and the allergy-related symptoms reported by the ragweed-allergic patients;
3. Recombinant production of the most important Amb a 1 isoform and its physicochemical and immunological characterization;
4. Recombinant production of profilin Amb a 8, physicochemical and immunological characterization, and evaluation of potential cross-reactivities;
5. Design and characterization of Amb a 8-derived peptides for Amb a 8 IgE epitope mapping.

IDENTIFICATION OF IGE SENSITIZATION PROFILES IN RAGWEED POLLEN ALLERGY

In order to identify IgE sensitization profiles, sera from 150 patients allergic to ragweed pollen were tested in ImmunoCAP to identify the presence of CCD-specific IgE (cross-reactive carbohydrate determinants). Next, the sera of CCD-negative patients (n=130) were evaluated in immunoblot with ragweed pollen extract transferred to a nitrocellulose membrane, followed by detection of IgE binding with antibodies labelled with radioactive isotopes. The results of this analysis revealed the existence of 19 different IgE sensitization profiles within this study population. These profiles showed 7 types of IgE signals in different combinations and intensities. Also, to determine the impact of the major allergen Amb a 1 on the reactivity profiles, immunoblot inhibition experiments were performed with 2 isoforms of Amb a 1, one natural (nAmb a 1.01) and the other recombinant (rAmb a 1.03). These experiments showed that the two isoforms have different IgE inhibition capacities, suggesting that they also have different IgE reactivities.

Analysis of the reactivity profiles revealed that the most common IgE signal on the blot is the double band around 38–40 kDa, which based on inhibition assays performed with the two isoforms Amb a 1 can be attributed to Amb a 1. This was also confirmed by other studies. The signals that were still visible after inhibition with Amb a 1 represent the other ragweed pollen allergens. Thus, the two bands observed below the molecular mass of 17 kDa could include and represent several minor ragweed pollen allergens, such as lipid transfer protein Amb a 6 (10 kDa), plastocyanins Amb a 7 (10 kDa) and Amb a 3 (11 kDa), Amb a 9 (10 kDa) polcalcin, or Amb a 8 (14 kDa) profilin. The 24 kDa band may represent the defensin Amb a 4, and the band from 55 kDa may be attributed to the recently discovered enolase, Amb a 12.

The association between allergic symptoms reported by patients and the type of IgE sensitization profile showed that patients with a complex reactivity profile experienced more allergic symptoms. Also, patients sensitized to Amb a 1 and other ragweed allergens showed more asthma-like symptoms than those who were sensitized only to the major allergen Amb a 1. All these findings suggest that polysensitization with several ragweed pollen allergens causes more allergic symptoms and induces more frequent asthma-like manifestations.

These results were also verified and confirmed by basophil degranulation assays, using humanized rat basophil leukaemia cells (RBL) which express the high-affinity receptor for IgE (FcεRI) on their surface. These cells were incubated with the serum of patients with a complex sensitization profile, the serum that was previously subjected to a process of Amb a 1-specific IgE removal. The results showed that the stimulation of basophils with ragweed pollen extract

induces the release of chemical mediators even in the absence of the Amb a 1-specific IgE and this proves that sensitization to other ragweed pollen allergens is also capable of inducing basophil degranulation, respectively clinical symptoms.

PRODUCTION AND CHARACTERIZATION OF RECOMBINANT AMB A 1.01 ISOFORM OF MAJOR ALLERGEN AMB A 1 FROM RAGWEED POLLEN

As could be observed in the previous study, the IgE sensitization profiles of ragweed pollen allergic patients are complex and heterogeneous, involving allergic responses to at least 11 allergenic molecules. However, a detailed characterization requires the individual evaluation of each allergen, which is currently impossible given that only Amb a 1 and Amb a 4 allergens are available for diagnosis. Obtaining pure molecular allergens can be achieved either by isolation from the protein extract, a costly method with a high risk of contamination, or by protein expression as recombinant allergens.

As previously mentioned, Amb a 1 is the major ragweed pollen allergen, and 5 isoforms have been identified and described so far, each with different IgE reactivities. For better management of ragweed allergy, it is important to evaluate the clinical impact of each isoform. However, this is limited by the tests available on the market, which use a mixture of the 5 isoforms, without allowing a distinct assessment of each of them.

Previous studies indicate that Amb a 1.01 isoform has the highest allergenicity, and yet it has only been obtained by purification from pollen extract and is not entirely pure. This situation suggests that the optimal solution for obtaining a pure allergen is to produce it as a recombinant protein. Obtaining Amb a 1 isoforms as functional allergens has proven to be difficult and it has not been completely achieved. Attempts to express these allergens in *Escherichia coli* cells produced only misfolded proteins with reduced IgE reactivity. So far, only recombinant Amb a 1.03 obtained in *Pichia pastoris* has demonstrated IgE reactivity comparable to the native isoform. Thus, the objective of this study was to produce Amb a 1.01 as a recombinant protein with similar characteristics to the natural counterpart in order to improve the molecular diagnosis for ragweed pollen allergy.

Therefore, two recombinant proteins representing the mature form of Amb a 1.01 isoform were expressed in insect cells. This protein expression system was chosen because Amb a 1.01 is a protein with a complex structure, with a glycosylation site and disulfide bridges. One of the proteins was designed with an N-terminal His-Tag and an amino acid sequence for His-Tag cleavage (rAmb a 1.01), while the other contained a C-terminal His-Tag (rAmb a 1.01 His+) which was not removed. It was observed that during protein expression,

only rAmb a 1.01 was secreted into the culture medium, while rAmb a 1.01 His⁺ remained intracellularly. Most likely, the rAmb of 1.01 His⁺ formed protein aggregates, a phenomenon encountered usually when the protein is misfolded, this incorrect folding being probably caused by the position of the His-Tag. Both recombinant proteins were purified by affinity chromatography and characterized physicochemically and immunologically in comparison to the natural isoform (nAmb a 1.01).

SDS-PAGE analysis showed that the recombinant rAmb a 1.01 has a slightly higher molecular weight than the natural allergen, but MALDI-TOF analysis confirmed that both allergens have a comparable molecular weight of about 40 kDa. In addition, circular dichroism (CD) analysis showed similar mean residual ellipticity values, suggesting that the secondary structure of the recombinant allergen is similar to that of the natural allergen. In contrast, rAmb a 1.01 His⁺ showed structural differences when compared to the natural isoform.

For the immunological characterization of the 2 recombinant isoforms and their natural counterpart 100 ragweed pollen allergic patients were tested in ELISA to identify their IgE binding potential. The results showed that the natural Amb a 1.01 had an IgE binding frequency of 99%, confirming its major allergen status. Recombinant protein rAmb a 1.01 showed a similar IgE binding frequency and the optical density values strongly correlated with those of nAmb a 1.01. In contrast, rAmb a 1.01 His⁺ showed a lower IgE binding frequency (86%) and a weaker correlation of optical density values with nAmb a 1.01. These results indicate that rAmb a 1.01 has immunological features similar to the natural allergen, while rAmb a 1.01 His⁺ may have a different secondary structure caused by incorrect folding, which impairs its ability to bind IgE.

For clinical relevance evaluation of the allergens, a very important test is the basophil activation test. It allows the assessment of each allergen's ability to trigger mediators release, mediated by specific IgE. Because this type of test is extremely sensitive, it is suitable to determine the association between allergen concentration and allergic response. This study revealed that nAmb a 1.01 and rAmb a 1.01 have comparable and intense allergenic activity, while the allergenicity of rAmb a 1.01 His⁺ was reduced, and detectable only in patients with high levels of IgE and after stimulation with high allergen concentrations. A potential explanation for reduced IgE binding could be a misfolding of rAmb a 1.01 His⁺ after urea denaturation, which hides or blocks the IgE epitopes.

Based on the results of the physicochemical and immunological evaluation, rAmb a 1.01 was found to exhibit a similar structure and IgE reactivity to nAmb a 1.01, suggesting correct folding of this recombinant protein, unlike rAmb a 1.01 His⁺. Thus, in the following

experiments, only the rAmb recombinant isoform of 1.01 was used. including for the production of specific allergen antisera after rabbit immunization.

The importance of the Amb a 1.01 isoform was also assessed by an inhibition assay in ImmunoCAP, in which patients' sera were preincubated with rAmb a 1.01 and pollen extract and then tested for IgE binding to the natural Amb a 1 allergen preparation found on the ImmunoCAP. Following this evaluation, rAmb a 1.01 showed a high potential for IgE binding inhibition suggesting that either the ImmunoCAP with nAmb a 1 contains large amounts of Amb a 1.01 or that patients are mainly sensitized to this isoform. In any case, this recombinant isoform proves to be a suitable candidate for the diagnosis of ragweed allergy, but more detailed studies including other isoforms of the major allergen Amb a 1 are needed.

This is the first study reporting the production of Amb a 1.01, as a functional and correctly folded recombinant protein with comparable characteristics to the natural allergen, thus becoming a valuable resource for improving molecular allergen diagnosis and immunotherapy to ragweed pollen allergy.

PRODUCTION AND CHARACTERIZATION OF RECOMBINANT AMB A 8 ALLERGEN FROM RAGWEED POLLEN

IgE sensitization profiles of ragweed pollen allergic patients frequently showed signals corresponding to other allergens in addition to the major allergen Amb a 1. Among these signals is the one corresponding to 14 kDa and is attributed to profilin Amb a 8. Therefore, this study aimed to determine the clinical importance of Amb a 8. However, for a better identification of its clinical relevance, it is necessary to test each patient for this allergen which might be possible by producing it as a recombinant protein. Also, being part of the profilin family, Amb a 8 shows a high degree of structural identity with other proteins from this family, so this study also aimed to identify its cross-reactivity with extracts and profilins from other allergenic sources. In addition, this study also aimed to identify the IgE binding epitopes of Amb a 8, necessary in future studies for the development of molecular immunotherapies for ragweed pollen allergy or even more, for profilin allergy.

Due to the fact that Amb a 8 it is a small protein with a simple structure, the recombinant allergen (rAmb a 8) was produced using the *E. coli* cells protein expression system. Because during the protein expression, rAmb 8 formed inclusion bodies, it required isolation and purification under denaturing conditions, using a large amount of urea which was subsequently removed by dialysis. The protein was further tested in SDS-PAGE and presented a molecular weight (approx. 15 kDa) similar to that reported in the literature, which

was also supported by the MALDI-TOF evaluation. Also, these tests showed that rAmb a 8 forms dimers, which was also observed in other profilins, including natural ones, proving that this dimerization is not caused by the recombinant production of the allergen. Circular dichroism measurements revealed that rAmb of 8 exhibits a proper folding.

The allergenicity of rAmb a 8 was determined by IgE binding analysis in ELISA with sera from 255 patients allergic to ragweed pollen. This allergen maintained its status of minor allergen with an IgE frequency of 27.17%. However, when tested in RBL assay it showed significant allergenic activity, inducing mediator release in 5 out of the 6 patients tested, with an intensity similar to or even higher than that induced by the major allergen Amb a 1. These results indicate that rAmb a 8 is capable of causing high basophil degranulation and thus inducing allergic symptoms. However, the exact clinical manifestations cannot be determined precisely because the patients were also sensitized to the major allergen Amb a 1. Nevertheless, this allergen showed clinical relevance in the allergic population included in this study.

Known as a highly conserved protein, Amb a 8 exhibited a high degree of cross-reactivity when tested with homologous profilins from mugwort (Art v 4), birch (Bet v 2), and timothy grass (Phl p 12) pollens.

All these immunological characteristics of Amb a 8 led to the necessity of a more detailed characterization, including the localization of IgE binding epitopes. Therefore, 5 peptides of approximately 24-35 amino acids covering the entire amino acid sequence of Amb a 8 were designed and synthesized. These peptides were tested with serum from patients allergic to Amb a 8 and displayed no IgE reactivity, suggesting that this allergen does not have linear epitopes. Subsequently, the peptides were conjugated to a carrier protein - KLH and injected into rabbits. The anti-peptide rabbit sera were then tested for binding to the complete allergen and further used in IgE binding inhibition tests. Some of these sera showed a high inhibition potential, suggesting that Amb a 8 contains mainly conformational epitopes. Notably, the rabbit sera specific for 2 of the Amb a 8 peptides exhibited a high IgE inhibition potential (approximately 80%), thus representing promising candidates for future molecular immunotherapy.

Overall, Amb a 8 profilin was successfully produced in *E. coli* cells, with physicochemical and immunological characteristics suggesting that it is a functional allergen with correct folding, which may represent an important element in the diagnosis and therapy of ragweed pollen allergy, although more detailed research is necessary.

CONCLUSIONS AND PERSONAL CONTRIBUTIONS

All the scientific objectives set for this study were successfully achieved. The study revealed that ragweed pollen sensitization profiles are heterogeneous and complex, involving 19 different IgE reactivity profiles. Analyses of clinical symptoms showed that patients with more complex sensitization profiles tended to experience more severe allergic symptoms. In the study, the two isoforms of Amb a 1, namely nAmb a 1.01 and rAmb a 1.03, showed different inhibition patterns, suggesting that their IgE reactivities are distinct. Also, although Amb a 1 is considered the major ragweed pollen allergen, it is not solely responsible for inducing allergic symptoms. This fact emphasizes that Amb a 1, although important, is not sufficient for a complete diagnosis and immunotherapy of ragweed allergy, and allergens such as Amb a 11, Amb a 4, Amb a 8 and Amb a 8, should be considered for an appropriate management of ragweed pollen allergy.

Also, the most allergenic isoform of the major allergen Amb a 1 has been successfully produced as a recombinant protein in insect cells. It presents physicochemical and immunological characteristics similar to those of the natural isoform, having an IgE reactivity of 99% and an intense allergenic activity. At the same time, profilin Amb a 8 was efficiently produced in *Escherichia coli* cells and presented the characteristics of a clinically relevant allergen for this study population, with an IgE frequency of 27% and a high allergenic activity. Moreover, Amb a 8 showed a high degree of cross-reactivity with homologous profilins from mugwort, birch and grass pollen, and identification of IgE binding epitopes for Amb a 8 showed that this allergen contains mainly conformational epitopes.

Personal contributions

Summarized below are some of my contributions to the study:

- Processing and storage of serological samples obtained from the patients included in the study and updating of the patients' database with information obtained from the questionnaire (e.g. symptoms, other sensitivities);
- Performing immunoblots with ragweed pollen extract and identification of the IgE reactivity profiles. Performing IgE binding inhibition immunoblots using two isoforms of major allergen Amb a 1 to determine if there are other allergens than Amb a 1 involved in patients' IgE response to ragweed pollen;
- Design of the first experimental model to capture and remove the Amb a 1 specific IgE from the serum of allergic patients. The serum was then tested in the basophil activation test

and it was observed that RBL cells incubated with the serum still degranulated after stimulation with ragweed extract;

- Design of the Amb a 1.01 and Amb a 8 DNA constructs to obtain the vectors necessary for protein expression;
- Expression, isolation and purification of two recombinant proteins representing the most important isoform of major allergen Amb a 1 (one with a C-terminal His Tag and the other with an N-terminal His Tag but which was eliminated after purification). It should be mentioned that this is the first time when this allergenic isoform is successfully produced as a functional recombinant protein.
- Expression, isolation and purification of ragweed profilin Amb a 8 in E. coli cells;
- Physicochemical and immunological characterization of the obtained recombinant allergens, showing that the recombinant Amb a 1.01 is similar to the natural isoform and can be used successfully in future diagnostic tests.
- Demonstrating that in our population the minor allergen Amb a 8 is clinically relevant and that it should be taken into consideration for ragweed allergy diagnosis and potential immunotherapy.
- Determining the potential cross-reactivities of the Amb a 8 allergen with homologous profilins from the most common sources of pollen in our area (birch, timothy grass and mugwort).
- Design and characterization of Amb a 8-derived peptides. Identification of the IgE binding and basophil degranulation potential and testing the serum obtained after rabbits' immunization to identify the IgE epitopes of Amb a 8. This is the first research approach of this type and it enabled to determine the peptides with the highest potential of blocking IgG induction, which is an important step in immunotherapy development.