

Allergen Preparation and Standardization: An Update



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Introduction

Allergy is defined as an exaggerated response of the adaptive immune system typified by immunoglobulin E (IgE) responses against the offending substance called 'allergen' [1]. The immunologic basis of allergic diseases is observed in two phases: sensitization and development of memory T and B cell responses along with IgE production [2]. Allergy manifests in form of various conditions such as anaphylaxis, urticaria, angioedema, allergic rhinoconjunctivitis, allergic asthma, serum sickness, allergic vasculitis, hypersensitivity pneumonitis, atopic dermatitis (eczema), contact dermatitis and granulomatous reactions, as well as the colorful spectrum of food or drug - induced hypersensitivity reactions [2]. Asthma, allergic rhinitis, atopic dermatitis and inhalant sensitization have been appropriately referred to as first wave of the epidemic of the 21st century [3,4]. During the last 60 years, there has been a rise in the epidemic prevalence of allergic disorders, which is expected to reach up to 4 billion in 2050s [5].

Allergic reactions are initiated by certain types of antigens, referred to as allergens, which have been broadly categorized into four classes as Inhalants (pollen, fungi, dust), Ingestants (food, drugs), Contactants (latex, plant trichomes) and Injectants (drugs/insects). Allergen immunotherapy (AIT) has been used to treat allergic disease since the early 1900s [6]. Allergen-specific immunotherapy (also known as allergy shots) identified as disease-modifying intervention for allergic diseases involves subcutaneous administration of gradually increasing quantities of the patient's relevant allergens until a dose is reached that is effective in inducing immunologic tolerance to the allergens [7]. Instead of subcutaneous, sublingual allergen immunotherapy has recently been also introduced. The term "allergen extracts" refers to solutions of proteins or glycol proteins extracted from source material not yet incorporated into a therapeutic allergen immunotherapy extract [8]. Vials of allergen immunotherapy extracts are prepared individually (specifically customized)

using saline buffers for each patient to reduce the risk of allergen cross-contamination, anaphylactic side effects, sensitization to unknown allergens in extract. Major allergens responsible for allergy in extract can be modified chemically or enzymatically in order to reduce their allergenic potential while still retaining the immunogenicity for achieving same tolerance as natural counterpart.

The collection of raw materials for allergen preparation should be performed by qualified personnel and appropriate measures should be taken to ensure correct characterization and quality check of source materials. For allergen immunotherapy trained Compounding personnel including, but not limited to, registered nurses, medical assistants, or physicians' assistants, be employed which strictly follow allergen extract preparation guidelines. Allergen immunotherapy efficacy and precision of diagnostic allergy skin testing are entirely dependent on the quality of the allergen extracts used [8]. Standardized allergen preparations have been recommended for use in diagnostic kits and immunotherapy as is evident by different position papers published [8-10]. The advantage of standardized extracts is that the biologic activity is more consistent and reproducible, therefore the risk of an adverse reaction caused by extract potency variability should be diminished.

Allergen Preparation

Allergen source materials are prerequisite and are broadly classified into following categories:

Pollen Allergens

The natural sources of inhalant allergens from plants are the pollens. The collection may be performed by vacuuming or drying flower heads followed by grinding using sterile mortar and pestle. The crucial step of cleaning is done by passing through sieves of different mesh sizes and storing in sealed containers at -20°C. The pollen samples are subjected to pollen

purity checking for actual pollen content. As recommended, samples with pollen content 90% or more and plant/floral parts less than 10% of the same species are accepted for extraction of antigens. In no case, fungal spores, other pollen and dust particles together should exceed more than 2%. Pollens may show large variation in quantitative composition depending on season and location of growth. So, in order to achieve a relatively constant composition, harvests from different years and collection sites should be pooled. An important point is to perform pollen collection during beginning and peak of flowering and that too in brown paper bags to avoid fungal contamination. Defatting using diethyl ether is done for selected pollens to get rid of the lipid matter.

Extract manufacturing procedures may vary including as buffer composition, extraction temperature and time, also the incorporation of stabilizers influence the final quality of the extract. Preferably, physiological buffers like Sodium Carbonate (pH 9.6); Phosphate buffered saline (PBS) (pH 7.2); Ammonium bicarbonate (pH 8.0); Tris buffered saline (TBS) (pH 7.5) are used. Pollen grains contain certain proteolytic enzymes which may degrade rapidly the allergenic content in the sample. So, to overcome this, protease inhibitors as PMSF (1mM phenyl methyl sulfonyl fluoride) and chelating agents as EDTA (5mM ethylene diamine tetra acetate) are added during extraction.

Fungal Allergens

The primary inoculum should be obtained from fungal culture banks like Indian Agriculture Research Institute (IARI), Delhi; Institute of Microbial Technology (IMTECH), Chandigarh and National Chemical Laboratory (NCL), Pune. Fungi are cultured under controlled conditions of temperature and growth media composition. Usually Sabouraud's broth with/without yeast extract is preferred. The mycelia mat or surface growth consisting of "mycelia - spore mass" is used for extraction of antigens instead of culture filtrate which mostly contain mycelia antigens. Owing to difficulties in maintaining a constant composition of fungal cultures, it is recommended that extracts should be derived from several independent cultures of the same species [11].

Insect Allergens

The optimal source for insect allergens is dependent on the natural route of exposure - Inhalation; Injectant (bite/sting); Contactant and Ingestant (accumulation of feces, vomitus and debris in house dust). Where whole insects or insect debris are inhaled, the whole insect body is selected as allergen source. On other hand, for biting or stinging insects, saliva or venom, respectively, is the proper allergen sources. In some cases, mixed species of cockroach, mosquitos' houseflies, moths and ants etc. are used for antigen extraction. Live insects are stored in deep freezers, lyophilized and crushed to be further used for antigen preparation [11].

Dust Mites

Two mite species namely *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are common indoor allergens in India. However recent surveys on dust mites have reported *Acarus*, *Glycophagus*, *Blomea* etc as allergenically important. It is worthwhile to mention that mites collected in different culture phases show variable allergenic reactivity [12]. Mites are highly difficult to be grown in bulk. The specific conditions required are low temperature (26 °C), high humidity and specific media as sprouting gram powder, dog meals, and human scales etc. Extracts based on whole mite culture (WMC) include material from mite bodies, eggs, larvae, and fecal particles as well as mite decomposition material. Separation of mites from growth medium is a tedious task [11].

Food Allergens

Protein rich food items of family Leguminosae as pulses, peanut, soybean and cereals are procured from local markets for antigen preparation in saline buffers which can be further stored. In case of fresh dairy products as milk, egg and fresh vegetables & fruits direct prick to prick is preferred.

Miscellaneous Allergens

Other miscellaneous allergenic materials include vegetable and farm dusts, and danders collected from source materials. Pigeon feathers and droppings also serve as antigenic source for some hypersensitive patients.

Extraction of Allergens

Antigen Extraction

Since allergens and antibodies are proteinaceous in nature, utmost focus needs to be towards preventing protein denaturation during allergen extraction procedure and further storage. Allergen extraction mimics physiological conditions present in the human airways (i.e pH and ionic strength). Elevated temperature, high ionic strength and organic solvents needs to be avoided [11]. In general, various steps involved in antigen preparation from various raw material(s) are described in detail. It is to be ensured that autoclaved lab ware, approximate pH and cold temperature conditions during mixing or transferring of suspension needs to be ensured for efficient preparation.

Grinding: It is necessary to crush the solid source material to fine powder to increase the surface area for efficient extraction. The solid source material can be crushed to fine powder using sterile pestle and mortar or using blender or juicer (for juicy fruits).

Defatting: It is done to obtain a clear supernatant of proteinaceous antigenic substances, free from lipid oily layer and non-specific irritants as resins, waxes, pigments. Fresh solvent ether, toluene or acetone in excess amount (3-4times) is added to the material to be defatted in a conical flask with manual shaking

and kept at 4°C. The upper oily layer is decanted, and process is continued until no colour is visible. The solid suspension needs to be cleared through filtration. To minimize the solvents Soxhlet Apparatus is also used for defatting. The material thus obtained is desiccated or air dried inside laminar hood for 24-48 hrs. and stored in plastic containers at -20°C till further processing for antigen extraction.

Extraction: A defined amount of defatted dried material powder is suspended in (w/v) alkaline buffer (pH 8.0) in an Erlenmeyer flask. Protease inhibitors such as 5mM ethylene diamine tetra acetate and 1mM phenyl methyl sulfonyl fluoride is added to the mixture and is subjected to continuous stirring on magnetic stirrer at 4 °C for 8-20hours as the case may be. For liquid samples volume by volume (v/v) measurements are estimated. As for 1:50 extraction, 50 ml of saline buffer is added to 1ml of liquid sample. Preferably extracting solutions are alkaline (pH 8.0) buffer saline or ammonium bicarbonate solution.

Clarification: The antigen extract solution, is then subjected to centrifugation at 10,000g for 20 minutes, at 4 °C for separating the soluble ingredients. The supernatant is retained and solid suspension is discarded.

Dialysis: Low molecular weight, i.e., below 5000 Da, non-antigenic material like resins, pigments etc. should be removed from the extract by dialysis. Any substance excluded from the final product should be proved to be nonallergenic [11]. For dialysis the extract supernatant is filled in a clipped cellophane tubing of defined size and immersed in buffered saline, which is subjected to magnetic steering at low temperature. Change of dialysis tube at defined intervals will ensure efficient removal of unwanted substances. For some substances (e.g. house dust, mustard, potato, spinach, beets, coffee, pepper) give nearly universally positive reactions unless dialyzed.

Sterilization: The supernatant obtained after centrifugation or dialysis is filtered through a sterile 0.45µm membrane filter fitted in sterile Millipore filter assembly and further transferred aseptically to sterile labeled antigenic glass vials. The entire process of sterilization is carried inside laminar hood. It is better to prepare small aliquots for which can be stored at 4 °C or lyophilized for further storage to avoid repeated freezing and thawing.

Lyophilization: The prepared antigenic extracts in small aliquots are lyophilized in Lyophilizer chamber and finally stored at -20 / -70 °C for future use to enhance its self-life.

Sterility Testing - Microbial assays for identifying bacterial and fungal contamination, if any in antigenic extracts should be carried out using suitable media.

Allergen Standardization

The two important factors for consideration during allergen standardization are:

- i. Selection of a reference extract and,
- ii. Selection of an assay or protocol to compare the manufactured extracts with the selected reference extracts [8].

In the United States, Food and Drug Administration (FDA) and Centre for Biologics Evaluation and Research (CBER) - a division of allergenic products and parasitology regulates the licensing of allergenic extracts for clinical use [13-16]. While more specifically for Allergen Immunotherapy, allergen extract preparation guidelines have been established by 2 entities: the US Pharmacopeia and an American Academy of Allergy, Asthma, and Immunology/American College of Allergy, Asthma, and Immunology/Joint Council of Allergy, Asthma, and Immunology Joint Task Force. The Allergy Immunotherapy practice parameter (AIPP) third update included the previously proposed, but now formally adopted, guidelines and the USP chapter 797 guidelines.

US-licensed extracts are supplied as aqueous, glycerinated, lyophilized, and acetone- and alum-precipitated formulations. The list of commonly used allergens which have been standardized includes - extracts for cat hair, cat pelt, *D pteronyssinus*, *D farinae*, short ragweed, Bermuda grass, Kentucky bluegrass, perennial rye grass, orchard grass, timothy grass, meadow fescue, red top, sweet vernal grass, and Hymenoptera venoms (yellow jacket, honeybee, wasp, yellow hornet, and white-faced hornet).

The potency of allergen extracts is assessed by using quantitative skin tests and stated in terms of bioequivalent allergy units (BAU). The quantitative test is called the intradermal dilution for 50 mL sum of erythema (ID50EAL). Standardized extracts with wide range of biological potencies are available as 10,000 and 100,000 BAU for grasses; 5,000 and 10,000 BAU for cat allergen; 5,000, 10,000, and 30,000 AU for dust mite; and 100,000 AU or 1:10 and 1:20 wt/vol for short ragweed, with the Amb a 1 concentration listed in FDA units on the label of the weight/volume extracts [8]. A point to mention that allergy unit (AU) is bioequivalent to BAU. For standardized allergen extracts, the potency of the manufacturer's extract is recognized by comparing the extract with the CBER's reference control using an in vitro ELISA assay [17]. The acceptable range for a 10,000- or 100,000-BAU/AU extract is based on a statistical test for equivalence to a CBER/FDA. Based on dose-response studies using US licensed allergen extracts, the probable effective dose ranges for standardized US-licensed allergenic extracts have been estimated from clinical trials primarily conducted in Europe [8].

In case of, nonstandardized extracts, maintenance dose is much more difficult to calculate. This is because nonstandardized extracts' weight by- volume (w/v) or protein nitrogen unit (PNU) labeling do not essentially correlate with biological potency. This concept is supported by data provided from one of the leading extract manufacturers (ALK-Abello) highlighting that major allergen content in Bermuda grass extracts ranged from 141

to 422 g/mL of Cyn d 1 [18]. The AIPP third update provides new dosing recommendations for Bermuda grass, imported fire ant, and nonstandardized extracts distinguishing between pollen (0.5 mL of a 1:100 or 1:200 vol/vol) and mold/fungi or cockroach (highest tolerated dose) extracts [18].

In Europe, manufacturers report allergen extract potency as units based on an in-house reference standard (IHRS), making it difficult to understand the exact doses used. One of the European allergen extract manufacturers use in-house reference standards that are based on titrated skin prick testing of allergic patients. Consequently, *in vitro* test (enzyme-linked immunosorbent assay [ELISA] inhibition) compare the potency of commercial batches with the in-house reference and potency is assigned as arbitrary units, which is not printed on packed vials. Quantifying the major allergen is not an acceptable criterion for standardization worldwide as the techniques, monoclonal antibodies used in ELISA and the patient natural exposure are variable and cannot be compared. Consequently, in 2001 European Union sponsored a study "CREATE" - Certified References for Allergens and Test Evaluation. Under this program manufacturers have implemented protocols which have overall IgE-binding potencies as their focus and supports the introduction of major allergen-based standardization for effective immunotherapy.

The objectives of CREATE was to produce purified recombinant allergens and compare these with their natural counterparts to serve as gold standard. Nine recombinant molecules representing eight major allergens were produced: rBet v 1, rPhl p 1, rPhl p 5a and rPhl p 5b, rOle e 1, rDer p 1, rDer p 2, rDer f 1 and rDer f 2. Recombinant allergens were compared with purified natural allergens for physico-chemical (identity, purity, folding, aggregation state, solubility and stability) to serve as Certified Reference Materials (CRM). Immunological parameters (IgE-binding potency, biological activity and dose-response behaviour in ELISA) were also assessed [19]. Two of the recombinant allergens- rBet v 1 and rPhl p 5a have qualified to be good reference materials for use in immunotherapy clinical trials.

In India, Drug Controller of India and the respective nodal agencies regulates the allergen manufacture for diagnostic and therapeutic purposes. Till date the emphasis is on Good Manufacture Practice (GMP) to be followed by allergen units. Allergen potency is expressed as w/v (weight/volume), protein estimation (PNU in µg/ml) and sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) protein profile is followed for maintaining/comparing the quality of antigen in different batches. Coordinated and ambitious efforts are required by allergists/Immunologists and commercial manufacturers to upgrade the quality control of allergen extracts following standard of World Health Organization and International Union of Immunological Societies (WHO/IUIS) protocols [20]. Infact the need to develop allergen certification centre in the country like Food and Drug Administration (FDA) and Centre for

Biologics Evaluation and Research, (CBER) USA has been realised much earlier. In recent times few of the government funded (Ministry of Health & Family Welfare) research institutes like National Institute of Biologicals (NIB), Indian Pharmacopoeia Commission (IPC) have been recognised for quality control of commercial allergen extracts and dissemination information on allergen preparation and standardization of allergen extracts which are classified as drugs.

During the last two decades, research projects were pursued to characterize the allergen extracts from diverse sources at Institute of Genomics and Integrative Biology and V.P.Chest Institute, Delhi. Indian flora is very rich and diverse in different parts of the country, hence a large number of pollens are predominant at different times in atmosphere [21]. About 30 species of fungi have been recorded from "air spora" responsible for type I hypersensitivity. Additionally, several allergens from insects, mites, animal epithelia etc also contribute in inducing and aggravating respiratory allergy diseases in predisposed individuals. The task of characterizing allergens in our country is comparatively difficult than Europe and U.S.A where only few allergens are prevalent.

Allergen Standardization – New Trends

Allergens as natural extracts are complex mixtures of major and minor allergens. Allergens are proteinaceous in nature and exhibit genetic variations/sequence polymorphisms which results in different isoforms. This heterogeneity affects the IgE binding capacity and impart signature peptide sequences to each allergen isoform [22]. Currently used methods for major allergen determination include single radial immuno diffusion, rocket immuno electrophoresis, ELISA (enzyme-linked immunosorbent assay). The quantitative aspects of these techniques are dependent on monoclonal antibodies as reagents which are prone to degradation with time, unsuitable for concomitant quantification of several allergens and are able to identify single epitopes and/or single isoallergens only.

Basophil histamine release test which relies on same principle as the skin test have also been recommended to demonstrate qualitative differences in biological activity or the composition of allergen preparations, which is undetectable by IgE inhibition assays. The limiting factor is that it is technically demanding and difficult to perform and also offers less precision. Besides the routine use of monoclonal and polyclonal antibodies for quantifying the potency of allergenic extract, other advanced physiochemical methods such as mass spectrometry (MS) have been used for allergen standardization. Using advanced MS techniques for allergen extract characterization and standardization offers advantages as various allergens of the extract can be studied simultaneously. High-resolution and accurate mass (HRAM) MS ability detect peptides with high selectivity and mass accuracy (<3 ppm, parts per million) and are able to recognise single amino acid substitution on the allergen surface.

Two reference materials (rBet v 1 and rPhl p 5a) and relevant ELISA assays are under validation for future establishment as international references under the guidance of the European Directorate for the Quality of Medicine (EDQM) after extensive biological and biochemical investigation. Currently, these two reference materials (rBet v 1 and rPhl p 5a) are under further validation by BSP090 project. IgE or IgG recognition could be affected even by a single amino acid substitution on the surface of the allergens.

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