Review

# Coordinated and standardized production, purification and characterization of natural and recombinant food allergens to establish a food allergen library

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Reliable diagnosis of food allergy is dependent on the analytes used. Approaches based on well-defined individual molecules of either natural or recombinant origin are likely to replace those based on food extracts in the future. Therefore, a library comprising well-characterized authentic natural and recombinant allergens was formed within the EC funded IP EuroPrevall. The advantages and disadvantages of including either natural or recombinant proteins are summarized, together with inclusion criteria such as purity and the implications of both allergenic and nonallergenic impurities for the performance of diagnostic assays. In order to harmonize quality criteria of the individual food allergens included in the library, as suite of characteristics and associated methodologies used to define them, was agreed. The application of these methods and impact on the quality and performance of the final purified food allergens included in the EuroPrevall allergen library is discussed.

**Keywords:** Allergen library / Component resolved diagnosis / Food allergen / Physicochemical characterization / Recominant protein

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## 1 Introduction

Usually harmless dietary proteins of animal or plant origin can cause food allergic reactions in predisposed humans evoking a range of symptoms including mild reactions and full-blown life-threatening anaphylactic shock. At present the only treatment available for food allergic individuals is to avoid the offending food, and have medication to treat a reaction in case of emergency. Reliable diagnosis of food allergy is essential [1] to reduce the unnecessary burden of exclusion diets imposed by misdiagnosis. It also permits adherence to dietary recommendations for the allergic consumer. More than 90% of food allergies are caused by only a dozen of food sources. However, out of hundreds of proteins, only a restricted number of proteins is able to induce an allergic reaction and the production of specific IgE antibodies [2–4]. Recent analyses of allergen databases revealed that 3 major and 14 minor protein families account for animal derived food allergens [4], while most plant food allergens belong to 4 structural protein families [5]. At present total protein extracts of foods are widely used in routine clinical diagnosis, despite the fact that several problems are associated with their use of extracts, the lack of standardization of preparations and batch-to-batch inconsistency representing two of the most important pitfalls [6–8].

When preparing total protein extracts from raw food sources the variation in allergen expression due to bio-variability has to be taken into account as it has been shown for apple allergens [9, 10]. Furthermore, some important allergens are present at low concentrations and may be missing from some extract preparations, contributing to their low diagnostic sensitivity, as it has been shown for Bet v 1 homologous food allergens from hazelnut [6, 11], from peanut [12] and recently from kiwi [13].

In addition, endogenous enzymatic processes in raw food sources may degrade allergens during extraction reducing the content of relevant allergens [14].

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**Abbreviations: LTP,** lipid transfer protein; **TLP,** thaumatin-like protein

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**Table 1.** Allergen library comprizing animal and plant food allergens

| Source | | IUIS Allergen designation | Protein family/Functional properties | Source | |
|---|---|---|---|---|---|
| | | | | Recombinant protein | Natural protein |
| Cow's milk | *Bos domestica* | Bos d 4 | α-Lactalbumin | | Cow's milk (raw) |
| | | Bos d 5 | β-Lactoglobulin | | Cow's milk |
| | | Bos d 8 | total casein | | Cow's milk |
| Goat's milk | Caprinus | | Goat's milk casein | | |
| Hen's egg | *Gallus domesticus* | Gal d 1 | Ovomucoid | | Hen's egg white |
| | | Gal d 2 | Ovalbumin | | Hen's egg white |
| | | Gal d 3 | Ovotransferrin | | Hen's egg white |
| | | Gal d 4 | Lysozyme | | Hen's egg white |
| | | Gal d 5 | Serum albumin | | Hen's egg yolk |
| Carp | *Cyprinus carpio* | Cyp c 1 | Parvalbumin | *E. coli* | |
| Cod | *Gadus morhua* | Gad m 1 | Parvalbumin | *E. coli* | Codfish muscle |
| Shrimp | *Penaeus aztecus* | Pen a 1 | Tropomyosin | *E. coli* | |
| Apple | *Malus domestica* | Mal d 1 | Bet v 1 homologue, PR-10 | *E. coli* | |
| | | Mal d 2 | Thaumatin-like protein (TLP), PR 5 | | Apple fruit |
| | | Mal d 3 | Non specific (ns) LTP; PR14 | | Apple peel |
| | | Mal d 4 | Profilin | *E. coli* | |
| Peach | *Prunus persica* | Pru p 1 | PR-10 | *E. coli* | |
| | | Pru p 3 | ns LTP | | Peach peel |
| Hazelnut | *Corylus avellana* | Cor a 1.04 | PR-10 | *E. coli* | |
| | | Cor a 11 | Cupin 7S vicilin, seed storage globulin | | Hazelnuts |
| | | Cor a 2 | Profilin | *E. coli* | |
| | | Cor a 8 | ns LTP; PR14 | *P. pastoris* | |
| | | Cor a 9 | Cupin 11S legumin, seed storage globulin | | Hazelnuts |
| Peanut | *Arachis hypogea* | Ara h 1 | 7S seed storage globulin | | Peanuts |
| | | Ara h 2,6,(7) | 2S albumin | | Peanuts |
| | | Ara h 3/4 | 11S seed storage globulin | | Peanuts |
| | | Ara h 8 | PR-10 | *E. coli* | |
| Celery | *Apium graveolens* | Api g 1.01 | PR-10 | *E. coli* | |
| | | Api g 4 | Profilin | *E. coli* | |
| | | Api g 5 | FAD-containing oxidase | | Celeriac |

All these problems can be overcome by replacing food extracts with highly purified allergen preparations an approach which has gained increasing interest in the recent past [15–17] and has been termed "component resolved diagnosis" (CRD). Several additional benefits have been attributed to the use of purified allergen molecules including (i) increased sensitivity of *in vitro* diagnosis; (ii) detection of geographic differences in patterns of sensitization to individual allergens; (iii) correlation of the clinical situation to patterns of sensitization; (iv) identification of individual allergens as biomarkers for severity or persistence of food allergies, and (v) identification of individual allergens as markers for cross-reactive allergies. For example, diagnosis of cherry, hazelnut, and apple allergy could be improved by the use of individual allergens, since different sensitization patterns were identified [18–20]. For these plant food allergies sensitization to lipid transfer proteins (LTPs) could be correlated with rather severe and persistent food allergic symptoms [20–22]. Several food allergic syndromes have been described by clinicians which can be explained by the cross reactivity of homologous allergens present in various food sources. For example, the latex-fruit syndrome is in part due to cross reactivity of chitinases, glucanases, and patatin [23].

For these reasons we have created a library, comprising the most important allergens, from major allergenic foods including those from foods which have to be labeled in the EU (EC Directive 2003/89/EC) such as cow's milk, hen's egg, fish (both salt water (*Gadus morhua*) and fresh (*Cyrpinus carpio*), an example shrimp (*Farfantepenaeus aztecus*), peanut, an example tree nut, hazelnut and celery. In addition allergens from two stone fruits from the Rosaceae family (apple, peach) and goat's milk were included.

This work has been undertaken as part of an EC funded project, EuroPrevall, which aims at defining the patterns, prevalence, cost, and basis of food allergy across Europe. Among various activities, the production of highly purified and well characterized allergens of either natural or recombinant origin was undertaken in order to develop *in vitro* diagnostics with improved predictive power.

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**Table 2.** Parameters and applied methods to assess the physico-chemical characteristics of purified natural and recombinant food allergens from the EuroPrevall allergen library.

| Parameter | Methods |
|---|---|
| Sequence verification | MALDI-TOF/Q-TOF MS, N-terminal sequencing |
| Isoforms | LC-MS, 2D-PAGE |
| Folding/Structure | CD, FT-IR, NMR spectroscopy |
| Aggregation stages | Gel permeation chromatography |
| Purity, glycosylation, proteolysis | SDS-PAGE (silver stain), Size exclusion chromatography, ES-MS |
| Biological function | *e.g.*, enzymatic activity, lipid-binding |
| IgE reactivity | RAST/EAST/Immunoblotting with human sera |
| Allergenic activity | Histamine release assay |
| Inhibition with natural extract | ELISA, EAST, CAP inhibition |
| Structure | 1D-NMR |
| Reference sera | 3 – 5 specific sera for IgE binding assays |

However, many earlier studies involving purified allergen components provided only limited information on structure and physico-characterization of the allergens even though their biological activity has been shown to depend on their structural integrity [24–27]. Therefore, in the approach taken in developing the EuroPrevall allergen library was to characterize the physicochemical and allergenic properties of individual food allergens. In this context, it was mandatory to compare wherever possible, recombinant and natural allergens. A team of experts in allergen purification and characterization have contributed to this allergen library, designing and optimizing the purification protocols and applying a range of state-of-the art analyses regarding protein integrity. This special issue of *Molecular Nutrition and Food Research* is dedicated to present the collection of purification and characterization data of 31 individual food allergens (see Table 1) developed by this group. This introductory paper describes the approaches taken to harmonizing the quality criteria which had to be met prior to inclusion of individual food allergens into the EuroPrevall library (see Table 2).

## 2 General considerations for purification strategies

Before isolating a food allergen, a fundamental decision has to be taken as to whether to isolate the allergenic molecule from the natural source or to produce it in recombinant form in a heterologous expression system. This decision should be made on a case-by-case basis taking into account the pros and cons of the two approaches and considering all that is known about the allergen in question as illustrated in Fig. 1. Any post-translational modification of a protein affecting its overall IgE binding capacity needs to be maintained throughout the purification process. If no critical modifications are known, stability is another key issue. A number of proteins are known to be present as various isoforms. If the range of isoforms contributes to the overall allergenic activity, the purification of the mixture of isoforms from natural sources would be appropriate in order to replicate the composition of the food as closely as possible. If hypoallergenic and hyperallergenic molecules are known the production of a single recombinant isoform with high allergenic reactivity can be considered [28].

If proteins are exposed to active proteases during the extraction and purification procedures, as is frequently the case when preparing proteins from plant tissues, their susceptibility to proteolysis needs to be addressed. For proteins that are degraded upon extraction from natural source materials, expression in heterologous systems is the method of choice [14]. Abundance is another key issue. If allergens are expressed in high quantities, suitable purification protocols can be optimized, while preparations of allergens expressed at low in natural sources can present difficulties. For proteins that are either degraded upon extraction from natural source materials and/or are of low abundance, expression in heterologous systems is an important alternative and maybe the method of choice.

In case post-translational modifications or an abundance of isoforms contributes to the overall allergenicity of a food, purification from natural sources might be preferred. For example, if proteolytic processing of proteins takes place in the natural source to generate a mature protein with full allergenic activity, as is known for seed storage proteins [29,30], purified natural allergens are superior to recombinant proteins.

If disulfide bridges are important to establish the IgE epitope structure required to obtain full biological activity of an allergen either naturally purified proteins with intact intra- or inter-molecular disulfide bonds maybe preferred or a specialist expression system able to form disulfide bonded proteins, such as *Pichia pastoris* have to be used [31].

In general, an expression system closely matching the natural host would reduce the probability of misfolding. Nevertheless, it is necessary to finally compare the purified product with the extract, and/or to compare the recombinant protein with the natural counterpart regarding their integrity. Examples for all these scenarios are given in the

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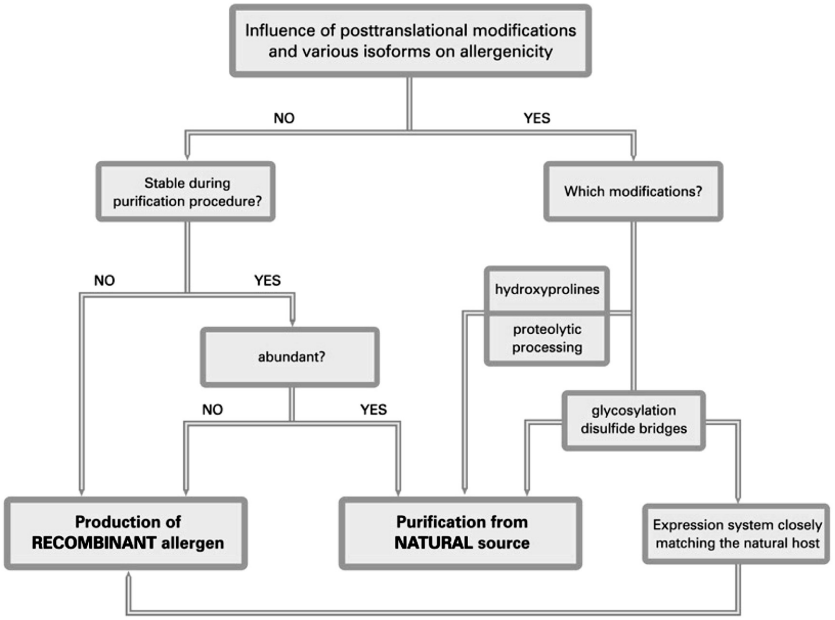


**Figure 1.** Decision tree for production of recombinant allergens *versus* purification of food allergens from natural sources.

present Special Issue of *Molecular Nutrition and Food Research*.

## 3 Allergens from the EuroPrevall allergen library

Approximately 10–15 foods account for more than 90% of food allergic reactions. In order to avoid unintended exposure to food allergens present in processed foods legislators all over the world have started to regulate allergen labeling for this consumer group at risk.

The EC legislation has listed the following food items that have to be declared: milk, egg, fish, crustaceae, cereals, nuts, soybean, celery, mustard, sesame seeds, molluscs, and lupine (Directive 2003/89/EC and amendment 2006/142/EC). The foods that the EuroPrevall studies are focused on include many of these (Table 1). Thus foods of animal origin include cow's and goat's milk, hen's egg, carp and codfish, and shrimp were selected. From fruits and vegetables apple and peach, hazelnut and peanut, and celery were chosen. Thus α-lactalbumin (Bos d 4), β-lactoglobulin (Bos d 5), and total caseins (Bos d 8) were purified from milk derived from a single genotyped cow [32]. In addition, the caseins from goat's milk were provided [32]. From hen's egg white ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), lysozyme (Gal d 4) were purified whereas serum albumin (Gal d 5) was purified from egg yolk [33]. The parvalbumins are known to be the major allergens in fish [34]. Therefore, the parvalbumins were purified from codfish (Gad m 1) and carp (Cyp c 1) and in addition the recombinant codfish parvalbumin was produced and purified [35]. The most important allergen from shrimp, tropomyosin, was submitted to this collection as purified recombinant protein (Pen a 1) [36]. For foods of plant origin, EuroPrevall studies are focused on hazelnut, peanut and celery, all allergenic foods which must be labeled together with those from fruits, namely apple and peach. Rosaceae fruits contain proteins homologous to the major birch pollen allergen Bet v 1, which are designated, Mal d 1, in apple and, Pru p 1, in peach. Both proteins were produced as recombinant proteins [37, 38]. Also the non-specific lipid transfer proteins are known as relevant allergens in fruits and both Mal d 3 and Pru p 3 were purified from fruit peel tissue [37, 38]. As a member of the profilin protein family, Mal d 4 was also produced in *E. coli* [37]. In addition, the thaumatin like protein, Mal d 2, was purified from apple fruit tissue [37].

Celery was included as a vegetable allergen source. The Bet v 1 homologue, Api g 1, and the profilin, Api g 4 were provided as recombinant proteins and a natural glycoprotein, the FAD-containing oxidase, Api g 5 was purified from the bulb [39].

The predominant allergens in nuts belong to the seed storage proteins. From hazelnut the 7S vicilin (Cor a 11) and the 11S legumin (Cor a 9) were purified from nuts [40]. In addition, hazelnuts also contain the Bet v 1 homologue, Cor a 1, an allergenic profilin, Cor a 2 and the lipid transfer protein, Cor a 8. Again, all three were included in the library as as recombinant proteins [41]. Like tree nuts legume seeds such as peanuts contain allergenic 7S (Ara h 1) and 11S seed storage proteins (Ara h 3 / 4), together with the 2S albumins as major allergens (Ara h 2 and Ara h 6), which were purified from natural sources [42]. Additionally the Bet v 1 homologue, Ara h 8, contributes to the allergenic profile of peanuts, which was produced as a recombinant protein [42].

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For some of the foods such as hazelnut, peach and celery this represents the production of the first comprehensive allergen panel and will support the development of novel diagnostics and in particular assess the utility of component resolved diagnosis.

## 4 Purification and characterization of food allergens

### 4.1 Purification of natural food allergens

Purification of individual proteins from foods needs to be optimized for each protein. The first step involves successful solubilization of the protein of interest from the food source whilst minimising co-extraction of unwanted substances such as polyphenols, sugars, lipids, and DNA which would otherwise hamper resolution in various subsequent purification steps. Enrichment of a protein from the initial extract can be achieved using selective precipitation steps upon addition of salt and/or pH adjustments together with various chromatographic separations. These all exploit the physicochemical properties of the desired protein, such as p*I*, ligand binding and its amphipathic properties. Throughout these protocols the native structure and preservation of allergenic activity needs to be checked by IgE binding assays and any contamination by other allergenic proteins has to be excluded. Final batches of purified food allergens with a purity of at least >95% have to be further checked for stability and optimal storage conditions. In some cases, especially with seed storage proteins, concentration of final batches is sometimes difficult since these proteins tend to precipitate and several buffer systems have to be tested to reach optimal conditions. Since natural food allergens usually contain a mixture of isoforms, the final batch has to be characterized regarding its purity and composition accordingly.

In some cases, existing purification protocols already available were used and the purification of the desired food allergen was straight forward as in the case of natural Api g 1, the celery Bet v 1 homologue [39]. In the case of egg allergens, commercially available, partially purified preparations were used as the starting material and a considerable improvement in purity was achieved [33]. For other proteins, protocols had to be established and modified. For example, purification of apple thaumatin-like protein (TLP) was hampered by the presence of pectin. Additional precipitation steps had to be performed in order to clear the extract for further purification [37].

### 4.2 Production and purification of recombinant food allergens

The selection of a suitable expression system for preparation of recombinant proteins depends on the expected yield, whether post-translational modifications are required, the need for correct formation of inter- and intramolecular disulfide bonds and the solubility of the product. In most cases, various prokaryotic expression systems of various types have proven to be effective. Much less frequently fungal systems such as *P. pastoris* or transgenic plant systems have been utilized. Recombinant food allergens for the EuroPrevall library were prepared using *E. coli* and yeast expression systems either as non-fusion or His-tagged proteins. Recombinant proteins expressed in *Pichia* were purified from cell culture supernatant. In the case of prokaryotic expression systems, the recombinant protein was extracted from the *E. coli* lysate and further purified.

While the risk of additional allergenic activity of co-migrating *E. coli* proteins is rather low the intrinsic allergenic activity of the recombinant protein has to be carefully checked and compared to the natural counterpart.

In the case of Api g 4, celery profilin, the expression yield could be increased four- to six-fold when changing from the non-tagged expression system to a His-tagged expression [39]. It seems that the His-tag protects the recombinant protein from proteolytic degradation. In the case of His-tagged recombinant Ara h 8, the Bet v 1 homologue from peanut, the product was found to be labile in solution and it was not stable at concentrations above 350 μg/mL. Cor a 1.04, the Bet v 1 homologue from hazelnut showed a tendency to form oligomers which had to be separated from the monomeric form [41]. For Mal d 1 and Pru p 1, the Bet v 1 homologue from apple and peach respectively, protein expression was performed at 25–30°C rather than 37°C. While lowering the temperature, the formation of inclusion bodies was avoided and thus the extraction of the recombinant protein could be performed under non-denaturing conditions [37, 38].

## 5 Criteria for inclusion of purified allergens into the library

The purified food allergens were further evaluated regarding their purity, authenticity, and correct folding (see Table 2). Purity of the final batches was assessed by SDS-PAGE and size exclusion chromatography. In order to evaluate the range of isoforms found in a naturally derived batch, 2-D electrophoresis was performed. MS analysis was also used to evaluate purity. The identity and the correct sequence of allergens were verified by MS and N-terminal sequencing, and in selected cases indicative fragments of the allergens were sequenced. MS was used as an additional criterion for identity, and to analyze potentially impurities, artefact formation and chemical modifications. Evidence of the folded state of the proteins was assessed by circular dichroism spectroscopy and in some instances fourier-transform infrared spectroscopy and 1D-NMR analysis.

The allergenic activity of purified proteins was verified by using sera from selected patients with established food

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allergy, performing IgE immunoblots, ELISA, and RAST. Inhibition assays were also used to compare the allergenic activity of natural allergen with their recombinant counterparts and purified proteins with allergens present in food extracts. Additionally, assays based on basophil cells were applied to measure basophil activation upon addition of allergens which induce cross-linking of specific IgE bound to high affinity receptors as an indicator for biological activity of allergens.

## 6 Conclusions

The EuroPrevall allergen library is based on a collection of of natural and recombinant food allergens meeting a set of harmonized quality criteria. This volume comprises the collection of protocols for the preparation of 31 allergens together with data on their authentication. These represent a blue print of how to produce and characterize high quality allergens suitable for diagnostic purposes as well as in-depth studies on for example the effect of processing conditions on the allergenic potential of food allergens. The protocols published in this special issue and the detailed physico-chemical characteristics of the component allergens will allow other researchers to produce batches of allergens of similar quality. The availability of such information will improve the comparability of studies utilizing purified allergens in future. In particular these high quality purified food allergens will contribute to improving conventional food allergy diagnostics as well as new technologies such as protein biochips.

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