



Production and analysis of recombinant tree nut allergens



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ARTICLE INFO

Article history:

Available online 31 July 2013

Keywords:

Tree nut
Allergy
Recombinant allergen
Epitope
Food allergy

ABSTRACT

Allergic reactions to tree nuts are a growing global concern as the number of affected individuals continues to rise. Unlike some food allergies, tree nuts can cause severe reactions that persist throughout life. The tree nuts discussed in this review include those most commonly responsible for allergic reactions: cashew, almond, hazelnut, walnut, pecan, Brazil nut, pistachio, and chestnut. The native allergenic proteins derived from tree nuts are frequently difficult to isolate and purify and may not be adequately represented in aqueous nut protein extracts. Consequently, defined recombinant allergens have become useful reagents in a variety of immunoassays aimed at the diagnosis of tree nut allergy, assessing cross-reactivity between various nuts and other seeds, mapping of IgE binding epitopes, and analyzing the effects of the food matrix, food processing, and gastric digestion on allergenicity. This review describes the approaches that can be used for the production of recombinant tree nut allergens and addresses key issues associated with their production and downstream applications.

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1. Introduction

Food allergies are adverse immunological (hypersensitivity) reactions to normally harmless proteins in foods [1]. The specific proteins which are recognized by IgE and elicit an immune response are known as allergens. The average human diet contains a wide variety of foods; however, only a few are responsible for the majority of food allergies [1]. The most common allergenic foods include milk, egg, wheat, soy, fish, peanuts, tree nuts, and shellfish. Often allergies to wheat, egg, milk, and soy are outgrown whereas allergies to peanuts, shellfish, and tree nuts typically persist throughout life and can cause severe type I (IgE-mediated) allergic reactions [2–5]. Tree nuts are typically eaten as snacks or incorporated into foods, and include almond, Brazil nut, cashew, chestnut, coconut, hazelnut, macadamia, pecan, pine nut, pistachio, and walnuts. The popularity and consumption of tree nuts varies among populations and geographical locations. The most commonly consumed tree nuts in the US, in rank order, are cashew, almond, pistachio, pecan, and walnut, whereas in Europe the most popular nuts, in rank order, are walnuts, almonds and hazelnuts [6–8]. Allergic reactions to tree nuts are rising; it is currently estimated that 0.6% of the US population and up to 1.4% of the European population are allergic to one or more tree nuts [9–11].

Food allergens are classified as either type 1 or type 2 according to their specific biochemical and immunological properties. Type 2 allergens are referred to as incomplete allergens. Reactivity to these food allergens often results from a primary sensitization by aeroallergens (e.g., pollens) [12–14]. The subsequent consumption of foods, including a variety of fruits and vegetables containing proteins homologous to the sensitizing allergen, triggers an IgE-mediated reaction [14,15]. Such reactions are frequently mild and localized to the oral cavity.

Type 1 allergens, in contrast, are known as complete food allergens as they both sensitize the patient, primarily through the gastrointestinal tract (GIT), and elicit allergic symptoms. Type 1 allergens are typically resistant to thermal denaturation and gastric digestion, consequently retaining a substantial proportion of their IgE-reactive epitopes [12,15,16]. Common foods containing type 1 food allergens include peanuts, tree nuts, egg, milk, and fish [15,17]. To date several type 1 allergens have been identified in tree nuts including hazelnut Cor a 8, 9, 11, 12, and 13, and walnut Jug r 1, 2 and 3. It should be noted that while type 1 allergens tend to predominate in peanut and tree nuts, these foods have been found to contain type 2 food allergens as well (e.g., chestnut Cas s 1, hazelnut Cor a 1 and 2, and almond Pru du 4), a likely result of patient sensitization to cross-reactive pollen allergens [15,17]. The higher incidence of type 1 food allergy in children is likely due to a greater degree of intestinal permeability and a less developed immune system [15]. Symptoms of type I food-induced allergy range from mild gastrointestinal reactions, such as abdominal pain, diarrhea, and vomiting, to severe life threatening respiratory reactions, such as systemic anaphylaxis [15]. It is estimated

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that 8–50 per 100,000 individuals experience an anaphylactic reaction annually [18–24]. In children, peanut and cashew nut are the most common eliciting agents [21,22]. Due to the severity of reactions to tree nuts and the growing number of individuals affected, extensive research has been performed to identify and characterize constituent IgE-reactive proteins. In addition, immunoassays aimed at investigating cross-reactivity between various nut and seed proteins, as well as structural studies, IgE epitope mapping, and analyses of the effects of associated food matrices, enzymatic digestion, and denaturation have been performed in hopes of determining common features which may predispose such proteins to be targeted by IgE. It is anticipated that the accumulating information will provide insights into the unique features which render tree nuts allergenic and may be useful for diagnostic and therapeutic purposes.

2. Tree nut allergens

To date, 32 tree nut proteins have been shown to be reactive with IgE from allergic patients (<http://www.allergen.org/>). Many of these proteins have been extensively studied at the molecular level. A current list of officially designated tree nut allergens is presented in Table 1. These allergens can be grouped based on their sequence, function, and structural similarities [25,26].

2.1. Seed storage proteins

One such group is the seed storage proteins, which are estimated to account for ~50% of the total protein in most nuts [27–31]. Seed storage proteins act as a carbon and nitrogen source during germination and serve as amino acid reserves for the developing seedling [32,33]. Those with allergenic properties include the 11S globulins (legumin-like proteins), 7S globulins (vicilin-like proteins), and 2S albumins [25,34]. The 11S globulins are members of the cupin superfamily characterized by a six stranded β -barrel conformation [35,36]. They are typically ~360 kDa hexamers with each subunit containing an acidic 42–40 kDa polypeptide that is disulfide-linked to a ~20 kDa basic polypeptide [26,33,37]. It is not uncommon for multiple 11S globulin isoforms to be expressed in nuts. Surprisingly, despite sequence similarity, not all isoforms in a given nut display similar IgE reactivity [33,38,39]. The 7S globulins are also members of the cupin superfamily. They are typically trimeric with a molecular mass of ~150 kDa comprised of 40–55 kDa polypeptides [25,33,40]. The 2S albumins are small ~13 kDa protein members of the prolamin superfamily. They contain eight conserved cysteine residues which form two intrachain disulfide bonds and two interchain disulfide bonds linking a ~9 kDa and ~4 kDa polypeptide chain [26,33,41–43].

2.2. Pathogenesis-related proteins

Several pathogenesis-related proteins (PR-proteins) have been identified as tree nut allergens. PR-proteins are produced in the plant upon attack by bacteria, fungi, or when abiotically stressed [44,45]. They range from 5–70 kDa, are stable at acidic pH, and tend to be highly resistant to proteolytic degradation [44,45]. Allergenic PR-proteins include chitinases, Bet v 1 homologues, and lipid transfer proteins (LTP) [46–50].

2.3. Structural proteins

Another group of tree nut allergens are the profilins. These are small cytosolic ~12–15 kDa proteins that bind actin in eukaryotic cells and perform an important role in regulating actin filament polymerization [51,52]. Profilins are highly conserved among

plants, displaying both structural and sequence similarities (70–85% amino acid sequence identity) [25]. These proteins have been described as pan-allergens because of the considerable cross-reactivity observed between profilins from different sources [25,35,53,54]. Oleosins are small ~15–26 kDa hydrophobic proteins that serve to stabilize oil bodies in desiccated seeds [55–57]. They have a highly conserved structure with three distinctive domains; a hydrophilic domain at the N-terminus, a central hydrophobic α -helical domain, and an amphipathic α -helical domain at the C-terminus [55,58]. Among the tree nuts, oleosin has only been identified as an allergen in hazelnuts [59].

2.4. Other allergenic proteins

In recent years several additional proteins have emerged as potentially important allergens in tree nuts including manganese superoxide dismutase (MnSOD), 60S acidic ribosomal protein P2, and cytosolic class I small heat shock protein. MnSOD is a homodimeric or a homotetrameric enzyme that is located in mitochondria and peroxisomes [60,61]. In plants, MnSOD plays an important role in preventing cellular damage by reactive oxygen species [60]. MnSOD was identified as a minor allergen in pistachio nuts though it has been suggested that the high degree of sequence similarity between MnSOD from different organisms could potentially lead to allergen cross-reactivity [62]. The 60S acidic ribosomal protein P2 is a small ~11 kDa ribosomal protein with a highly conserved structure that is involved in mRNA translation [63–66]. Originally identified as minor allergens in several fungi [67–70] they were recently recognized as an IgE-reactive protein in almond nut [71]. Finally, the cytosolic class I small heat shock proteins are produced following abiotic stress [72,73] and are reactive with IgE in some chestnut-allergic sera (unpublished data, <http://www.allergen.org/>).

3. Purification of native tree nut allergens

The growing number of allergens identified in tree nuts is mainly due to advances in protein purification and molecular biology techniques. Initially, studies to identify tree nut allergy and constituent IgE-reactive proteins were performed using aqueous nut extracts in immunoblotting assays, radioallergosorbent tests, or skin prick tests [74–83]. Further characterization has been accomplished by application of a wide variety of biophysical techniques such as high performance liquid chromatography, mass spectrometry, nuclear magnetic resonance, X-ray crystallography, amino acid sequencing, circular dichroism spectroscopy, and enzymatic activity assays [37,84–93]. Application of these techniques has led to the identification, characterization and, in some cases, purification of specific allergens, which can subsequently be used in immunoassays for the detection of IgE from tree nut allergic individuals.

Whereas native allergens offer the conformationally and antigenically optimum choice for analysis, the characteristics of some tree nut allergens complicate their use in analytical studies [85,94]. First, native allergens are often difficult to purify to homogeneity even when applying multistep protocols [28,30,95–98]. Enzymatic degradation during *in vivo* (*in planta*) synthesis, fractionation or purification, the frequent presence of multiple isoforms, strain/cultivar differences, variations reflecting different developmental stages, and environmental influences on growth, can all impact the degree of homogeneity within and between samples [85,94]. A lack of homogeneity can interfere with the downstream use in diagnostics and immunoassays. Finally, some native allergens are present at low concentrations in nuts necessitating the use of excessive starting material and complex purification

Table 1
Tree nut allergens (detailed information available at <http://www.allergen.org/>).

Tree nut	Allergen	Reactivity	Type/function	Molecular weight	Native/recombinant	Expression system/vector	Ref.
<i>Anacardium occidentale</i> (Cashew)	Ana o 1	Major allergen, 10 of 20 patients reactive in immunoblots	7S vicilin-like protein/seed storage protein	50 kDa	Recombinant	<i>E. coli</i> /pMAL-c2	[106]
	Ana o 2	Major allergen, 13 of 21 patients reactive in immunoblots	11S legumin-like protein/seed storage protein	55 kDa	Recombinant	<i>E. coli</i> /pMAL-c2	[119]
	Ana o 3	Major allergen, 21 of 26 patients reactive in immunoblots	2S albumin/seed storage protein	14 kDa	Recombinant	<i>E. coli</i> /pMAL-c2	[120]
<i>Bertholletia excelsa</i> (Brazil nut)	Ber e 1	Major allergen, 8 of 9 patients reactive in immunoblots	2S sulfur-rich albumin/seed storage protein	9 kDa	Both	<i>P. pastoris</i> /pPIC9	[78,90,103]
	Ber e 2	Minor allergen, patient serum was reactive in immunoblots	11S globulin/seed storage protein	29 kDa	Native		[78,176]
<i>Carya illinoensis</i> (Pecan)	Car i 1	Major allergen, 22 of 28 patients reactive in dot blots	2S albumin/seed storage protein	16 kDa	Recombinant	<i>E. coli</i> /pMAL-c4X	[107]
	Car i 4	Major allergen, 16 of 28 patients reactive in dot blots	11S legumin-like protein/seed storage protein	55.4 kDa	Recombinant	<i>E. coli</i> /pMAL-c4X	[121]
<i>Castanea sativa</i> (European chestnut)	Cas s 1,	Major allergen, 8 of 8 patients reactive in immunoblots	PR-10/pathogenesis-related protein	22 kDa	Native		[177]
	Cas s 5	Major allergen, 4 of 4 patients reactive in immunoblots	Chitinase/ pathogenesis-related protein		Native		[178]
	Cas s 8	Minor allergen, recognized by pool of 6 patients in immunoblots	Non-specific lipid transfer protein/pathogenesis-related protein	12–13 kDa	Native		[46]
	Cas s 9	Information unavailable	Cytosolic class I small heat shock protein	17 kDa	Recombinant		unpublished
<i>Corylus avellana</i> (Hazelnut)	Cor a 1	Major allergen, 65 of 65 patients reactive in immunoblots	PR-10/pathogenesis-related protein	17 kDa	Both	<i>E. coli</i> /pTYB11	[47,48]
	Cor a 2	Minor allergen, 7 of 17 patients reactive	Profilin/structural protein	14 kDa	Recombinant	<i>E. coli</i> /pET30a	[117]
	Cor a 8	Major allergen, 7 of 7 patients reactive in immunoblots	Non-specific lipid transfer protein/pathogenesis-related protein	9 kDa	Both	<i>E. coli</i> /pET-16b <i>P. pastoris</i> /pPICZalphaA	[49,98,117]
	Cor a 9	Major allergen, 12 of 14 patients reactive in 2-dimensional proteomics	11S legumin-like protein/seed storage protein	40 kDa	Native		[98,179]
	Cor a 11	Minor allergen, 31 of the 65 patients reactive in immunoblots	7S vicilin-like protein/seed storage protein	48 kDa	Both	<i>E. coli</i> /pET100D	[98,180]
	Cor a 12	Major allergen, 118 of 185 patients reactive in RAST, immunoCAP, and immunoblot	Oleolin/structural protein	17 kDa	Recombinant	<i>E. coli</i> /pET19b	[59]
	Cor a 13	Major allergen, 118 of 185 patients reactive in RAST, immunoCAP, and immunoblot	Oleolin/structural protein	14–16 kDa	Recombinant	<i>E. coli</i> /pET19b	[59]
	Cor a 14	Minor allergen, 5 of 15 patients reactive in immunoblots and immunoCAP	2S albumin/seed storage protein	15–16 kDa	Both	<i>E. coli</i> /pPAL7	[95]
	Jug n 1	Information unavailable	2S albumin/seed storage protein	18 kDa	Recombinant	unpublished	Ling et al. unpublished
	Jug n 2	Information unavailable	7S vicilin-like protein/seed storage protein	55 kDa	Recombinant	unpublished	Ling et al. unpublished
<i>Juglans regia</i> (English walnut)	Jug r 1	Major allergen, 12 of 16 patients reactive in immunoblots	2S albumin/seed storage protein	15–16 kDa	Recombinant	<i>E. coli</i> /pGEX <i>E. coli</i> /pTH19*	[102,105]
	Jug r 2	Major allergen, 9 of 15 patients reactive in immunoblots	7S vicilin-like protein/seed storage protein	44 kDa	Recombinant	<i>E. coli</i> /pGEX	[122]
	Jug r 3	Major allergen, 42 of 46 patients were in immunoblots	Non-specific lipid transfer protein/pathogenesis-related protein	9 kDa	Native		[50]
	Jug r 4	Major allergen, 21 of 37 patients reactive in immunoblots	11S legumin-like protein/seed storage protein	55 kDa	Recombinant	<i>E. coli</i> /pMAL-c2X	[115]
<i>Pistacia vera</i> (Pistachio)	Pis v 1	Major allergen, 19 of 28 patients reactive in immunoblots	2S albumin/seed storage protein	7 kDa	Recombinant	<i>E. coli</i> /pET24b	[38]
	Pis v 2	Major allergen, 14 of 28 patients reactive in immunoblots	11S legumin-like subunit/seed storage protein	32 kDa	Recombinant	<i>E. coli</i> /pET24b	[38]
	Pis v 3	Minor allergen, 7 of 19 patients reactive in immunodot blots	7S vicilin-like protein/seed storage protein	55 kDa	Recombinant	<i>E. coli</i> /pMAL-c2	[114]
	Pis v 4	Minor allergen, 10 of 25 patients reactive in immunoblots	Manganese superoxide dismutase	25.7 kDa	Recombinant	<i>E. coli</i> /pET-32b	[62]
	Pis v 5	Minor allergen, 10 of 28 patients reactive in 2-D immunoblots	11S legumin-like subunit/seed storage protein	36 kDa	Native		unpublished
<i>Prunus dulcis</i> (Almond)	Pru du 3	Minor allergen, 5 of 31 patients reactive in immunodot blots	Non-specific lipid transfer protein/pathogenesis-related protein	9 kDa	Recombinant	<i>E. coli</i> /pMAL-c4X	unpublished
	Pru du 4	Minor allergen, 8 of 18 patients reactive in immunodot blot and ELISA	Profilin/structural protein	14 kDa	Both	<i>E. coli</i> /pMAL-c2	[99]
	Pru du 5	Minor allergen, reactive with pooled patient sera in immunodot blots	60s acidic ribosomal protein P2	10 kDa	Recombinant	<i>E. coli</i> /pMAL-c2	[71]
	Pru du 6	Major allergen, 9 of 18 patients reactive in immunodot blots	11S legumin-like protein/seed storage protein	57–60 kDa	Both	<i>E. coli</i> /pMAL-c4X	[39]

* Indicates a unique vector that is not available commercially.

strategies to obtain acceptable yields. For example, 20 g of starting material yielded ~500 µg of purified native almond profilin, Pru du 4 [99].

4. Production of recombinant tree nut allergens

For many applications, recombinant allergens offer an attractive alternative to circumvent these issues. They have demonstrated their utility in a variety of immunoassays and show potential for use in immunotherapy for tree nut allergy [85,100,101]. Through the application of molecular genetic techniques, large quantities of molecularly defined homogenous allergen preparations can be obtained. A variety of expression systems are now available to help ensure that recombinant allergens most closely resemble the native form. For example, analyses of recombinant 2S albumin from walnut (Jug r 1) and Brazil nut (Ber e 1) by serological IgE reactivity, basophil activation assay, circular dichroism, and chemical/temperature denaturation assays have demonstrated that these recombinant allergens are, by these measures, equivalent to their native counterparts [102–104]. For many of the tree nut allergens, the production of their recombinant forms has become a relatively simple and straightforward process.

4.1. cDNA production and PCR amplification of allergen genes

The products named in this section are the ones commonly used in our laboratories as well as by others. Alternatives may also be available.

Recombinant tree nut allergens are often generated by screening cDNA libraries with allergen-specific antisera such as patients' sera, lab animal polyclonal antibodies, or monoclonal antibodies. The libraries are created from nut tissues collected at various stages of development (near mature, immature, and somatic embryos) to insure the presence of the desired mRNA. Freshly collected tissues are chopped, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. Total RNA can be extracted in TRIzol (Life Technologies, Carlsbad, CA, USA) or by using the RNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA, USA). mRNA can be isolated from the total RNA with a Poly-Atract kit (Promega, Madison, WI, USA). The synthesis of cDNA and construction of the library can be performed with the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene Inc, Cedar Creek, TX, USA). The resultant library is plated and protein expression is induced using nitrocellulose membranes soaked in isopropyl- β -thiogalactopyranoside (IPTG). Incubation of the membrane with the antibody of interest allows the identification of immunopositive plaques. Positive plaques are manually picked, purified, amplified with M13 primers by means of PCR, and sequenced. Many of the recombinant tree nut allergens have been generated using this approach including those of walnut [105], cashew [106], almond [99], and pecan [107]. For detailed information on the isolation of RNA, mRNA, generation of cDNA, cDNA library construction, PCR, primer design, amplification, or rapid amplification of cDNA ends (RACE), refer to protocols provided by the specific manufacturers or [109,110].

The approach outlined above works well if the specific sequence of the targeted gene is known (facilitating PCR amplification) or has been determined as a result of screening the cDNA library with an allergen-specific antibody followed by sequencing of reactive plaques. However, if the nucleotide sequence of a suspected allergen gene is unknown and if antisera are not available, PCR amplification using degenerate primers can be employed. For this purpose, regions of conserved DNA sequence in homologous proteins of related plant species are identified. Typically, a mixture of primers incorporating the major sequence variances is produced in the hopes that at least some of the primer members will display

sufficient homology to the unknown target sequence in the cDNA library to initiate the PCR reaction [110,111]. If nucleotide sequences are unavailable, minimally degenerate primers can be designed to best match the possible nucleotides encoding the conserved amino acids of homologous proteins for use in PCR amplification [112]. Once a portion of the target cDNA is sequenced, additional primers can be generated based on the actual gene sequence and paired with primers directed against the vector sequence to obtain the full length allergen gene sequence. In those instances where only a portion of the allergen gene is amplified by degenerate PCR and a cDNA library from the desired nut is not available, 5' or 3' rapid amplification of cDNA ends (RACE) can be performed to derive the missing genetic sequence [110,113]. Gene-specific primers corresponding to a segment of the known region are paired with a poly(A) tail (3' end) targeting primer or a tag sequence associated with the 5' end to amplify the full length allergen gene [110,113]. This approach has been successfully used to amplify the MnSOD, Pis v 4, and the 7S vicilin, Pis v 3, allergen genes from pistachio nut [62,114], the 11S globulin, Jug r 4, from walnut [115], the lipid transfer protein, Cor a 8, the pathogenesis-related protein PR-10, Cor a 1, and the oleosins, Cor a 12 and 13, from hazelnut [49,59,108].

4.2. Cloning, expression, and purification of recombinant tree nut allergens

Once the allergen gene has been amplified by PCR and sequenced, it can be cloned and expressed (for detailed protocols refer to [109]). A wide variety of expression systems are available (bacteria, yeast, insect, plant, or Chinese hamster ovary cells). The selection of a suitable system for the recombinant allergen depends on several factors including, desired yield, need for post-translational modifications, ease of purification, cost, and size of the allergen. Whereas insect and mammalian cells typically generate proteins that are more properly folded and glycosylated, low yields, extended culture times, and greater expense have limited their use. Moreover, since most tree nut allergens are naturally unglycosylated [33,40], the insect and mammalian expression systems offer little advantage in this respect [116]. Yeasts (*Pichia pastoris* and *Saccharomyces cerevisiae*) also glycosylate expressed proteins and facilitate disulfide bond formation and have the advantage of lower cost and higher rates of secretion as compared to mammalian and insect cells. However, their ability to glycosylate expressed tree nut allergens offers little benefit as indicated above. To date, yeast (*P. pastoris*) has only been used to express two tree nut allergens (Ber e 1 and Cor a 8) [103,117].

Consequently, most allergens have been expressed in *Escherichia coli* using a variety of different expression vectors as shown in Table 1. Bacteria often produce high yields of protein in a short amount of time, are cost effective, and easy to culture, which makes them an ideal choice for many applications. Each vector has its own set of features (Table 2). The use of tagged or fusion proteins facilitates rapid and efficient purification by affinity chromatography to produce highly purified recombinant proteins. Detailed information on purification and fusion protein cleavage is provided by the respective manufacturers.

One popular vector is pMAL which generates an N-terminal fusion protein containing maltose binding protein (MBP) associated with the allergen of interest (Table 2) (New England BioLabs Inc, Beverly, MA, USA). MBP fusion proteins can easily be purified from the bacterial lysates through binding to immobilized amylose resin followed by elution with a maltose-containing buffer (New England BioLabs Inc, Beverly, MA, USA). Once purified, a site-specific protease such as thrombin, factor Xa, enterokinase, or Genenase I™ can be used to cleave the MBP fusion protein from the allergen. The addition of MBP also helps enhance the solubility of the fused

Table 2
Vectors used to express recombinant tree nut allergens.

Expression system/ Vector	Fusion partner	Location	Purification	Tag removal
<i>E. coli</i> /pMAL	Maltose binding protein (MBP)	N-terminal	Amylose resin followed by elution with a maltose-containing buffer	Site-specific protease such as thrombin, factor Xa, enterokinase, or Genesee I TM protease
<i>E. coli</i> /pGEX	Glutathione S-transferase (GST)	N-terminal	Immobilized glutathione followed by elution with glutathione	Site-specific protease such as thrombin, factor Xa, or PreScission TM protease
<i>E. coli</i> /pPAL7	Prodomain of subtilisin	N-terminal	Immobilized subtilisin protease	On-column cleavage facilitated by fluoride buffer activation of subtilisin protease
<i>E. coli</i> /pET	6–10 aa histidine (His) tag	N- or C-terminal	Immobilized divalent cations (e.g., Ni ⁺⁺ , Co ⁺⁺) followed by elution with imidazole-containing buffer	Tag removal typically unnecessary due to small size
<i>E. coli</i> /pTYB11	Intein and chitin binding domain	N-terminal	Immobilized chitin	On-column cleavage via intein at low temperatures in the presence of thiols such as DTT
<i>P. pastoris</i> /pPIC9	None		Various chromatography steps	None
<i>P. pastoris</i> /pPICZalphaA	None		Various chromatography steps	None

proteins, an important issue in the production of several recombinant tree nut allergens [118]. For example, the 11S and 7S seed storage allergens are naturally hexameric and trimeric proteins respectively. When expressed without a fusion partner, these molecules tend to aggregate and are relatively insoluble as compared to their native counterparts [39,119]. pMAL has been used to express allergens from almond (Pru du 4, 5, and 6) [39,71,99], cashew (Ana o 1, 2, and 3) [106,119,120], pecan (Car i 1 and 4) [107,121], walnut (Jug r 4) [115], and pistachio (Pis v 3) [114].

The pGEX vector generates a fusion protein containing N-terminal glutathione S-transferase (GST) which can be purified using immobilized glutathione followed by elution with soluble glutathione (Table 2) (GE Healthcare Biosciences, Pittsburgh, PA, USA). If desired, cleavage of the allergen from GST can be achieved using a site-specific protease like thrombin, factor Xa, or PreScissionTM protease depending on the vector used. pGEX has been used to express allergens from walnut (Jug r 1 and 2) [105,122].

More recently, the pPAL7 vector has been used to express the recombinant hazelnut allergen, Cor a 14 [95]. This vector generates an N-terminal fusion protein containing the prodomain of subtilisin and allows for the simultaneous capture and cleavage of the fusion protein (Table 2) (Bio-Rad, Hercules, CA, USA). The fusion protein is captured by immobilized subtilisin protease and on-column cleavage occurs by fluoride buffer activation of subtilisin protease to release the allergen for collection.

Another popular vector used for the expression of tree nut allergens is the pET vector system (Table 2) (Novagen, EMD Millipore, Billerica, MA, USA). This vector contains a short 6 to 10 aa histidine (His) tag which can be located at either the N- or C-terminal of the allergen depending on the pET vector used. The His tag facilitates capture through interaction with immobilized divalent cations (e.g., Ni⁺⁺, Co⁺⁺). An imidazole-containing buffer is used for elution. An important advantage of using His tags is that it minimizes the possibility that bulky fusion partners might interfere with natural oligomerization or antibody reactivity in downstream immunoassays. Also, because of its small size, a tag removal step is typically unnecessary [123]. pET vectors have been used to express allergens from hazelnut (Cor a 2, 8, 12, and 13) [49,59,117], and pistachio (Pis v 1, 2, and 4) [38,62]. Additional pET vectors are available that lack a fusion tag or contain GST, HSV, T7, Dsb, Trx, or a combination of tags (Novagen, EMD Millipore, Billerica, MA, USA). To date these vectors have not been used to express recombinant tree nut allergens. For a comprehensive review of tagged fusion proteins please refer to Ref. [123].

Although bacteria are the most popular expression system for tree nut allergens, they do have certain drawbacks such as the inability to replicate the post-translational modifications associated with some native nut allergens, particularly disulfide bond formation and glycosylation [116,124]. The expression of recombinant allergens typically occurs in the cytoplasm of bacterial cells, which is a reducing environment not conducive to disulfide bond formation [124]. This can be problematic as most tree nut allergens, such as the seed storage proteins (11S legumin, 2S albumin) and PR-protein, LTP, have several disulfide linkages which may be necessary for proper folding, epitope expression, and resistance to denaturation [26,33,41,125].

To facilitate disulfide bond formation, a short leader peptide can be genetically attached to the N-terminus of the newly synthesized recombinant protein to target it to the oxidizing environment of the bacterial periplasm. Most commercial vectors are available with this adaption. Although periplasm expression can facilitate the production of folded, soluble, disulfide bonded recombinant proteins, it often lowers overall expression levels and can result in unwanted leakage (and loss) of the recombinant protein into the culture medium [116].

Alternative approaches to the disulfide bond issue are found in other genetically modified strains of bacteria [116,124]. For a comprehensive review of strain engineering for improved expression of recombinant proteins in bacteria please refer to [124]. One such strain is Rosetta-gami B which contains mutations in thioredoxin reductase (*trx*B) and glutathione reductase (*gor*) genes resulting in the formation of disulfide bonds in the bacterial cytoplasm (Novagen, EMD Millipore, Billerica, MA, USA). We have recently used Rosetta-gami B cells to express recombinant almond allergens that are conformationally and serologically more similar to their native counterparts as compared to molecules derived from traditional unmodified *E. coli* strains (unpublished data).

A few tree nut allergens are glycosylated. Recently, it was discovered that N-linked glycosylation of proteins can occur in the enteropathogenic bacteria *Campylobacter jejuni* due to the *pgl* gene cluster [124,126]. Transformation of the gene cluster into *E. coli* has been shown to facilitate the production of glycosylated recombinant proteins [126]. Additionally, genetically modified strains that co-express chaperone proteins (GroEL/GroES, DnaK/DnaJ, IbpA/IbpB, Skp, FkpA) are available to help minimize aggregation and improve recombinant protein folding [124]. These expression systems have not yet been used for the production of recombinant tree nut allergens.

By judicious selection of bacterial expression systems, recombinant allergens can be produced that are structurally and functionally similar to their native counterparts in most cases.

5. Applications of recombinant tree nut allergens

The use of recombinant tree nut allergens continues to expand. To date, they have been used in a variety of immunoassays aimed at assessing patient IgE reactivity, investigating cross-reactivity between various nut and seed proteins, assisting in structural studies, epitope mapping and characterization, and analyses of the effects of food matrix, enzymatic digestion, and denaturation on allergenicity.

Recombinant proteins have played a major role in the identification and characterization of a wide variety of tree nut allergens (Table 1). Amplification of an allergen gene directly through the cDNA production, cloning, and expression allows researchers to bypass purification of the native protein. This approach is ideal when appropriate techniques are unavailable to identify/isolate the native allergen from the whole nut extract. Once obtained the recombinant allergen can be used to assess patient reactivity, characterize IgE binding epitopes, and study the effect of mutations on IgE binding. Several IgE binding allergens have been identified using this approach including: cashew (Ana o 1, 2 and 3) [106,119,120], hazelnut (Cor a 8 and 14) [49,95], pecan (Car i 1 and 4) [107,121], walnut (Jug r 1, 2, and 4) [105,115,122], pistachio (Pis v 1, 2, and 3) [38,114], and almond (Pru du 6) [39]. Collectively, the cloned and expressed allergens represent 11S globulins (Ana o 2, Car i 4, Jug r 4, Pis v 2, and Pru du 6), 7S globulins (Ana o 1, Jug r 2, and Pis v 3), 2S albumins (Ana o 3, Car i 1, Cor a 14, Jug r 1, and Pis v 1), and a lipid transfer protein (Cor a 8).

Recombinant proteins are becoming an increasingly important tool for the diagnosis of tree nut allergy as they can provide standardized quality controlled samples for *in vitro* analysis of IgE reactivity. The importance of this is illustrated by the results of a study in which nine commercially available hazelnut extracts used in immunoassays were shown to display a wide variation in the concentrations of some constituent allergen proteins [127]. Some, such as Cor a 8, were undetectable in several extracts [127]. A variety of precisely defined recombinant tree nut allergens have been developed, in part, to address this problem and some (Ber e 1, Cor a 1, and Cor a 8) are now commercially available to clinicians

for *in vitro* allergen diagnosis (ImmunoCAP assays, Phadia AB, Uppsala, Sweden). Their clinical application, known as recombinant allergen-based component resolved diagnostics (CRD), focuses on analyzing IgE reactivity to specific allergenic proteins to accurately identifying the eliciting molecules [128]. This approach has been successfully used, for example, to evaluate IgE reactivity of hazelnut-sensitized individuals in Spain, Switzerland, and Denmark to reveal significant geographical differences in the recognition of Cor a 1.04 and Cor a 8 [129]. Additionally, recombinant allergens, such as Cor a 1.04, have been successfully used to augment conventional hazelnut allergen extracts to enhance the scope of IgE detection and aid in diagnosis [130]. The analysis of clinical histories, double-blind placebo-controlled food challenges, and *in vitro* CRD using recombinant allergens has lead to the identification of several marker allergens which are associated with the severity of tree nut allergy. Studies focused on hazelnut have shown that specific allergenic proteins can serve as marker proteins in diagnosing not only the symptom-eliciting nut allergen but in predicting the severity of the patient reaction (Cor a 8, 9, 12, 13, and 14 are associated with severe objective symptoms, and Cor a 1, and 2 with milder subjective symptoms) [49,59,131–133]. A similar observation was made with the recombinant almond allergen, Pru du 4, in which the reactive patients experienced milder reactions upon consumption of almonds and suffered from pollinosis [99]. These studies demonstrate the usefulness of recombinant proteins for *in vitro* allergen testing as they provide a well-defined, stable reagent for diagnosis.

Once a tree nut allergen has been identified and cloned, epitope mapping studies are typically performed to identify the IgE binding regions. To date, most studies have focused on the identification of sequential (linear) epitopes which are defined as short contiguous stretches of amino acids (typically 8–15 residues) [16,134,135]. These epitopes are relatively easy to identify by probing short synthetic overlapping peptides, representing the entire amino acid sequence, with serum from sensitized individuals [134]. Sequential epitopes are prevalent on type I food allergens as they can remain intact, unlike many conformational epitopes, even after extensive food processing and gastric digestion [134]. Sequential IgE binding epitopes have been described in pecan (Car i 1 and 4) [107,121], cashew (Ana o 1, 2, and 3) [106,119,120], walnut (Jug r 1 and 4) [102,136,137], hazelnut (Cor a 9) [137], and almond (Pru du 6) [39].

Several studies have focused on comparing the sequential epitopes identified on tree nut allergens with the hopes of establishing patterns of recognition and common features, which may predispose such proteins to be targeted by IgE. Those identified on the 11S globulins have been the most extensively studied. Comparison of these epitopes (walnut Jug r 4, hazelnut Cor a 9, cashew Ana o 2, almond Pru du 6, and pecan Car i 4) with each other and with homologous allergenic nuts and seeds (peanut Ara h 3 and soybean Gly m 6) showed that many display a high degree of sequence conservation and/or are localized to homologous regions, designated as allergenic 'hot spots' [39,121,137]. Mapping of these epitopes onto three-dimensional models of the allergens revealed that several were located in regions that had a similar conformation and charge distribution [121,137,138]. Many of the epitopes were found to cluster together on the surface of the allergens to form extended IgE-binding areas. It has been suggested that such sequential epitope clusters may be part of larger conformational epitopes on the allergen [138]. The pattern was similar for the 7S allergens (cashew Ana o 1, walnut Jug r 2, hazelnut Cor a 11, and peanut Ara h 1) in that there was epitope sequence conservation and clustering [139]. A majority of the sequential epitopes identified on the 2S albumins (cashew Ana o 3, pecan Car i 1, and walnut Jug r 1) were also found to have positional homology when compared on the primary structure [107]. Three-dimensional modeling

of the 2S albumin epitopes identified two immunodominant IgE binding regions, which were located in highly charged areas on the molecular surface [107].

In contrast to the abundant information on sequential allergen epitopes, few conformational IgE binding epitopes have been identified on tree nut allergens. This situation is largely due to the technical difficulty in identifying conformational epitopes which typically require two or more peptide segments held in a specific orientation as a result of tertiary or quaternary structure [1,140,141]. One approach to conformational epitope identification is to generate a set of chimeric molecules in which segments of the allergen of interest are exchanged, through genetic engineering, with homologous segments of a non-reactive homologue. The resultant chimeric molecules are then screened for maintenance or loss of antibody reactivity. For example, to identify conformational IgE binding epitope regions on the Brazil nut 2S albumin allergen, Ber e 1, segments were exchanged with the sunflower seed homologue and a conformational epitope was located in a helix-loop-helix region [103]. A similar approach was taken to identify a conformational epitope-bearing segment on the cashew 11S globulin allergen, Ana o 2, wherein the soybean 11S globulin, Gly m 6, served as the exchange partner [142,143]. In this study, immunoassays using a murine monoclonal antibody that competed with patient IgE antibodies indicated the presence of a conformational epitope in a segment spanning amino acids 20–43 of the acidic polypeptide [143]. This result was supported by analyzing constructs with varying degrees of N-terminal truncation and performing alanine scanning mutagenesis coupled with molecular modeling to verify the location of the epitope and identify critical residues responsible for antibody binding [143].

Several new approaches are being applied to epitope mapping studies on recombinant tree nut allergens with a focus on identifying the more difficult conformational epitopes. These include negative stain immunoelectron microscopic analysis of individual allergen-mAb immune complexes [143], phage display to identify allergen mimotopes (unpublished), and hydrogen deuterium exchange coupled with mass spectrometry (i.e., epitope footprinting) [144–146].

There have been a number of computer based algorithms designed to predict B cell epitopes, including ABCPred, BcePred, BEPI-TOPE, BepiPred, CEP, discotope, immune epitope database (IEDB), and the structural database of allergenic proteins (SDAP) [147–149]. Predictions are based on the properties of the amino acid sequence (hydrophilicity/hydrophobicity, charge, secondary structure) and the predicated three-dimensional structure, which is often generated by homology modeling (exposed surface area) [147,150]. In theory, these algorithms should be able to identify potentially immunogenic/epitope regions on protein [149,150]. However, studies have shown that there is often little correlation between the experimentally derived and computed data [150,151]. These findings highlight the importance of experimental data for the valid identification of epitopes on proteins.

It is often observed that individuals who are allergic to one tree nut, also display IgE reactivity to other nuts, a situation which could result from either co-sensitization or true cross-reactivity [75,152–157]. Recombinant tree nut allergens provide a useful tool for investigating this phenomenon on a molecular level. Studies have analyzed cross-reactivity between tree nuts and other seeds, including peanut and sesame. Studies on peanut-allergic patients with and without walnut allergy demonstrated that the observed sensitization to both nuts is due to the recognition of unique IgE binding epitopes on the walnut allergens Jug r 1, 2, and 4 and not cross-reactivity between homologous peanut epitopes [158]. To investigate cross-reactivity between the 11S globulins from walnut and sesame seed, inhibition immunoblotting assays were performed. IgE binding to recombinant Ses i 6 was significantly re-

duced when serum was pre-incubated with recombinant walnut Jug r 4 [159]. Similarly, chicken antibodies (IgY) raised against recombinant Jug r 4 recognized both walnut Jug n 4 and sesame Ses i 6 in immunoblotting assays indicating that these 11S globulins display cross-reactive epitopes [159].

Two particular nut seeds with a well-documented history of cross-reactivity are pistachio and cashew, both members of the Anacardaceae family [75,81,152,160]. Studies comparing the recombinant pistachio allergen, Pis v 3, and cashew homologue, Ana o 1, found that in all of the pistachio/cashew allergic patients tested, IgE binding to either allergen was blocked when sera was pre-incubated with either recombinant allergen [114]. A panel of murine monoclonal antibodies raised against cashew Ana o 1, also recognized recombinant Pis v 3 in dot blot assays suggesting that antibody reactivity to these allergens is likely focused on the conserved regions [114].

Whereas many studies have assessed digestibility and the effect of the food matrix on allergenic proteins in whole nut protein extracts, few have focused on the stability of specific tree nut allergens [161–166]. Recombinant tree nut allergens have been used to assess the effect of the food matrix, food processing, and gastric digestion of tree nut allergens. For example, studies done on the Brazil nut allergen, Ber e 1, found that exposure of mice, by intraperitoneal injection, to recombinant Ber e 1 alone did not induce a detectable IgG or IgE antibody response. However, co-administration of the allergen with the total lipid, sterol-rich, or polar lipid fractions, resulted in a strong antibody response [167]. This suggests that the food matrix plays an important role in the sensitization to Brazil nut allergens [167]. Analysis of the *in vitro* digestibility of recombinant Ber e 1 has been assessed using simulated gastric fluid and was found to exhibit a similar degree of resistance to digestion as the native allergen [168]. Also, the stability of both the native and recombinant allergen to thermal and chemically induced unfolding was found to be similar when analyzed by circular dichroism spectroscopy [168]. These findings suggest that cells of the gut mucosal immune system, which are presumably responsible for the sensitization to this allergen, likely come into contact with the intact allergen [168]. This information is important in furthering our understanding of the mechanism by which tree nut allergy is induced and how the food matrix may affect the allergenicity of a protein.

6. Limitations of recombinant tree nut allergens

Recombinant allergens are relatively easy to produce and have provided important insights into the properties of tree nut proteins which stimulate the production of IgE leading to type I hypersensitivity. However, there are several limitations associated with their production and use. An important limitation, especially for the *E.coli*-based expression systems, is the lack of post-translational modifications which may affect the overall conformation of the recombinant allergen and thus its antibody reactivity [94]. As indicated above, several tree nut allergens, in particular the 11S and 7S seed storage allergens, are enzymatically cleaved into subunits in the developing seed, a process which facilitates their oligomerization and enhances solubility [25,26,33,85,169]. This process is not readily mimicked in current expression systems, which can lead to protein aggregation and, presumably, protein misfolding. Many tree nut allergens also contain intra- and intermolecular disulfide bonds which influence folding and stability (e.g., 11S globulins, 2S albumins, chitinase, and LTP) [33,85]. Classical *E. coli*-based expression vectors are deficient in this capacity [85,116]. Additionally, the glycosylated state of the native tree nut allergen should be considered when expressing the recombinant version. Fortunately, most tree nut allergens naturally lack glycosylation and thus can be considered for expression in multiple

systems [33,40]. However, some tree nut allergens, such as the 7S seed storage allergen, are glycosylated. To date, there have been no reports of the unglycosylated recombinant homologues being deficient in antibody reactivity as a consequence.

Many recombinant allergens are produced with fusion tags (GST, MBP, or His) to aid in the downstream purification of the recombinant protein. Whereas small tags, such as His, likely do not interfere in subsequent antibody binding assays, others may produce larger steric effects and potentially block epitopes on the allergen [123]. Ideally, it would be desirable to cleave and remove the tag once the recombinant allergen has been purified or to express the recombinant allergen in an unfused state. This approach has been successful for some recombinant tree nut allergens (e.g., pecan Car i 1, pistachio Pis v 1, and hazelnut Cor a 8, 12, 13, and 14) [38,59,95,107,117]. However, others, such as the seed storage allergens, have been found to aggregate and become insoluble (almond Pru du 6, cashew Ana o 2, pistachio Pis v 4, and walnut Jug r 1) when expressed without their solubility enhancing fusion partner or have been resistant to enzymatic cleavage to remove the fusion partner [39,62,105,119]. Overall, the stringent selection of the optimum expression system and vector (discussed in Section 3.2) can help avoid some of these pitfalls so that recombinant tree nut allergens that most closely resemble their native counterparts can be produced.

7. Conclusions and future perspectives

Tree nut allergy is a growing global concern as the number of affected individuals continues to increase. Due to the difficulties associated with purifying many native tree nut allergens, recombinant allergens have become indispensable reagents in the laboratory and for the clinical diagnosis of tree nut allergy. Such reagents allow for standardized diagnosis and detection of allergen specific IgE, as well as provide important information about the varying geographical distribution of reactivity to certain tree nut allergens. Additionally, these allergens can be used to better understand the cross-reactive nature observed in certain nuts and the mapping of IgE binding epitopes. Such information is critical for furthering our understanding of tree nut allergy and the mechanism by which sensitization may occur.

To date, tree nut allergens have not been used in immunotherapy. Whereas there are several therapeutic approaches potentially available (oral immunotherapy, sublingual immunotherapy, epicutaneous immunotherapy, and allergen specific immunotherapy), to date, these have been used exclusively to treat pollen allergy. Several clinical trials have been performed on milk, egg, peanut, and pollen-related food allergies but have yet to be approved for the treatment [170–175]. Future work on tree nut allergens will likely focus, in part, on the application of recombinant proteins for the desensitization of tree nut-allergic individuals. As the number of recombinant allergens available continues to increase and their IgE binding epitopes are fully evaluated, the development of hypo-allergenic forms which have been genetically engineered to reduced IgE binding capacity can be anticipated.

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