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Published in:
Critical Reviews in Biotechnology

Link to article, DOI:
10.1080/07388551.2020.1772194

Publication date:
2020

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Larsen, J. M., Bang-Berthelsen, C. H., Qvortrup, K., Sancho Vega, A. I., Hansen, A. H., Andersen, K. I. H., Thacker, S. S. N., Eiwegger, T., Upton, J., & Bøgh, K. L. (2020). Production of allergen-specific immunotherapeutic agents for the treatment of food allergy. *Critical Reviews in Biotechnology*, *40*(6), 881-894. https://doi.org/10.1080/07388551.2020.1772194

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Production of allergen-specific immunotherapeutic agents for the treatment of food allergy

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Word count: 10411

Keywords: Food allergy, immunotherapy, extracts, recombinant production, food processing, synthetic peptides.

Abstract

Allergen-specific immunotherapy is emerging as a viable avenue for treatment of food allergy. Clinical trials currently investigate raw or slightly processed foods as therapeutic agents, as trials using food-grade agents can be performed without the strict regulations to which conventional drugs are subjected. However, this limits the ability of standardization and may affect clinical trial outcomes and reproducibility. Herein, we provide an overview of methods used in the production of immunotherapeutic agents for the treatment of food allergy, including processed foods, allergen extracts, recombinant allergens, and synthetic peptides, as well as the physical and chemical processes for the reduction of protein allergenicity. Commercial interests currently favor producing standardized drug-grade allergen extracts for therapeutic use, and clinical trials are ongoing. In the near future recombinant production could replace purification strategies since it allows manufacturing pure, native allergens or sequence-modified allergens with reduced allergenicity. A recurring issue within the field is the inadequate reporting on production procedures, quality control, product physicochemical characteristics, allergenicity and immunological properties. This information is of vital importance in assessing therapeutic standardization and clinical safety profile, which are central parameters for development of future therapeutic agents.

Introduction

Food allergy is a global health problem of growing concern [1,2] affecting approximately 2-4% of adults and 5-8% of children [1]. The disease arises due to the inhibition or breakdown of immunological tolerance resulting in the generation of immunoglobulin E (IgE) towards specific proteins in food, so-called allergens. Ingestion of the specific food by sensitized individuals triggers the immediate release and synthesis of inflammatory mediators, which initiate a cascade of immunological events leading to an array of symptoms ranging from mild to life threatening. Mild symptoms may be restricted to one organ such as the gastrointestinal tract (pain, nausea, vomiting, and diarrhea) or skin (itchiness, urticaria, and angioedema) and progress to more severe reactions affecting the respiratory and cardiovascular systems (dyspnea, wheezing, and tachycardia) [3]. Often accidental, unintended ingestion of a food by allergic patients causes acute life-threatening or even fatal anaphylaxis. This perpetual risk contributes to significantly decreasing the quality-of-life experienced by patients [4].

Allergen-specific immunotherapy (IT) is emerging as a potential avenue for the treatment of food allergy [5–7], but lacks standardized treatment agents and regimens for effective and long-lasting treatment outcomes. The first IT drug for use in food allergy was approved in 2020, when the FDA approve the standardized peanut protein extract Palforzia for the treatment of peanut allergy in children [8]. Still, safer IT agents, evaluation of long-term effectiveness, and IT targeting other patient groups and food allergies are need. The overall goal of IT is to suppress the pathogenic immune response driven by allergen-specific IgE with the contribution of type-2 immune cells, and enhance immune regulatory mechanisms [9,10]. This has predominantly been achieved by exposing patients to the offending food via the oral route (oral IT, OIT) using very small dosages not eliciting clinical symptoms followed by gradually increasing dosages. The ability to eat increasing doses of the allergen due to repeated, gradual exposure is called desensitization. However, this OIT approach to desensitization using food is commonly associated with severe adverse gastrointestinal reactions, and can cause anaphylaxis. Thus, efforts have been made to produce IT agents in which food allergens have been modified to reduce IgE-binding, abolish IgE-crosslinking capacity, or decrease sensitization potential (i.e. the particular agent has reduced allergenicity). Furthermore, IT agents have been produced for administration via non-oral routes (e.g. subcutaneous, epicutaneous, and sublingual administration) to reduce the risk of eliciting food allergic reactions associated with OIT.

Herein, we review methods for the production of IT agents. We will focus on agents that are currently applied in the clinic, in clinical trials, or tested in pre-clinical animal models. We draw perspectives from IT strategies in the treatment of non-food allergies, as well as selected methods to produce novel agents with reduced allergenicity. Please refer to figure 1 for an overview of IT agents, and the materials and processes used in the production covered by the present review.

Raw whole foods

IT using whole foods or slightly processed foods have demonstrate clinical efficacy in human trials [5]. Raw foods have predominantly been used for treatment of cow's milk allergy and hen's egg allergy. The sources of the milk and egg products are usually not indicated, nor the type of milk used (e.g. skimmed, whole milk etc.), and processing methods such as pasteurization or homogenization are frequently not stated (please refer to trials in [5]). The amount of protein in the raw food is commonly measured to allow a quantified administration, yet unknown origin, allergen content, food matrix composition, and specific processing methods may influence replication. Many clinical trials in peanut allergy have used commercially available peanut flour [5], on which limited information on processing and product characteristics are provided.

Cooked and processed foods

It is generally recognized that cooking changes or destroys conformational epitopes in food proteins, thereby decreasing the risk of allergic elicitation upon ingestion, while retaining the IT potential. Clinical trials have been conducted using foods that have undergone normal cooking processes, including boiled or fried hen's eggs, baked cow's milk or hen's eggs, and wheat porridge and boiled noodles [11]. Although cooked foods may have beneficial characteristics for disease management and therapy [12], the widespread lack of detailed descriptions of the actual cooking process and origin of the food create unknown variation in these IT agents. Additionally, the general absence of product characterization beyond protein concentration complicates reproduction of these complex products and the associated trial outcomes. For this reason, more standardized IT drugs may allow more consistent trials with enhanced reproducibility (please refer to trials in [11]).

Industrially processed cow's milk and hen's egg products have been used as IT agents, including dehydrated and powdered products [13–19]. The processing methods of these products are largely undescribed in the clinical studies, but some are known to be commercially available [13,15] allowing for some standardization dependent on manufacturer's product quality control.

Allergen extracts

Allergen extracts represent a way to produce an IT agent, which can form the basis for further modification of allergenicity and therapeutic properties (discussed in later sections), or as an agent without food matrix for the administration via non-oral routes, including subcutaneous IT (SCIT), epicutaneous IT (EPIT), and orally in the form of sublingual IT (SLIT). Extracts are produced by separating the relevant allergens from the raw material (containing non-antigenic components like lipids, sugars, and phenols) using physical processing (e.g. grinding) and/or chemical extraction (e.g. defatting). In some cases, this is followed by several purification steps requiring specialized chromatographic techniques. The choice of procedures is based on an assessment of protein physicochemical characteristics (e.g. pI, solubility, post-translational modifications). Early studies using SCIT with peanut protein extract report only the use of an aqueous extract of defatted peanuts, but provide no information on methods for defatting, extraction procedure, or other preparation [20,21]. Similarly, no information on methods used for the preparation of peanut extracts is available for drugs currently undergoing clinical testing for OIT [22,23] (AR101, Aimmune), EPIT [24–26] (Viaskin, DBV Technologies), or SLIT (SAR-439794, NCT03463135, Sanofi/Design Corp) IT, which likely can be ascribed to company trade secrets. A number of investigators conducting SLIT trials report the use of a commercially available aqueous extracts (Greer Laboratories) that have been prepared from raw peanuts using a 0.50% NaCl and 0.54% $NaHCO_3$ buffer at pH 6.8-8.4 [27–30]. Another study reported extraction of defatted peanut using TRIS/HCl at pH 8.5 and 10% (w/v) before downstream chemical modification and testing in animal model [31]. Finally, two SLIT studies report on the extraction of hazelnut [32] and peach [33]. Hazelnut was defatted using diethyl ether followed by extraction using 4% Tween-20 at 50% (w/v) for 1 h at 4°C. Peach peelings were extracted using 0.03 M NaCl at pH 1.2 at 10% (w/v) for 2 h at 4°C. There is limited information on the production and quality control procedures. The potency of

an extract is influenced by several factors, including food cultivars, growing conditions, processing, and extraction method [34–37]. Correct allergen dosing regimens are important for the efficacy and safety during treatment. However, different allergen extracts have been found to vary significantly in potency and composition [38]. In an attempt to reduce variation in the practice, allergen IT guidelines are in place for control of product composition and long-term quality, and for production issues [39–41]. Still, in-depth product characterisation is needed in future studies, including assessment of product composition and native conformation of allergens following the purification process. These efforts are also important, since extracts may lose lipid-soluble allergens and change conformations with unknown importance for treatment efficiency.

Purified natural allergens

Purified allergens from natural food sources have been identified as an alternative to the varying and less well-characterised allergen extracts. However, until now only pre-clinical studies in animal models reported on commercially available purified proteins, for which the preparation method and protein characteristics were not disclosed [42,43]. Nevertheless, technical advances in the field of molecular biology have enabled cost-effective production of recombinant allergens (reviewed in the following sections) in higher yields and with decreased allergenicity, to provide a potentially safer choice as compared to purified natural allergens.

Recombinant allergens and peptides

Biotechnological methods for recombinant protein production allow for the generation of pure and well-defined allergens for IT. Furthermore, the technology enables production of allergen-derived peptides (e.g. T cell epitopes), as well as production of modified allergens with reduced allergenicity (e.g. mutations in IgE-binding sites, sequence shuffled allergens, or fusion proteins) [44]. The sections below provide an overview of the methods used in production of recombinant food allergens. Most of the recombinant allergens have been produced for in vitro or pre-clinical use, but some have been tested in clinical trials for the treatment of fish [45] and peanut (EMP-123) allergy [46].

Selection of Expression Host

Selecting the right expression host is crucial for successful production of functional allergens. Therefore, much effort has focused on identifying the most efficient expression host for expression of each individual allergen. This section shortly summarizes the different organisms previously used for production of food allergens, and advantages and disadvantages associated with each organism are addressed. An overview of recombinantly produced food allergens can be found in Table 1, along with the employed host and vector system for each allergen.

The most widely used expression host for recombinant proteins is *Escherichia coli* (*E. coli*). This host has been used to express a variety of food allergens, including allergens from peanut (Ara h 1, Ara h 2, Ara h 3, Ara h 5, Ara h 6) [47–53], apple (Mal d 1) [54], cow's milk (Bos d 5) [55], hen's egg (Gal d 1) [56], soy (Enolase, TPI, MDH, Gly m 3) [57,58], cod (Gad m 1) [59], carp (Cyp c 1) [59–62], pistachio nut (MnSOD) [63], hazelnut (Cor a 8) [64] and wolf herring (parvalbumin) [65]. Most studies do not explicitly describe the reason for choosing *E. coli* as the host organism, but most likely this is due to the ease of engineering, the high growth rate, low maintenance, and ability to produce proteins on a large scale [66].

Several different strains of *E. coli* have been reported in the above-mentioned studies, with the *E. coli* BL21 (DE3) in conjunction with the T7 expression vector remaining the most frequently used system. In addition, multiple variations of this strain, including the *E. coli* BL21 (DE3) CodonPlus-RIL, *E. coli* BL21 (DE3) CodonPlus-RIPL, *E. coli* BL21 Star (DE3) and *E. coli* BL21 (DE3) pLysS have been successfully used as expression systems for production of allergenic proteins. The BL21 and BL21 Star (DE3) represent codon-optimized strains expressing higher levels of the rarest tRNAs, as these are often more widely present in allergens [48]. The BL21 Star (DE3) strain expresses a higher level of protein due to higher stability of mRNA, making it highly relevant for expression of allergens, which are usually non-toxic. Relative to the former, BL21 (DE3) pLysS generally shows lower expression of the protein due to addition of the T7 lysozyme. In earlier studies *E.coli* K strains such as JM109 and TB1 were used for the production of allergens from soy and hen's egg, but are not used any longer due to newer and better strains [58,67].

The second most commonly prokaryotic cell type used is the gram-positive *Lactococcus lactis* (*L. lactis*). The main advantage of *L. lactis* compared to *E. coli* is its ability to secrete the produced proteins directly into the medium by the use of signaling peptides, such as the SP310 reported by

Glenting et al., 2007 [68]. Additionally, *L. lactis* is generally recognized as safe, grows rapidly, relatively easy to engineer, and can produce disulfide bonds, hereby enabling correct folding of allergens to preserve conformational epitopes. The main disadvantage of *L. lactis* remains the same as for *E. coli;* both host systems are unable to insert post-translational modifications [68]. *L. lactis* has been used to produce the food allergens Ara h 2 (peanut) and Bos d 5 41-60 peptide (cow's milk). The peanut allergen was produced using the common MG1363 strain, while the cow's milk allergen was produced using the NZ9000 strain, the latter of which is superior at forming correct disulfide bonds [68–70].

The yeast *Pichia pastoris* has also been used to produce various food allergens such as Cor a 8 (hazelnut), Ber e 1 (Brazil nut) and Ara h 6 (peanut). The main advantage of using this species rather than bacterial hosts is that it can introduce certain post-translational modifications, producing allergens that are almost identical to their native analogs. Furthermore, yeasts are easily engineered, regarded as a good production organisms, and can be engineered to secrete the proteins [64,71,72]. The main disadvantage of *P. patoris* is a slower growth rate compared to bacteria.

Higher organisms have also been used for production of food allergens, including Oleosins from peanut (Ara h 10, Ara h 11 and Ara h 14) in Sf9 insect cells, Ara h 1 from peanut in the algae cells *Chlamydomonas reinhardtii,* and Mal d 2 from apple in the plant cells *Nicotiana benthamiana* [73–75]. Notably, such cell types are attractive for allergen production due to their ability to perform post-translational modifications resulting in protein conformations that are very close to those present in the native congeners [73,75]. In addition, the *C. reinhardtii* is simple and inexpensive to maintain while the Sf9 cells allow easy purification [73,75]. The main disadvantage of the above-mentioned eukaryotes remains slow growth rate, and the Sf9 cells also require high maintenance and fresh reinfection for each production batch [73]. Generally, production host selection is based on multiple parameters such as production yield, purification and downstream processing aspects. Other aspects that are affecting the host selection are price and protein/peptide functionality in patient settings such as post-translational modifications and native conformation.

Selection of expression vector

The choice of expression vector can also have an effect on protein expression, with different cell types needing different expression vectors. For *E. coli*, the most widely used expression system for allergen production is the pET-system (Table 1). This vector is widely studied and known to produce high protein yields using the bacteriophage T7 promoter [66]. Several different variations of the pET plasmids have been used, each containing minor modifications. Other used vectors, for example pMW172, pTTQ18βlac, pGEX-4T-2 and pMAL-c2, are all protein production vectors, with pGEX-4T-2 and pMAL-c2 being used to make protein fusions in the form of glutathione S-transferase (GST) or maltose-binding protein (MBP) respectively. This strategy of combining the allergen with a fusion partner has been used when the allergen is difficult to solubilize or are ending up in inclusion bodies. Two different vectors pSEC and pAMJ399 have been used in *P. lactis* [69,70,74]. Both of these are good expression vectors with inducible promoter, allowing a high control of expression [68,76]. In *P. pastoris*, the use of vector pPIC or pPINK allows for methanol-induced expression of the proteins [64,71,72]. The pPIC system integrates genes into the chromosome, while the pPINK expresses the proteins from the plasmid. Expression in *C. reinhardtii* can be done using the pJAG15 vector that integrates the gene of interest into the chloroplast chromosome. Protein production from the promoter in this vector is light dependent, making it inducible [75]. The expression in *N. benthamiana* is done using the TMV-expression vector pBSG735A66 [74,77]. For the expression of the peanut proteins Ara h 10, Ara h 11 and Ara h 14, an Invitrogen expression system was used for the recombinant protein production in insect Sf9 cells. The recombinant expressed Oleosins were encoded on a BaculoDirect vector [73].

After a the host has been chosen can plasmids for the selected host can be evaluated based on different parameters such as downstream processing and purification. A tag can be added to the plasmid encoding the recombinant allergen and thereby simplifying the purification process and/or increase the yield. Many plasmid systems offer a number of different tags in the beginning or the end of the gene encoding the allergen sequence of interest. Many use constructs with protein fusions or affinity tags such as His-Tag in N or C-terminus. A general overview of tags has been reviewed elsewhere [78]. Additionally, tags make the evaluation of expression more simple as it makes it easier to monitor by Western Blot or ELISA.

Production, recovery and purification of recombinant food allergens

Most studies describing strategies for the production of recombinant food allergens use either inducible expression systems in *E. coli* under the control of the *lac* operator induced by IPTG [79], or production in *P. pastoris* under the control of the AOX1 promoter induced by methanol [80]. Cells are allowed to grow to appropriate cell densities and growth phases before initiation of recombinant production leading to optimized production yields [79]. For production in *E. coli,* 0.5-1.0 mM IPTG is typically added as inducer around mid-exponential phase and expression is performed at 30-37°C for 2-5 hours before harvesting. These conditions have been used for production of major allergens from fish [60,81,82], shellfish [83,84], hen's egg [67,85], peanut [47,49,50,72] and tree nuts [86–93]. Some studies reported increased yield and solubility of recombinant food allergens by lowering expression temperature [48,65,94–97] or inducer concentration [63,95,98].

For production in *P. pastoris*, fed-batch fermentation has been used, reaching high cell densities over several days after which cells are harvested and resuspended in media containing 0.5% methanol. The lack of repressing carbon sources and the presence of methanol induces expression from the AOX1 promoter, and expression is continued for 2-5 days under continuous feeding with methanol. High cell densities and methanol-limitation have been shown to increase yields [80,99], and in the *S. cerevisiae* fusion system, the α-factor pre-pro-peptide has been used to facilitate effective secretion of the recombinant allergens [100]. This technique has been utilized for production of recombinant allergens from Brazil nut [101,102], hazelnut [64,103], peanut [72], avocado [104], watermelon [105], peach [106], apple [107] and mustard [108].

Recombinant food allergens produced by *E. coli* are found intracellularly, and a cell disruption step is necessary to recover any protein produced by this method. Disruption of cells is typically done biochemically using lysozymes, most often combined with sonication, which shears cells with ultrasonic frequencies [109]. This combination has allowed efficient recovery of recombinant food allergens from peanut [47,49,72], hazelnut [110] and walnut [111,112], while sonication alone also has been used to recover several recombinant food allergens [86–88,94]. Other strategies use cryopulverization [45,63,65,81,82,92,113] and homogenization [89,93,96,114,115] for cell disruption. Recombinant allergens with high solubility and allergens fused to solubility-enhancing tags such as Maltose-Binding Protein (MBP), Thioredoxin or

Glutathione S-transferase (GST) [116], are purified from isolated intracellular supernatant. However, recombinant allergens from peanut [49,50], apple [54] and pistachio [63] have in some studies formed aggregates as insoluble inclusion bodies [117]. These had to be solubilized using denaturing conditions and subsequently undergo renaturation typically through gradual removal of denaturants using dialysis [118]. Recombinant food allergens produced by *P. pastoris* are secreted into the supernatant, and samples are typically concentrated prior to purification to reduce sample volumes using either ultracentrifugation [101,102,119] or ammonium sulfate precipitation [103,119–122]. Notably, lyophilization [119,123] has been used to concentrate recombinant peach allergen Pru p 3 [106].

Recombinant food allergens are purified using fast protein liquid chromatography (FPLC) techniques. The use of different stationary and mobile phases allow the separation of proteins based on characteristics such as ligand specificity, size, charge and hydrophobicity [124–126]. Chromatography is the most frequently used purification technique and requires at least two purification steps separating proteins based on different characteristics to give the final recombinant allergens in high purity [86–88,95,101–103,127]. Recombinant allergens with fusion tags produced in *E. coli* can be selectively designed for affinity purification. Polyhistidine tags (His tags) inserted into the primary sequence provides a 'handle' with exceptionally high binding to stationary phases typically used in metal affinity chromatography (IMAC). Due to strong interactions between the histidine imidazole rings and transition metal ions immobilized on resin, the entire protein is immobilized on the stationary phase under native and even denaturing conditions, thereby allowing impurities to elute, after which reducing pH or increasing imidazole concentration of the mobile phase liberates the desired protein [128]. Solubility fusion tags such as MBP and GST have dual functions, as they can also be used for affinity purification. Amylose resin is used for binding of MBP fusion proteins, while GST fusion proteins require glutathione resin for binding [129,130]. Affinity purification with fusion tags have been used for purification of recombinant food allergens from peanut [47–50,94], oyster [83], hen's egg [67], cashew [86–88], pecan [89,93], hazelnut [91,92], walnut [93,111,131,132] and almond [133,134]. While affinity purification is effective for isolating recombinant food allergens, tags can influence structure and allergenicity [94,135,136]. Cleavage sites recognized by specific proteases such as thrombin and Factor Xa are therefore often incorporated between the fusion tag and the allergen, thereby enabling tag removal

after purification. This strategy has proven effective for removal of fusion tags from several recombinant food allergens [49,67,72,86,88,89,93,111]. However, removal of solubility tags may lead to insoluble species that aggregate, whilst other cases report on enzymatic resistance towards removal of fusion partner or unspecific degradation by proteases [87,131,134]. Immobilized specific monoclonal antibodies have also been used for affinity purification of Bos d 5 (BLG, cow's milk) and Mal d 3 (apple) without the use of fusion tags [114,137]. Some studies report additional purification steps (after cleavage of fusion tags) using size-exclusion chromatography (SEC) [86–88,95,103], where proteins are separated based on their ability to move through gel-particles-features dictated by protein molecular weight and conformation [124,125].

Affinity chromatography and SEC represent the most frequently used purification techniques for recombinant food allergens produced in *E. coli*. Contrary, recombinant food allergens produced by *P. pastoris* are typically purified using ion-exchange chromatography (IEC) in combination with either hydrophobic interaction chromatography (HIC) or SEC. IEC separates proteins based on the ionic interaction strength between charged protein side chains and the stationary phase (matrix) [124,125]. Anion-exchange matrices, which bind negatively charged proteins, have been used for purification of recombinant allergens including Ber e 1 [101], Cor a 1 [103], Cor a 8 [122], Ara h 9 [127], Prs a 1 [104] and Mal d 3 [107]. As proteins are eluted with increasing salt concentrations or pH changes, a dialysis step is often required after IEC to remove salts. Subsequently can HIC or SEC be used for further purification and removal of salt. While SEC separates proteins based on size and conformation, HIC separates proteins based on their hydrophobic interactions with the solid phase [124,125]. Proteins are eluted by decreasing the concentration of salts, and reversed-phase high-performance liquid chromatography (RP-HPLC) have reported for the purification of recombinant Ber e 1 [102], Pru p 3 [106] and Sin a 3 [108]. A combination of IEC and HIC have also been used for purification of recombinant parvalbumins [45,81], Mal d 1 [54], and Pru p 1 [96] produced by *E. coli*.

Synthetic peptides

To date, allergen extracts and recombinant allergens have comprised the main focus in the development of new products for allergen-specific IT. However, in recent years increased interest has emerged towards therapeutically active small synthetic peptides [138]. Peptides can easily be

produced by solid phase peptide synthesis (SPPS) in sufficient quantities and quality for clinical trials [139]. Therapeutic strategies focus on the production of peptides that can be recognized by T cell cells (epitopes), but are too small (15-22 residues) to cross-link membrane bound IgE and elicit immediate allergic reactions. Preclinical studies in mice have reported efficacy of T cell epitope IT for hen's egg (Gal d 1 and Gal d 2) and shrimp (tropomycin), which was found to suppress allergen-specific type-2 immune responses and promote immune regulatory mechanisms [43]. Ara h 1 and Ara h 2 T-cell epitopes with no IgE-binding and basophil activation capacity have been identified [140,141]. These peptides are part of the PVX108 IT vaccine candidate for peanut allergy, currently in phase I clinical trial (Australia New Zealand Clinical Trials Registry #ACTRN12617000692336). A recently disclosed patent application describes small synthetic peptides of cow's milk Bos d 5 (A and B variant) targeting T cells for treatment of CMA (patent EP3135125A1), however currently no clinical trials have been reported.

Another IT approach using synthetic peptides is contiguous overlapping peptides" (COPs), where peptides covers the entire primary sequence of the parent native allergen [142]. COPs are intended as hypoallergenic products that should exhibit reduced binding to IgE without compromising T cell recognition. This approach has not yet been applied in the IT of food allergy.

Polymerized allergoids

An allergoid is an allergen extract that has been chemically modified to reduce its allergenic potential by structural denaturation and cross-linking of the allergens (polymerization). Thus, the allergoid exhibits reduced IgE binding capacity but retains T cell stimulatory properties. Chemical production of allergoids has been investigated for several decades, and most often includes acetylation and formylation of native allergen extracts [143]. The formylation process is as chemical reaction between the extracted proteins and the formyl-group of an aldehyde, where formaldehyde has been shown to react primarily with the amino group of the N-terminal amino acid, and the side-chains of arginine, cysteine, histidine, and lysine residues, thereby forming methylol adducts that are partially dehydrated to Schiff-bases [144]. In a second step, these groups form stable cross-linking methylene bridges with other amino acid residues such as tyrosine, arginine, asparagine, glutamine, histidine, and tryptophan allowing cross-linking between allergen molecules and/or other proteins in the extract. The pharmacological properties of the modified allergen (i.e. the

allergoid) relative to the unmodified variant is influenced by the number, position and local environment of each reactive amino acid within the protein, and on production procedures including the proportion of aldehyde, the concentration of reactant, as well as the pH and additives present in the solution [144]. The formylation process has mainly been used on pollen extracts, but a pre-clinical study on hen's egg Gal d 2 derived allergoids has demonstrated reduced allergenicity [145].

Transglutaminase has been used to generate allergoid polymers of cow's milk Bos d 5, which has been shown to exhibit reduced allergenicity in pre-clinical studies [146,147]. The transglutaminase enzyme catalyzes formation of a covalent link between the ε-amino group of lysine and the γ-carboxamide group of glutamine resulting in resistant intramolecular and intermolecular protein bridges, leading to polymerization [148]. This process is often used in the food industry to obtain specific dairy product characteristics, but may be applied to other allergens such as hen's egg Gal d 2 [149].

Monomeric allergoids

A "monomeric" allergoid is an allergen with a chemical induction of point modifications at strategic sites that preserves the immunogenicity and T cell linear epitopes, but reduces the affinity to IgE [150]. The most clinically employed method is the so-called carbamylation reaction, where potassium cyanate is used to transform ε-amino groups in lysine into ureido groups. Carbamylated allergoids have proven to be a safe and effective for the treatment of dust mite and grass-pollen allergy [151,152], but its application in food allergy IT remains to be explored. In addition, lysine succinylation in hen's egg Gal d 2 has been studied in a pre-clinical model of atopic dermatitis [153].

Alkylation and acylation of allergens also represent viable approaches to produce monomeric allergoids. The process often involves the reduction of allergen disulfide bonds, followed by alkylation of the free cysteine residues leading to changes in the allergen secondary structure. This approach was recently reported to reduce the allergenicity of Brazil nut 2S albumin (Ber e 1; patent US20070190069A1), peanut Ara h 2 and Ara h 6, and peanut protein extract [31,154,155]. Reduced and alkylated peanut extract (HAL-MPE1) is currently undergoing clinical phase 1 trials for the treatment of peanut allergy (NCT02991885, ClinicalTrials.gov).

Hydrolysis

Cleavage of the allergen backbone by hydrolysis generates smaller allergen peptides with compromised tertiary structures and reduced allergenicity due to the partial elimination of IgE binding epitopes. Enzymatic hydrolysis of cow's milk proteins has for decades been used in the production of infant formula for the management of cow's milk allergy in small children, although the prevention and treatment capacities of such products are debated [156]. Variations in enzymes, pH, salt concentrations, and processing have been used to generate products with different properties [157]. No other food allergies have been targeted using enzymatically hydrolyzed products in humans, but pre-clinical studies have demonstrated IT potential of hydrolyzed cashew protein extract and hen's egg white [43]. Chemical hydrolysis using cyanogen bromide (hydrolysis of C-terminal after a methionine residue) has been applied to cow's milk Bos d 9 [158], however its IT applicability remains to be investigated.

Evaluation of IT agents

IT agent production quality control and mechanism of action studies is commonly absent in reports of product development, pre-clinical studies, and clinical trials. Methods for the study of physicochemical characteristics, including SDS-PAGE, immunoblotting, HPLC, circular dichroism, can be used to evaluate consistent product composition [159–162]. Assessment of allergenicity and immunological properties can be used to evaluate consistent product functionality, and possibly mechanisms of action. This can involve several *in vitro* and *ex vivo* methods, including IgE binding assays using ELISA or Western blot, IgE cross-linking in basophil or mast cell assays, and T cell proliferation and cytokine assays, which in addition to *in silico* method are common in the evaluation of protein allergenicity [163]. A similar array of methods can be used for mechanism of action studies of IT agents in animal models [43,164,165], and during clinical trials [6,166].

Conclusions

Clinical trials demonstrate that foods work as IT agents in the treatment of food allergy, but can be associated with adverse allergic reactions and variable treatment efficacies. There are currently efforts to develop drug-grade agents based on allergen extracts to improve IT standardization and reproducibility. The recombinant production of food allergens has received

much attention with the application of different production methods, including bacterial, fungal and insect cell expression systems. Recombinant technology allows production of both native and modified allergens with reduced allergenicity. The future development of hypoallergenic IT agents may be of particular importance, as deficiencies in the clinical safety of food allergy treatment approaches is a major problem in current IT [167]. This highlights an important role of recombinant technology in the future production of IT agents, since it allows the development of well-defined, well-characterized, and standardized drugs. We find that current reports on the production of IT agents commonly lack detailed information on production procedures and agent characterization, limiting the ability to the compare agents, and evaluate IT strategies with improved safety and efficacy profile.

Author contributions

KLB and JU conceived review topic. JML, KLB, CHBB and KQ outlined manuscript draft. JML drafted the abstract, introduction, IT evaluation, and conclusion sections, and sections on whole, cooked and processed food. AIS drafted the sections on extracts and purified allergens. CHBB, KIHA, and SSNT drafted the section on recombinant allergens and peptides. KQ and AHH drafted the sections on synthetic peptides, allergoids, and hydrolysis. JML edited the final draft. All authors made substantial intellectual contributions and revised the manuscript critically. All authors reviewed and approved the final version of the manuscript, and agreed to be accountable for all aspects of the work.

Declaration of interest statement

JU is and has been an investigator for Regeneron, DBV Technologies and Aimmune Therapeutics, and investigator lead OIT studies funded by SickKids foundation. TE is an investigator for Regeneron and DBV Technologies, and investigator lead OIT studies funded by SickKids foundation. The remaining authors declare no competing interest in regard to publication of this manuscript.

Funding

This work is part of the ALLEVIATE project funded by Innovation Fund Denmark (grant number 6159-00005B). KQ and AHH were additionally supported by the Novo Nordisk Foundation. The foundations had no involvement in the preparation of the manuscript or decision to publish.

Figure text

Figure 1 – Source materials and processes in the production of various immunotherapeutic agents for the treatment of food allergy. Whole foods, cooked and processed foods are food-grade immunotherapeutic agents. Physical and chemical processing of foods allows the production of standardized and modified drug-grade protein extracts. Recombinant and chemical technologies can be used to produce drug-grade well-defined native allergen, modified hypoallergenic allergens or allergen-derived peptides for immunotherapy.

Tables

Table 1 – Overview of recombinant produced food allergens with their expression hosts and vectors.

Allergen	Originates from	Expression host	Expression vector	Ref.
Ara h 1	Peanut	*C. reinhardtii (algae)*	pJAG15	[75]
Ara h 1	Peanut	*E. coli* BL21 (DE3) pLysS	pET32a	[51]
Ara h 1, 2, 5, 6	Peanut	*E. coli* BL21 (DE3) *E. coli* BL21 (DE3) CodonPlus-RIL	pET16b	[48]
Ara h 2	Peanut	*E. coli* BL21 (DE3)	pET28a(+)	[50]
Ara h 2	Peanut	*E. coli* BL21 CodonPlus (DE3)-RIL *E. coli* Origami (DE3) *E. coli* AD494 (DE3)	pET16b pET32a	[49]

Ara h 2	Peanut	*L. lactis* MG1363	pAMJ399	[68]
Ara h 2 peptide	Peanut	*E. coli* BL21 Star (DE3)	pET28a	[47]
Ara h 3	Peanut	*E. coli* BL21 (DE3)	pET24	[53]
Ara h 6	Peanut	*E. coli* BL21 CodonPlus (DE3) -RIL *P. pastoris (yeast)*	pET32 Ek/Lix pPink-HC	[72]
Ara h 10, 11, 14	Peanut	Sf9 cells (insect)	BaculoDirect	[73]
Ber e 1	Brazil nut	*P. pastoris (yeast)*	pPIC9	[71]
Bos d 5	Cow's Milk	*E. coli* DH5a *E. coli* JM109	pTTQ18βlac	[55]
Bos d 5 (peptide 41-60)	Cow's Milk	*L. lactis* NZ9000	pSEC	[69,70]
Car i 1	Pecan	*E. coli BL21 (DE3)*	pMAL-c4x	[89]
Car i 4	Pecan	*E. coli BL21 (DE3)*	pMAL-c4x	[93]
Cor a 8	Hazelnut	*E. coli* BL21 Star (DE3) *P. pastoris (yeast)*	pET-16b pPICZxA	[64]
Cra g 1.03	Oyster	*E. coli*	pGEX-4T-3	[83]
Cyp c 1	Carp	*E. coli* BL21 (DE3)	pET17b	[60]
Cyp c 1	Carp	*E. coli* BL21 (DE3)	pET17b	[61]
Cyp c 1	Carp	*E. coli* BL21 CodonPlus (DE3) -RIPL	pET17b	[59]
Cyp c 1	Carp	*E. coli*	pET28b+	[62]
Enolase, TPI and MDH	Soy	*E. coli* BL21 (DE3)	pET15b	[57]
Gal d 1	Hen's egg	*E. coli*	pTrC-his A	[56]
Gal d 1	Hen's egg	*E. coli* JM109	pGEX-4T-2	[67]
Gad m 1	Cod	*E. coli* BL21 CodonPlus (DE3) -RIPL	pET17b	[59]
Gly m 3	Soy	*E. coli* TB-1	pMAL-c2	[58]
Jug r 2	Walnut	*E. coli* BL21	pGEX-4T-3	[111]

Jug r 1	Walnut	*E. coli BL21*	pGEX	[112]
Mal d 1	Apple	*E. coli* BL21	pMW172	[54]
Mal d 2	Apple	*N. benthamiana (plant)*	pBSG735A66 linearised	[74]
MnSOD	Pistachio	*E. coli* BL21 Star (DE3)	pET32b(+)	[63]
Parvalbumin	Wolf herring	*E. coli* BL21 (DE3)	pET28a	[65]
Profilin	Watermelon	*P. pastoris*	pPICZαA	[105]
Prs a 1	Avocado	*P. pastoris strain GS115*	pPIC9	[104]
Pru du 4	Almond	*E. coli BL21 (DE3)*	pMAL-c2	[133]
Pru du 6.01, Pru du 6.02	Almond	*E. coli*	pMAL-c4x	[134]
Pru p 3	Peach	*P. pastoris*	pPIC9	[106]
Sin a 3	Mustard	*P. pastoris*	pPICZαA	[108]
Sin a 4	Mustard	E. *coli* BL21-CodonPlus (DE3)	pET11b	[108]

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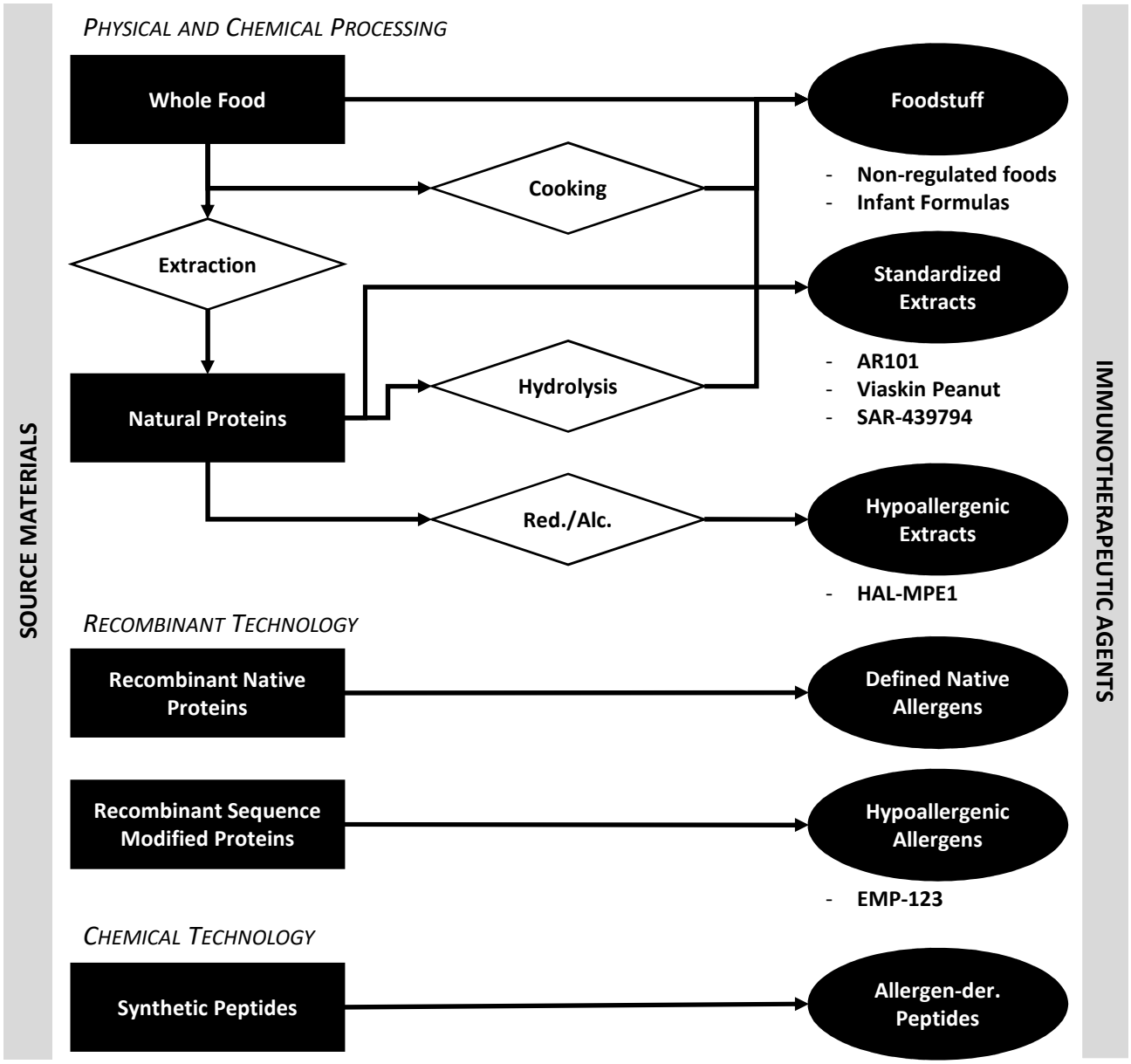
FIGURE 1


SOURCE MATERIALS
PHYSICAL AND CHEMICAL PROCESSING
Whole Food
Cooking
Extraction
Natural Proteins
Hydrolysis
Red./Alc.
Foodstuff
- Non-regulated foods
- Infant Formulas
Standardized Extracts
- AR101
- Viaskin Peanut
- SAR-439794
Hypoallergenic Extracts
- HAL-MPE1
RECOMBINANT TECHNOLOGY
Recombinant Native Proteins
Defined Native Allergens
Recombinant Sequence Modified Proteins
Hypoallergenic Allergens
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