

Expression, purification, characterization, and patient IgE reactivity of new macadamia nut iso-allergen

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ABSTRACT

Structural and functional information about food allergens is essential for understanding the allergenicity of food proteins. All allergens belong to a small number of protein families. Various allergens from different families have been successfully produced recombinantly in *E. coli* for their characterization and applications in allergy diagnosis and treatment. However, recombinant hexameric 11S seed storage protein has not been reported, although numerous 11S legumins are known to be food allergens, including the recently identified macadamia nut allergen Mac i 2. Here we report the production of a macadamia nut legumin by expressing it in *E. coli* with a substrate site of HRV 3C protease and cleaving the purified protein with HRV 3C protease. The protease divided the protein into two chains and left a native terminus for the C-terminal chain, resulting in a recombinant hexameric 11S allergen for the first time after the residues upstream to the cleavage site flipped out of the way of the trimer-trimer interaction. The 11S allergens are known to have multiple isoforms in many species. The present study removed an obstacle in obtaining homogeneous allergens needed for studying allergens and mitigating allergenicity. Immunoreactivity of the protein with serum IgE confirmed it to be a new isoform of Mac i 2.

1. Introduction

The negative impact of increasingly more prevalent food allergies [1–4] on the agriculture and food industry and allergic patients and their families has become a serious problem worldwide. Research on the disease mechanisms and treatments for allergies [5] as well as on the allergenicity of food proteins and its mitigation are needed. In many parts of the world, various food groups are generally or officially [6] recognized as primary allergen sources. Food allergens are predominantly proteins; in each allergen source, a few of the allergens cause most allergic reactions. Known proteins are classified into about eighteen thousand protein families [7], and most food allergens belong to a small number of families. From plant sources, the most notable protein families containing known food allergens are those with 2S albumins, 7S vicilins, 11S legumins, profilins, pathogenesis-related proteins, and non-specific lipid transfer proteins.

Tree nuts constitute one of the nine food groups (i.e., milk, eggs, fish,

Crustacean shellfish, tree nuts, peanuts, wheat, soybean, and sesame) considered major allergen sources in the United States [8]. If a tree nut protein is an allergen, it is very likely that its orthologue protein in another tree nut or in another major plant allergen source has also been identified as a food allergen. Thus, the allergen research field is very interested in characterizing problematic proteins and elucidating the allergenicity of food proteins. However, what makes some proteins food allergens remains elusive.

Food allergens isolated from natural sources and recombinant food allergens have been widely used in different research. Purified allergens have the advantage over raw food because they can also be used in component-resolved diagnosis [9–11] and Immunotherapy [12]. They are indispensable reagents for studying the allergenic properties of food proteins. Allergens from notable allergen families have been successfully expressed for the studies of their immunological, physical, and chemical properties and medical applications. Examples include 2S allergens (e.g. peanut allergen Ara h 2 [13]), 7S allergens (e.g. peanut allergen Ara h 1

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[14] and pecan allergen Car i 2 [15]), profilins (e.g. Ara h 5 [16]), pathogenesis-related proteins (e.g. Ara h 8 [17]), and non-specific lipid transfer proteins (e.g. pea allergen Pis s 3 [18]). To our knowledge, however, no recombinantly produced 11S allergen had a hexameric structure resembling the native proteins from natural sources. Here, we report the expression, purification, and characterization of an isoform of the recently identified macadamia nut allergen Mac i 2, and its reactivity with serum IgE molecules in patient sera.

2. Materials and methods

Kanamycin and Isopropyl β -D-1-thiogalactopyranoside (IPTG) were purchased from GoldBio (St Louis, MO). They are used at final concentrations of 50 μ g/mL and 1 mM, respectively. Protein standards for gel filtration column calibration were obtained from GE Healthcare (Piscataway, NJ). Other chemicals for cloning, bacterial growth, and protein purification were obtained from Fisher Scientific (Pittsburgh, PA). Materials for protein characterization were from Sigma Aldrich (St. Louis, MO) unless stated otherwise. All chemicals used in this study were of the highest grade available. Milli-Q water was purified in-house with a MILLI-Q ADVANTAGE A10 SYSTEM (Millipore, Bedford, MA) and was used throughout. Prestained protein standards were purchased from Bio-Rad (Hercules, CA) and Fisher Scientific.

2.1. Plasmid construction

A gene block encoding an 11S legumin of macadamia nuts based on the predicted mRNA in GenBank (access number XM_042662590.1) was custom synthesized by IDT DNA Technologies (Coralville, IA) with codon optimization and flanking cloning sites (*Afe* I at the 5' end and *Xho* I at the 3' end). Also, adapter sequences ctcaaaactcaaacagccat and gtgtctcaaaatctctgatgttac were present at the 5' and 3' end of the gene block, respectively. The gene block was PCR amplified with primers ctcaaaactcaaacagccat (forward) and gtaacatcagagattttgagacac (reverse) and digested with *Afe* I and *Xho* I. The digested product was purified using an agarose gel and inserted into pTGS4, a homemade vector having the backbone of pRSFduet-1 (Merck Millipore, Billerica, MA) with slight modification and changed cloning sites as shown in Fig. S1 of the supplemental material and will be available via Addgene (<https://www.addgene.org>). The ligation product was transformed into competent *Escherichia coli* dH5 α and spread on LB plates containing kanamycin. Five mL LB media containing kanamycin were inoculated with colonies for DNA miniprep the next day. QIAprep Spin Miniprep Kits (QIAGEN Valencia, CA) were used following the manufacturer's protocols. Positives with the correct coding sequence for Mac i 2 in the vector were identified by DAN sequencing performed by Elim Biopharm (Hayward, CA). The expression plasmid was named pRSF-Maci2.

2.2. Mac i 2 expression

pRSF-Maci2 was transformed into competent *Escherichia coli* BL21 (BL21 StarTM (DE3), Thermo Fisher Scientific, Waltham, MA), and a colony was picked and used to start a 50 mL BL culture containing kanamycin for overnight growth at 37 °C in a shaking incubator. Larger (1 L) cultures were started by adding 25 mL of the overnight culture. The shaker temperature was set to 18 °C when the OD at 600 nm of the cultures reached about 1. The expression of Mac i 2 was induced by adding IPTG 1 h later. The bacteria were harvested the next day after 14–18 h of incubation by spinning at 3900 g. The pellets were resuspended in a His-Binding buffer (buffer HB, 20 mM imidazole, pH 8.0, 500 mM NaCl) and stored at –20 °C.

2.3. Mac i 2 purification

The frozen cell suspension was thawed at room temperature, and the cell suspension was sonicated in the presence of 1 mM benzamidine. The

sample was centrifugated at 18 °C and 20,000g for 40 min. The protein was purified with two steps of chromatography with an AKTA FPLC system (GE Healthcare). The clear lysate was loaded into a 5-mL HisTrap FF Crude column (GE Healthcare) pre-equilibrated with 25 mL of buffer HB and washed with 25 mL of buffer HB, followed by 25 mL of a His-Wash buffer (45 mM Imidazole, pH 8.0, 500 mM NaCl). The protein was eluted with a His-Elution buffer (300 mM Imidazole, pH 8.0, 500 mM NaCl). The HisTrap column purified Mac i 2 was further purified with size exclusion chromatography (SEC) using an XK 26/70 column packed (to a bed volume of 344 mL) with prep grade Superdex 200 (GE Healthcare). The column was pre-equilibrated and run with buffer A (10 mM Tris, pH 7.4, 500 mM NaCl).

2.4. Protease processing of the recombinant Mac i 2

Human rhinovirus (HRV) 3C protease with GST and His-Tag was purchased from Life Technologies (Carlsbad, CA). The Mac i 2 was digested with HRV 3C protease by adding the protease (2 units/mg of Mac i 2) to the SEC purified Mac i 2 and incubated at room temperature overnight. The digestion product was passed through a glutathione column to remove the protease and then purified again with the Superdex 200 column.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses

500 μ L uninduced and induced bacteria were collected by centrifugation at 5000 rpm using a microcentrifuge. The pellets were resuspended in 50 μ L of 10 M urea and incubated at 96 °C for 10 min after being mixed with 50 μ L of a 2X SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) containing 100 mM β -mercaptoethanol. Other samples at different Mac i 2 production stages were similarly denatured at 96 °C in the SDS sample buffer. SDS-PAGE was carried out using homemade gradient polyacrylamide gels (4–20%) with a HEPES-Tris running buffer (100 mM HEPES, 100 mM Tris, pH 8.0, 3 mM SDS). Gels were stained with previously described [19] protocols and documented with an ImageQuant LAS 400 Imager (GE Healthcare).

2.6. Mac i 2 recognition by patient serum IgEs

Nineteen sera (#1–19 corresponding to de-identified patient codes 2114, 6924, 6544, 6972, 6871, 2029, 2194, 2085, 6989, 6218, 6080, 6222, 6316, 6642, 6569, 6702, 6683, 6634, and 5875, respectively) were collected from patients with macadamia nut allergies diagnosed by positive oral food allergy challenge. The patients were enrolled in the food allergy study at Stanford University under institutional review board approval with informed consent (IRB approval certificate 8629).

IgE binding of Mac i 2 was evaluated by Western blot. The trimeric and hexameric MAC i 2s were mixed together and separated in the gradient gels. The protein bands in the gels were transferred to PVDF membranes for immunoblot. The membranes were blocked with 10% nonfat dry milk (w/v) in TBST (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) at room temperature. The blocked membranes were incubated with individual serum diluted 1:25 with TBST containing 1% nonfat milk (TBSTM) for 60 min. The membranes were washed 3 times with TBST, 5 min each. Purified goat antibodies raised against human IgE and conjugated with horseradish peroxidase were purchased from LGC Seracare (Milford, MA) and used as secondary antibodies. The membranes were incubated with the secondary antibody (diluted 1:5000 with TBSTM) and washed three times as described above. A SuperSignal West Femto Substrate (Thermo Scientific) was used to detect the reaction of Mac i 2 with patient serum IgE molecules. An ImageQuant LAS4000 imaging system was used to record the chemiluminescence signals.

3. Results and discussion

3.1. Mac i 2 expression and purification

In plants, the 11S seed storage proteins are known to be expressed from a single gene, although many isoforms can exist in a single cultivar [20]. Post-translational cleavage of the protein precursor by a legumain is required for protein maturation [21]. Legumains are asparaginyl endopeptidases that cleave the Asn-Gly peptide bond within a well-conserved legumain substrate motif (NGXEET) among a wide variety of plant species [22]. The cleavage leaves the protein as two separate chains, an N-terminal acidic chain and a C-terminal basic chain. The acidic and basic chains form different structural domains connected by a disulfide bond [20]. Structural studies revealed that the hexameric 11S seed storage proteins could be viewed as a dimer of two trimers. For each of the six protein subunits, the C-terminal residues of the acidic domain are flexible and not involved in the trimer-trimer interactions [20,23,24]. In contrast, the Gly residue at the N-terminus of the basic domain is buried between the trimer-trimer interface. This makes it impossible to assemble the native hexameric protein without the post-translational protease cleavage. Hence the recombinant expression of an isoform of the 11S protein from soybean resulted in a trimeric proprotein [25].

Furthermore, the 11S protein is known to have many isoforms in various species [26,27], complicating the purification and characterization of the natural protein. The 11S legumin of macadamia nut has recently been identified as a food allergen (and named Mac i 2) by identifying serum IgE reacting proteins from macadamia nut extract [28]. Seven peptides were determined by de-novo and MS/MS sequencing, and sequence alignment with 11S proteins from other species identified it as a legumin [28]. An mRNA sequence of a macadamia 11S protein is available in the GenBank (XM_042662590.1). We sought to obtain recombinant hexameric 11S food allergens and used the newly identified macadamia allergen to develop the method. A signal peptide was predicated in the protein sequence deduced from the mRNA sequence, and the mature protein sequence is shown in Fig. S2. A sequence coding the 11S protein with an HRV 3C protease substrate incorporated at the place of the natural legumain substrate site (Fig. S2) and a His-Tag at the C-terminal was synthesized with codon optimization. The protein was successfully expressed, as shown in Fig. 1.

Two steps of chromatography were carried out to purify the recombinant protein. The C-terminal His-Tag was used to purify the protein by immobilized metal affinity chromatography (IMAC), and the IMAC purified protein was further purified with SEC (Fig. 2A). In the second step of the purification, the protein was eluted with an apparent molecular weight of ~152 kDa based on the calibration of the Superdex 200 column (Fig. 2B), consistent with a trimeric protein with a theoretical molecular mass of 57 kDa. The purified protein showed a single band by SDS-PAGE and Coomassie Blue staining (Fig. 2C). The purity of the protein was estimated to be 87% using the ImageJ program, and the yield of the protein is about 3.5 mg per liter of bacterial culture.

HRV 3C protease was used to cleave the protein at the 3CP substrate site introduced between the N- and C-terminal domains of the predicted native protein. After cleavage, the amino acids at the N-terminal of the basic domain of Mac i 2 would be the same as those in the natural allergen, making it possible to produce a natural trimer-trimer interface to facilitate the formation of hexamers.

After cleavage, the HRV 3C protease was removed by a glutathione column with a syringe, and the flowthrough was purified/concentrated using a HisTrap column. The digested protein was further purified with the Superdex 200 column (Fig. 2A), and it was eluted with an apparent molecular weight of ~359 kDa based on the calibration of the Superdex 200 column, consistent with it being a hexamer rather than a trimer. The cleavage results are shown in (Fig. 2C). The purity of the protein was estimated to 92% using the ImageJ program, and the yield of the protein is about 1.9 mg per liter of bacterial culture.

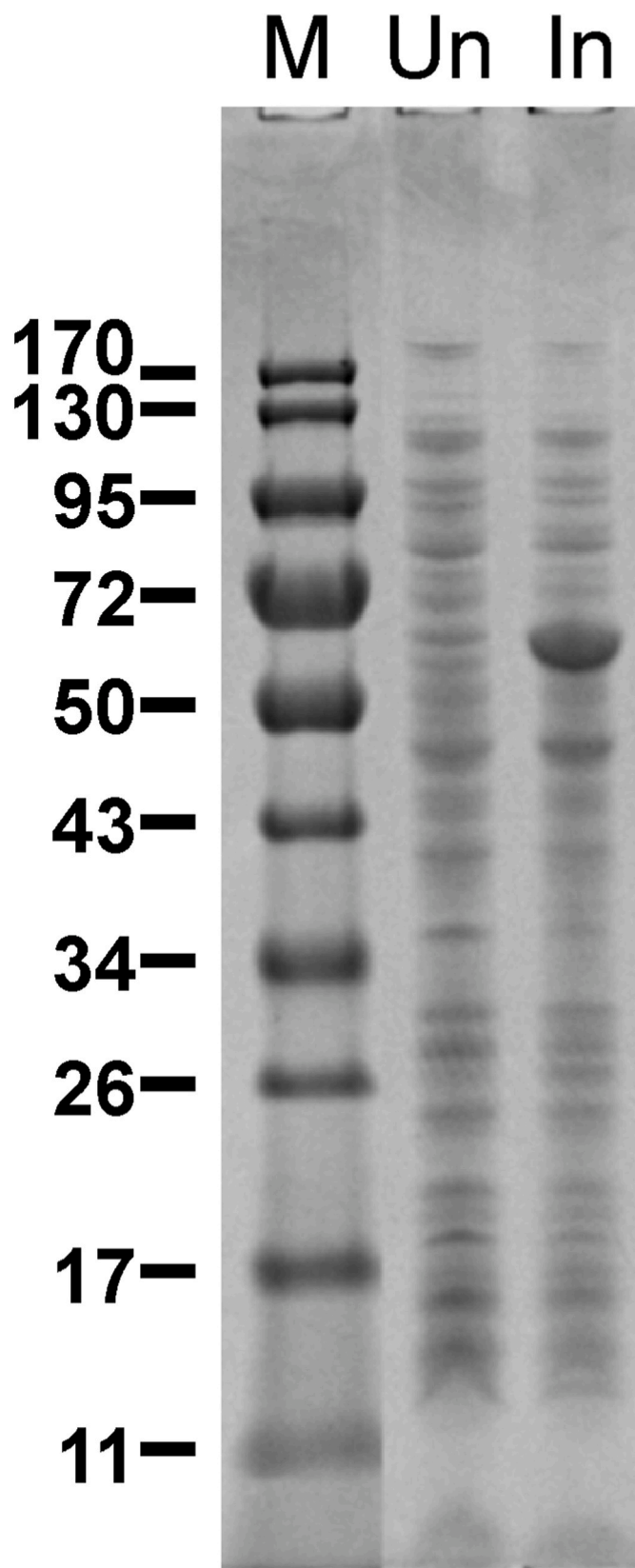


Fig. 1. Expression of macadamia nut allergen Mac i 2. Lane “M” was loaded with protein standards with the molecular sizes of the reference proteins (in kDa) indicated on the side of the gel image. Lane “Un” was loaded with the uninduced sample, and lane “In” was loaded with the induced sample. The strong band resolved from the induced sample shows the expression of the target protein.

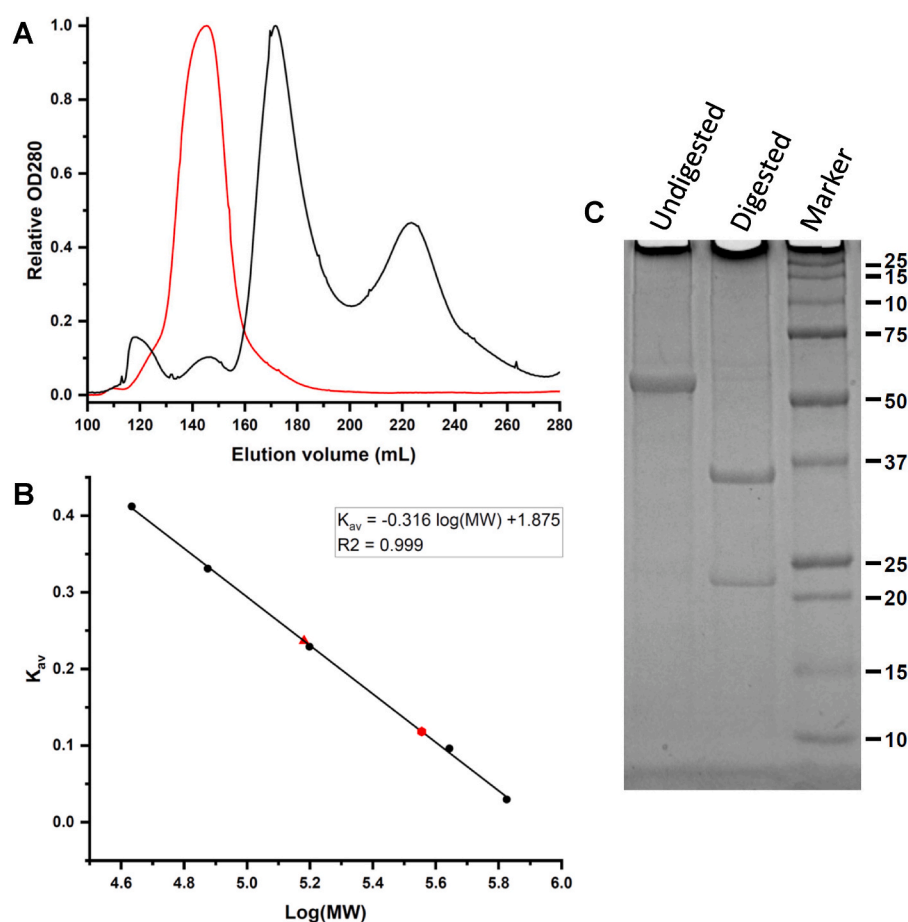


Fig. 2. Purification and In vitro processing of Mac i 2. **A**, The elution profiles of the SEC purification of the recombinant Mac i 2 before (dark) and after (red) digestion. **B**, Calibration of the SEC column with thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), and ribonuclease A (13.7 kDa). The K_{av} s of the SEC peaks of the undigested and digested samples were calculated, and their positions were indicated with a red triangle and a red hexagon, respectively, on the fitted line of the calibration. **C**, SDS-PAGE analysis of the undigested and digested recombinant Mac i 2. Lane M was loaded with protein standards with the molecular sizes of the reference proteins (in kDa) indicated on the side of the gel image.

3.2. Mac i 2 recognition by patient serum IgEs

The recently identified macadamia nut 11S allergen Mac i 2 with seven stretches of peptide sequences known is not the same as the 11S legumin predicted from the mRNA data. As many food allergens have multiple isoforms, we hypothesized that the 11S protein predicted from the mRNA is a different isoform of Mac i 2 and used patient sera from 19 macadamia nut allergic subjects. All the patients were diagnosed with macadamia nut allergies by double-blind placebo-controlled food challenges.

The purified trimeric protein and the hexameric protein after HRV 3C protease digestion were separately prepared in the SDS-loading

buffer and analyzed by SDS-PAGE (Fig. 2). They were then mixed and electrophorized in multiple SDS-gels lanes separated by standard protein markers. Each serum was used to assay the reactivity of its IgE content with the recombinant Mac i 2. For each serum, the membrane contained just one lane with the mixed proteins and half of a marker lane on each side. Images were taken in the chemiluminescence mode. To determine the marker positions, images were taken in the digitization mode with trans-illumination without adjusting the imager or moving the membranes. The two images were overlaid, and the size correlation was made by marking the positions of the reference bands. No serum recognized any of the marker bands, which can be considered as one kind of negative control, indicating that there were no IgE molecules in any of

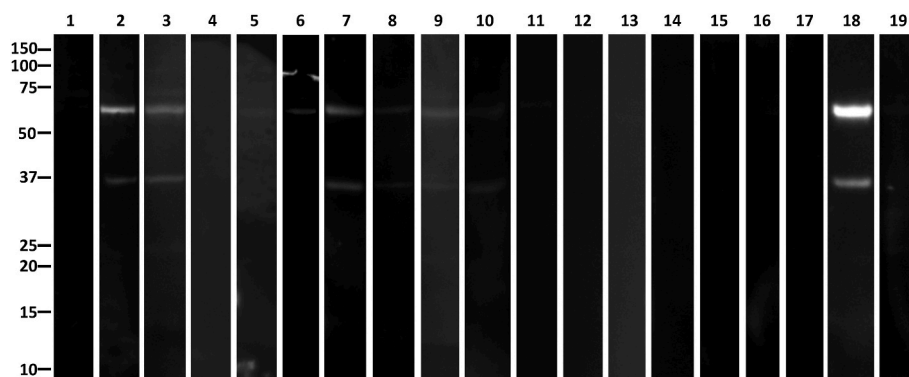


Fig. 3. Sera IgE recognition of recombinant Mac i 2. For immunoblot analysis, PVDF membranes with protein bands from a mixture of undigested and digested Mac i 2) were blotted with individual serum from 19 macadamia-allergic patients. The serum number is labeled at the top of the blot. The positions of the protein standard bands visualized by taking images in the trans-illumination mode of the ImageQuanta 4000 are shown on the left side of the chemiluminescence images.

the sera that nonspecifically bind a wide range of proteins or too much of the primary or secondary antibody was used, and the results are shown in Fig. 3. The data suggested that the 11S legumin expressed in this study represents a new isoform of the macadamia nut allergen Mac i 2 as 7 (2, 3, 7, 8, 9, 10, 18) of the 19 sera can be seen to react with the recombinant 11S protein. Serum 5 also reacted with the recombinant Mac i 2 though the reaction was not as strong. It could be seen that more of the sera reacted to the N-terminal acidic chain at ~37 kDa than the C-terminal basic chain between 20 and 25 kDa, a phenomenon consistent with the observations for the IgE recognitions of the acidic and basic chains of peanut allergen Ara h 3 [29].

These results showed the successful expression of an 11S food allergen in bacteria for the first time. The information and methodology can be used to obtain valuable reagents for studying the properties of the food allergens in the legumin family and the disease mechanisms and treatments for allergies.

4. Conclusion

Recombinant allergens are highly desirable because of the problems related to the inconsistent quality of natural allergen extracts, and the replacement of natural allergen extracts by recombinant allergens and their mixtures may assure high-quality products for diagnosis and treatment of allergies [30]. Recombinant allergens can also be used in the sensitive diagnosis of allergies and reduce the rate of misdiagnosis due to cross-reactivity [31]. However, because of the requirement of post-translational legumain processing, the hexameric 11S allergens' recombinant production has not been reported. The legumain in tree nuts cleave the peptide between Asn and Gly of the substrate sequence of N↓GXEET [32], and the residues C-terminal to the cleavage site are buried in the hexameric structure [20,23]. We sought to express single-chain 11S proteins and obtain hexameric allergens by *in vitro* protease cleavage.

The substrate specificity of HRV 3C protease is LEVLFQ↓GP, and the proline residue at the P2' position can be changed to any residues other than a tryptophan [33]. Thus, HRV 3C protease is ideal for mimicking the effect of the natural post-translational protease modification of the 11S seed storage proteins. In this study, a hexameric 11S legumin was recombinantly produced for the first time. The macadamia protein was produced as a trimer, and it was purified by a combination of IMAC and SEC with a yield of 3.5 mg/L and a purity of 87%. The trimeric protein was processed with HRV 3C protease, and hexameric macadamia legumin was obtained with a yield of 1.9 mg/L and a putity of 92%. Serum IgE reactions identified the protein as a new isoform of Mac i 2.

Author statement

Y.Z., conceptualization; Y.Z. and C.L. designed project; Y.Z., and S.R. B. carried out the experiments and generated data; S-C.L., S.C., and K.C. N., resources, expertise, and feedback.; Y.Z., drafted the manuscript; Y. Z., C.L., S.R.B., and K.C.N. review & editing.

Declaration of competing interest

The authors declare no conflicts of interest associated with this publication.

Mention of trade names or commercial products in this publication is solely to provide specific information and does not imply recommendation or endorsement by USDA. USDA is an equal opportunity provider and employer.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pep.2022.106211>.

References

- [1] R.S. Gupta, C.M. Warren, B.M. Smith, J.L. Jiang, J.A. Blumenstock, M.M. Davis, R. P. Schleimer, K.C. Nadeau, Prevalence and severity of food allergies among US adults, *JAMA Netw. Open* 2 (2019).
- [2] R.S. Gupta, E.E. Springston, M.R. Warrier, B. Smith, R. Kumar, J. Pongracic, J. L. Holl, The prevalence, severity, and distribution of childhood food allergy in the United States, *Pediatrics* 128 (2011) e9–e17.
- [3] S.H. Sicherer, A. Munoz-Furlong, H.A. Sampson, Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: a 5-year follow-up study, *J. Allergy Clin. Immunol.* 112 (2003) 1203–1207.
- [4] A.M. Kumfer, S.P. Commins, Primary prevention of food allergy, *Curr. Allergy Asthma Rep.* 19 (2019) 7.
- [5] L.E. Moore, P.H. Stewart, R.D. deShazo, Food allergy: what we know now, *Am. J. Med. Sci.* 353 (2017) 353–366.
- [6] USFDA, Food Allergies: what You Need to Know, 2010.
- [7] S. El-Gebali, J. Mistry, A. Bateman, S.R. Eddy, A. Luciani, S.C. Potter, M. Qureshi, L. J. Richardson, G.A. Salazar, A. Smart, E.L.L. Sonnhammer, L. Hirsh, L. Paladin, D. Piovesan, S.C.E. Tosatto, R.D. Finn, The Pfam protein families database in 2019, *Nucleic Acids Res.* 47 (2019) D427–D432.
- [8] U.S Congress, Food Allergy Safety, Treatment, Education, and Research Act of 2021, APR. 23, 2021.
- [9] R. Nugraha, T. Ruethers, A.C. Taki, E.B. Johnston, S. Karnaneedi, S.D. Kamath, A. L. Lopata, Recombinant tropomyosin from the pacific oyster (*Crassostrea gigas*) for better diagnosis, *Foods* 11 (2022).
- [10] M. Popielarz, A. Krogulska, The importance of component-resolved diagnostics in IgE-mediated cow's milk allergy, *Allergol. Immunopathol.* 49 (2021) 30–41.
- [11] M. Volpicella, C. Leoni, M.C.G. Dileo, L.R. Ceci, Progress in the analysis of food allergens through molecular biology approaches, *Cells* 8 (2019).
- [12] S.P. Lee, Y.S. Shin, S.Y. Kang, T.B. Kim, S.M. Lee, Recent advances in allergen-specific immunotherapy in humans: a systematic review, *Immune Netw* 22 (2022) e12.
- [13] G.A. Mueller, R.A. Gosavi, A. Pomes, S. Wunschmann, A.F. Moon, R.E. London, L. C. Pedersen, Ara h 2: crystal structure and IgE binding distinguish two subpopulations of peanut allergic patients by epitope diversity, *Allergy* 66 (2011) 878–885.
- [14] M. Chruszcz, S.J. Maleki, K.A. Majorek, M. Demas, M. Bublin, R. Solberg, B. K. Hurlburt, S. Ruan, C.P. Mattisohn, H. Breiteneder, W. Minor, Structural and immunologic characterization of Ara h 1, a major peanut allergen, *J. Biol. Chem.* 286 (2011) 39318–39327.
- [15] Y. Zhang, B. Lee, W.X. Du, S.C. Lyu, K.C. Nadeau, L.J. Grauke, Y. Zhang, S. Wang, Y. Fan, J. Yi, T.H. McHugh, Identification and characterization of a new pecan [*Carya illinoensis* (wangenh.) K. Koch] allergen, car i 2, *J. Agric. Food Chem.* 64 (2016) 4146–4151.
- [16] Y. Wang, T.J. Fu, A. Howard, M.H. Kothary, T.H. McHugh, Y. Zhang, Crystal structure of peanut (*Arachis hypogaea*) allergen Ara h 5, *J. Agric. Food Chem.* 61 (2013) 1573–1578.
- [17] B.K. Hurlburt, L.R. Offermann, J.K. McBride, K.A. Majorek, S.J. Maleki, M. Chruszcz, Structure and function of the peanut panallergen Ara h 8, *J. Biol. Chem.* 288 (2013) 36890–36901.
- [18] I.V. Bogdanov, Z.O. Shenkarev, E.I. Finkina, D.N. Melnikova, E.I. Rumynskiy, A. S. Arseniev, T.V. Ovchinnikova, A novel lipid transfer protein from the pea *Pisum sativum*: isolation, recombinant expression, solution structure, antifungal activity, lipid binding, and allergenic properties, *BMC Plant Biol.* 16 (2016) 107.
- [19] Y. Zhang, W.X. Du, C. Fregevu, M.H. Kothary, L. Harden, T.H. McHugh, Expression, Purification, and Characterization of Almond (*Prunus dulcis*) Allergen Pru du 4, *J. Agric. Food Chem.* 62 (2014) 12695–12700.
- [20] T. Jin, S.M. Albillos, F. Guo, A. Howard, T.J. Fu, M.H. Kothary, Y.Z. Zhang, Crystal structure of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin, *J. Agric. Food Chem.* 57 (2009) 8643–8651.
- [21] K. Muntz, F.R. Blattner, A.D. Shutov, Legumains - a family of asparagine-specific cysteine endopeptidases involved in propolypeptide processing and protein breakdown in plants, *J. Plant Physiol.* 159 (2002) 1281–1293.
- [22] C.D. Dickinson, E.H. Hussein, N.C. Nielsen, Role of posttranslational cleavage in glycinin assembly, *Plant Cell* 1 (1989) 459–469.
- [23] T. Jin, F. Guo, Y.-w. Chen, A. Howard, Y.-Z. Zhang, Crystal structure of Ara h 3, a major allergen in peanut, *Mol. Immunol.* 46 (2009) 1796–1804.
- [24] T. Jin, C. Wang, C. Zhang, Y. Wang, Y.W. Chen, F. Guo, A. Howard, M.J. Cao, T. J. Fu, T.H. McHugh, Y. Zhang, Crystal structure of cocosin, A potential food allergen from coconut (*cocos nucifera*), *J. Agric. Food Chem.* 65 (2017) 7560–7568.

- [25] M. Adachi, Y. Takenaka, A.B. Gidamis, B. Mikami, S. Utsumi, Crystal structure of soybean proglycinin A1aB1b homotrimer, *J. Mol. Biol.* 305 (2001) 291–305.
- [26] R.A. Badley, D. Atkinson, H. Hauser, D. Oldani, J.P. Green, J.M. Stubb, The structure, physical and chemical properties of the soy bean protein glycinin, *Biochim. Biophys. Acta* 412 (1975) 214–228.
- [27] Y.-S. Yan, X.-D. Lin, Y.-S. Zhang, L. Wang, K. Wu, S.-Z. Huang, Isolation of peanut genes encoding arachins and conglutins by expressed sequence tags, *Plant Sci.* 169 (2005) 439–445.
- [28] S. Kabasser, K. Pratap, S. Kamath, A.C. Taki, T. Dang, J. Koplin, K. Perrett, K. Hummel, C. Radauer, H. Breiteneder, A.L. Lopata, M. Bublin, Identification of vicilin, legumin and antimicrobial peptide 2a as macadamia nut allergens, *Food Chem.* 370 (2022), 131028.
- [29] P. Rabjohn, E.M. Helm, J.S. Stanley, C.M. West, H.A. Sampson, A.W. Burks, G. A. Bannon, Molecular cloning and epitope analysis of the peanut allergen Ara h 3, *J. Clin. Invest.* 103 (1999) 535–542.
- [30] R. Valenta, A. Karaulov, V. Niederberger, Y. Zhernov, O. Elisyutina, R. Campana, M. Focke-Tejkl, M. Curin, L. Namazova-Baranova, J.Y. Wang, R. Pawankar, M. Khaitov, Allergen extracts for in vivo diagnosis and treatment of allergy: is there a future? *J. Allergy Clin. Immunol. Pract.* 6 (2018) 1845–1855, e1842.
- [31] P. Gunasekara, S.M. Handunnetti, S. Premawansa, E. Witharana, I.P. Ratnayake, P. Kaluarachchi, C. Karunatilake, R.K.S. Dias, G.A.S. Premakumara, W. Dasanayake, S.L. Seneviratne, R. de Silva, Diagnosis of *Apis dorsata* venom allergy: use of recombinant allergens of *Apis mellifera* and a passive basophil activation test, *Clin. Mol. Allergy : CM* 20 (2022) 11.
- [32] S.M. Albillos, T. Jin, A. Howard, Y. Zhang, M.H. Kothary, T.J. Fu, Purification, crystallization and preliminary X-ray characterization of prunin-1, a major component of the almond (*Prunus dulcis*) allergen amandin, *J. Agric. Food Chem.* 56 (2008) 5352–5358.
- [33] X. Fan, X. Li, Y. Zhou, M. Mei, P. Liu, J. Zhao, W. Peng, Z.B. Jiang, S. Yang, B. L. Iverson, G. Zhang, L. Yi, Quantitative analysis of the substrate specificity of human rhinovirus 3C protease and exploration of its substrate recognition mechanisms, *ACS Chem. Biol.* 15 (2020) 63–73.