

Accepted Manuscript

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PII: S0963-9969(16)30484-7
DOI: doi: [10.1016/j.foodres.2016.10.043](https://doi.org/10.1016/j.foodres.2016.10.043)
Reference: FRIN 6476

To appear in: *Food Research International*

Received date: 18 July 2016
Revised date: 23 October 2016
Accepted date: 24 October 2016



Please cite this article as: Zhang, Y., Du, W.-X., Fan, Y., Yi, J., Lyu, S.-C., Nadeau, K.C. & McHugh, T.H., Identification, characterization, and initial epitope mapping of a pine nut allergen, *Food Research International* (2016), doi: [10.1016/j.foodres.2016.10.043](https://doi.org/10.1016/j.foodres.2016.10.043)

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Identification, Characterization, and initial epitope mapping of a pine nut allergen

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Abbreviations: CD, circular dichroism;

Abstract

The aims of this study were to predict, identify and characterize pine nut allergens. Korean pine (*Pinus koraiensis*) vicilin was predicted to be a pine nut allergen. Recombinant Korean pine vicilin was expressed in *E. coli* and purified. Natural Korean pine vicilin isolated from pine nuts (which displayed multiple bands in SDS-gels due to posttranslational digestion) and its full length recombinant counterpart were used to test whether it is a food allergen. The recognition of the protein (and its fragments) by patient serum IgE was analyzed by Western blot. The study included fourteen patients diagnosed with clinical pine nut allergy. Twenty nine percent of the patient sera recognized both the natural and recombinant pine nut vicilin, indicating that Korean pine vicilin is a bona fide food allergen. The serum recognition patterns of the naturally occurring protein fragments suggested that some of linear IgE epitopes may be mapped to the fragment boundaries. The chemical and thermal stability of the recombinant protein was investigated. It underwent a thermal transition with a $T_m=76.6$ °C. The transition was accompanied by an increase in the amplitude of the circular dichroism signal at 220 nm. Urea induced unfolding of the recombinant protein had a C_m of 4.6 M.

Key words: copper protein / IgE / pine nut allergy / seed storage protein / food allergy

1. Introduction

The prevalence of food allergy in industrialized countries has increased dramatically in recent years and food allergies have become a serious health concern globally (Jackson, Howie, & Akinbani, 2013; Wang, & Sampson, 2007). In the United States, eight food groups (milk, egg, fish, shellfish, tree nuts, peanuts, wheat, and soybeans) (Usfda, 2015a) are responsible for more than 90% of the food allergy cases. Seeds from a number of different trees are referred as tree nuts, albeit not all of them are nuts in the botanical sense. The seeds of pine trees are commonly referred to as pine nuts which are cataloged in the list of tree nuts by the US Food and Drug Administration (Usfda, 2015b). Peanuts and tree nuts account for most of the near-fatal and fatal food allergy cases in the United States (Sampson, Mendelson, & Rosen, 1992; Yunginger, Sweeney, Sturmer, Giannandrea, Teigland, Bray, Benson, York, Biedrzycki, Squillace, & Et Al., 1988). To date, there is no cure for food allergies (Kobernick, Chambliss, & Burks, 2015; Ponce, Diesner, Szepefalusi, & Eiwegger, 2016; Sathe, Liu, & Zaffran, 2016).

Pines (family *Pinaceae*, genus *Pinus*) are widely distributed around the globe. They are among the most important forest trees for the world's annual timber supply and paper industries (Liang, Wang, Liu, Ji, Fang, Liu, Carter, Webb, & Dean, 2007). Culinary use of pine nuts has a long history in Asia and Europe. Pine nut is one of the most expensive nuts in today's market and it is considered a delicacy in many parts of the world (Fao., 1998). More than 80% of pine nuts consumed in the US are imported, primarily from China (Geisler, & Huntrods, 2013) where the nuts of Korean pine are extensively harvested in the northeastern part of the country.

Pine nuts contain a considerable amount of numerous vitamins (e.g. vitamin A, B1 and B2), minerals (e.g. potassium, magnesium, copper), and dietary fiber (Nergiz, & Dönmez, 2004; Usda, 2008). Pine nut protein content is as high as 31.6% of the total dry weight of the edible portion

(Nergiz et al., 2004; Venkatachalam, & Sathe, 2006). Unfortunately, pine nuts are reported to cause allergic reactions in certain patients (Añó, Maselli, Sanz Mf, & Fernandez-Benitez, 2002; Garcia-Menaya, Gonzalo-Garijo, Moneo, Fernandez, Garcia-Gonzalez, & Moreno, 2000). Recently, the 6 kDa albumin of stone pine (*Pinus pinea*) were characterized as a pine nut allergen (Cabanillas, Cheng, Grimm, Hurlburt, Rodriguez, Crespo, & Maleki, 2012) and it has been officially recognized as a pine nut allergen (Pin p 1) by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee. In addition, three peptides (of 6, 9, and 10 amino acids, respectively) derived from a 50 kDa stone pine protein were recognized by the sera of a subgroup of patients. These peptides were matched to a white spruce (*P. glauca*) vicilin (Cabanillas et al., 2012).

Most food allergens have been defined by separating proteins in a food extract on an SDS gel, isolating a band that was recognized by immunoglobulin E (IgE) molecules in the sera of food allergy patients, and identifying the protein in the band by N-terminal amino acid sequence analysis, sequencing with mass-spectrometry, or other methods. We thought that targeted identification of uncovered food allergens may accelerate the process of understanding the allergenicity of food allergens. Knowing that the 7S vicilins from a number of nuts [including peanut (Ara h 1), hazelnut (Cor a 11), cashew nut (Ana o 1), pistachios (Pis v 3), and walnuts (Jug n 2 and Jug r 2)] are allergens, we hypothesized that Korean pine vicilin is a food allergen and focused on a strategy to directly test whether it would bind to IgE antibodies in sera from patient with pine nut allergy. Previously, we have utilized protein samples purified from pine nuts to characterize Korean pine vicilin (Jin, Albillos, Chen, Kothary, Fu, & Zhang, 2008a) and determine its crystal structure (Jin, Wang, Chen, Fu, Kothary, Mchugh, & **Zhang**, 2014). However, in Korean pine nuts, besides the removal of a signal peptide, most of the mature vicilin

is processed by peptidases at two additional sites of digestion, making it more challenging to investigate the allergenicity of this protein. Here, we describe the expression and purification of recombinant Korean pine vicilin, the identification of this protein as a food allergen, and the initial mapping of linear IgE epitopes.

2. MATERIALS AND METHODS

2.1 Plasmid construction

For the purpose of determining the peptide sequence of Korean pine vicilin, the plasmid pBlue-vicilin was constructed previously by isolating the coding sequence of Korean pine vicilin and inserting it into a cloning vector (Jin et al., 2014). In the present study, the coding sequence for mature vicilin was PCR amplified from pBlue-vicilin using primer I (ccaggatccgggcgagaagaagagcga, forward) and primer II (cctggtacctcaagaagagctccttggtc, reverse). The PCR product was digested with *Bam*HI and *Kpn*I and inserted into pTAP1S digested with the same enzymes by DNA ligation using quick ligase (New England BioLabs, Ipswich, MA). pTAP1S is a home-made vector with the backbone of pRSFduet-1 (Merck Millipore, Billerica, MA) and a modified multi-cloning-site sequence. The resulting plasmid was named pRSFpinkv and transformed into *E. coli* DH5 α . pRSFpinkv plasmid was prepared from the isolated colonies and sequenced to confirm correct insertion of the vicilin coding sequence in frame with that of an N-terminal His-tag.

2.2 Protein expression and purification

pRSFpinkv was transformed into *E. coli* BL21(DE3)* (Invitrogen, Carlsbad, CA) for protein expression. The transformed bacteria were grown in 1 liter of LB medium containing 50 mg/L kanamycin (Gold Biotechnology, St. Louis, MO) at 37°C in a shaking incubator. When the

OD₆₀₀ of the culture reached 1.0, the incubator temperature was reduced to 18°C and the culture was incubated for an additional 30 min. IPTG (Gold Biotechnology) was added to the medium to a final concentration of 1 mM and vicilin expression was induced for 16 hours. The cells were collected by centrifugation and the bacterial pellet was stored at -80 °C for future use.

The pellet from a one-liter culture was re-suspended in 50 mL of nickel binding buffer (buffer NB, 20 mM imidazole, pH 7.9, 200 mM NaCl) containing 1 mM benzamidine. Benzamidine was obtained from SIGMA (St. Louis, MO). Cells were sonicated for 8 minutes in a beaker surrounded by a mixture of ice and water, followed by centrifugation at 20,000g for 30 min at 4°C. The supernatant was loaded onto a 5-ml HisTrap FF crude column (GE Healthcare, Piscataway, NJ). The column was washed with 5 bed volumes of buffer NB and 5 bed volumes of buffer NB plus 45 mM imidazole, pH 7.9. The protein was then eluted from the column with buffer NB plus 280 mM imidazole, pH 7.9. The fractions of the HisTrap column elution containing the over-expressed protein were analyzed by SDS-PAGE, pooled, and loaded onto a XK 26/70 Superdex-200 Prep grade column (GE Healthcare). The XK 26/70 Superdex-200 Prep grade column was pre-equilibrated with 200 mM NaCl, 10 mM Tris-HCl, pH 7.9 and protein was eluted with the same buffer. All chromatographic steps were carried out at room temperature (23°C) using an FPLC system (GE Healthcare). Samples of the target protein after each step of purification were boiled in 1X SDS sample buffer (50 mM Tris-HCl, pH6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) containing 100 mM β-mercaptoethanol for 5 minutes and separated by electrophoresis with 4-20% gels and stained using the Colloidal Coomassie Brilliant Blue (CBB) G-250 Staining protocols (Dyballa, & Metzger, 2009). The images of the stained gels were collected with an ImageQuant LAS4000 imaging system (GE Healthcare). The oligomeric state of the purified protein was determined by calculating its molecular mass based

on its peak position in the elution profile of the Superdex 200 column. The column was calibrated with protein standards (Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; conalbumin, 75 kDa; and Ovalbumin, 43 kDa) purchased from GE Healthcare.

2.3 Circular dichroism (CD)

The buffer of the purified protein was changed to a buffer containing 5 mM phosphate (pH 7.2) and 100 mM NaCl by repeated concentration-and-dilution using an Amicon® Ultra centrifugal filter device with 30 kDa molecular weight cut off for proteins (Millipore). Far-UV spectra were collected using a J-815 CD spectrometer (Jasco, Tokyo) and a quartz cuvette with a light path length of 2.0 mm. Using 2.4 μ M protein sample, a wavelength scans were performed in the 195-260 nm region with a speed of 50 nm/min at 20 °C. The spectra were averaged for 10 scans. Heat induced structural transition of the recombinant vicilin was studied by monitoring CD signal at 220 nm as a function of increasing temperature (1 minutes per degree).

2.4 Urea induced denaturation

Two stock solutions of recombinant Korean pine vicilin were prepared. Both solutions contained 12 μ M of the protein, 5 mM phosphate (pH 7.2) and 100 mM NaCl. One of them additionally contained 9 M of urea. Twenty nine additional samples were prepared with various ratios of the two stock solutions to obtain samples at 0.3 M intervals of urea concentration from 0.3 to 8.7 M. The samples were thoroughly mixed and equilibrated at room temperature for 24 hours. The CD signal of these samples (at 220 nm) were measured at 20 °C every second for half a minute. The free energy of denaturation was calculated by fitting the data to a two-state transition using Origin Pro9 software (OriginLab Corp., Northampton, MA).

2.5 Patient Sera

Sera (#1: 5720, #2: 5137, #3: B5279, #4, 5561; #5, 2154; #6, 2552; #7, 2912; #8, 5875; #9,

5981; #10, 5980; #11, 6695; #12, 6519; #13, 6175; #14, 6461) were collected from patients with a history of IgE-mediated pine nut allergy and with a positive oral food challenge to pine nut. The patients were enrolled in the food allergy study at Stanford University under institutional review board approval with informed consent.

2.6 IgE Recognition of Vicilin

Korean pine nut protein extract, vicilin purified from pine nut, and purified recombinant pine nut vicilin were boiled in 1X SDS sample buffer containing 100 mM β -mercaptoethanol for 5 minutes and separated by electrophoresis on 4-20% gels. Multiple SDS-PAGEs were performed for protein detection by CBB staining and for IgE binding analysis by western blot. For the latter, proteins separated by SDS-gels were transferred to PVDF membranes using a Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad, Hercules, CA). The membranes were blocked in TBST (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% tween 20) containing 5% nonfat dry milk for 30 min at room temperature. The membranes were then incubated in 5 mL TBST with 1% nonfat milk and 20 μ L of individual human sera at 4°C overnight. After washed three times with TBST for a total of 15 minutes, the membranes were incubated with 5 mL TBST with 1% nonfat milk containing anti-human IgE secondary antibody conjugated to peroxidase (Sigma, 1:5000) for 60 minutes at room temperature. The membranes were washed again 5 x 5 minutes with TBST. Indirect detection of the bound IgE was performed using ECL Western Blotting substrate (Pierce, Rockford, IL, USA) and the ImageQuant LAS4000 imaging system.

3. Results and discussion

3.1 Protein expression, purification

Recombinant Korean pine vicilin expressed in *E.coli* at 18 °C was highly soluble in aqueous solutions. It was purified with immobilized metal affinity and size exclusion chromatography. The molecular mass of the purified protein was estimated to be 121 kDa based on the calibration of the Superdex gel filtration column (**Figure 1**). Thus the recombinant protein was likely to be a trimer in solution as the estimated molecular mass is approximately three times that of the calculated molecular mass of the monomer. This is consistent with the oligomeric state of the native protein purified from pine nuts estimated based on size exclusion chromatography and determined by analyzing its crystal structure (Jin, Albillos, Chen, Kothary, Fu, & Zhang, 2008b; Jin et al., 2014) and vicilin from other species (Shewry, Napier, & Tatham, 1995).

3.2 Protein Characterization

Circular dichroism spectroscopy indicated that recombinant Korean pine vicilin was a well-structured protein. Although assigning a number to the exact percent content of each secondary structure type from CD data is often a challenging task, it can be seen from **Figure 2a** that recombinant Korean pine vicilin is composed mainly of β -sheets as indicated by the spectra features with a maximum negative signal ~ 215 nm. This is also consistent with the secondary structure of the native Korean pine vicilin purified from pine nut (Jin et al., 2008b; Jin et al., 2014). The thermal stability of the recombinant pine nut vicilin was also studied by CD signal as a function of temperature. Monitoring its CD signal at 220 nm as a function of temperature did not reveal a transition from regular secondary structures to random coil. On the contrary, an apparent two-state thermal transition with a 220 nm CD signal increase was observed (**Figure 2b**). Fitting the data to a two-state transition resulted a $T_m = 76.6 \pm 0.1$ °C. The CD signal of native Korean pine vicilin also increased at ~ 75 °C (Jin et al., 2008b). Thus, these also suggested

that the structure of the recombinant vicilin was the same as that of the native protein purified from pine nuts. There was no observable precipitation after the transition, but the transition was not a reversible process as can be seen from the CD spectra before and after the thermal transition (**Figure 2a**). However, the CD signal at 220 nm after the temperature scan was not as strong as that at higher temperature in the temperature scan, indicating that heat induced structural changes were not locked in place when the temperature of the sample was brought back to 20 °C. Further investigation is needed to understand the mechanism of this transition. These data indicated that the recombinant Korean pine vicilin generated in this study is likely to have the same overall three dimensional structure as the native protein purified from pine nut and can serve as a valuable reagent for pine nut allergy research.

3.3 Chemical stability of recombinant Korean pine vicilin

Thermal studies described above indicated the recombinant protein has high thermal stability. To further assess its stability, the folding-unfolding equilibrium of the protein was studied by measuring its urea-induced transitions (**Figure 3**). The data points seemed to indicate a two-state transition between folded and unfolded states of Korean pine vicilin without a measurably populated stable intermediate state. The details of finding the free energy of unfolding of a protein can be found elsewhere (see e.g., ref (Pace, 1986)). Briefly, the Gibbs free energy (ΔG) of the equilibrium transition at a given denaturant concentration can be calculated using the concentrations of the protein in the folded [F] and unfolded [U] states $\Delta G = \ln([U]/[F])$. The free energy of transition is a linear function of the denaturant concentration to the first approximation:

$$\Delta G = \Delta G_0 - mC$$

where ΔG_0 is the free energy of the transition when there is no denaturant, C is the concentration

of the denaturant, and m is a constant. Generally, the CD signal is the sum of those of the folded and unfolded protein, both of which are linear functions of the denaturant concentration. At the middle point of the transition when $C = C_m$, the folded and unfolded states are equally populated, $[U] = [F]$, $\Delta G = 0$, and m equals to $\Delta G_0/C_m$. Thus, C_m , m , and ΔG_0 can be found by non-linear fitting of the CD data. The fitting results indicated that the transitions occurred with a midpoint concentrations of 4.7 ± 0.3 M and the free energy of unfolding, ΔG_0 , was 2.1 ± 0.2 kcal/mol. In addition, **Figure 3** indicated that at low urea concentration, the CD signal becomes stronger as the urea concentration increases. This was reminiscent of the CD signal changes with increasing temperature, though the mechanisms of the changes remains to be investigated.

3.4 Identification of Korean pine vicilin as an allergen

The patients whose sera were used in this study had an average age of 10.5 (range 6–15). The mean diameter of results of their skin prick tests was 4.64 mm (range 3-7). All these patients had a positive DBPCFC with pine nut. Eight of the patients also had an allergy to another tree nut, 3 to cashew (*Anacardium occidentale*), 2 to hazelnut (*Corylus avellana*), 2 to walnut (*Juglans regia*), and 1 to pistachio (*Pistacia vera*). The other six patients also had an allergy to one or more other foods [1 to both milk and eggs, 2 to eggs only, 1 to milk only, 1 to sesame (*Sesamum indicum*), and 1 to chick peas (*Cicer arietinum*)]. The clinical information of the patients is shown in **Table 1**.

Most food allergens belong to a very limited number of protein families. Korean pine vicilin is very likely to be a food allergen because it belongs to the cupin superfamily which contains the 7S vicilin and 11S legumin proteins known to be food allergens from seeds of many plants. We sought to determine directly whether Korean pine vicilin is a food allergen. Previously, we have purified Korean pine vicilin and determined its crystal structure (Jin et al., 2014).

However, the separation of Korean pine vicilin and convicilin requires multiple steps of purification and extra care was needed to avoid cross contamination. To aid the immunological analysis, we additionally purified Korean pine vicilin in its recombinant form. Purified recombinant and native Korean pine vicilin were separated with an SDS gel along with a 10 M urea Korean pine nut extract and stained with CBB (**Figure 4**). Identical samples were analyzed by western blot with 14 patient sera (**Figure 5**). Serum 1, 2, 6, and 13 clearly recognized the recombinant Korean pine vicilin and the full length vicilin purified from pine nut. These sera also recognized a protein band with the same size as the full length vicilin in the 10 M urea pine nut extract. In addition, sera 1 and 2 respectively recognized a band in the 10 M urea pine nut protein extract corresponding to the band they recognized in the purified natural vicilin lane. The recognition pattern of serum 13 is the same as serum 1, while serum 6 recognized both the fragments recognized by the other sera. These data indicated that Korean pine vicilin is a food allergen. The image of the membrane blotted with other sera did not reveal any association with vicilin. Their recognition patterns and the signal intensity were different (data not shown). As examples, the results for serum 3 and serum 5 are shown in **Figure 5a** and **5b**, respectively, indicating that these sera did not recognize denatured Korean pine vicilin. Whether these and additional clinically relevant samples recognize conformational IgE epitopes of Korean pine vicilin warrant future studies. As the recognition patterns of the sera were different, the result for one serum could also be considered as positive and/or negative control for the experiments with other sera. No serum from normal subject was included for experimental control.

3.5 Mapping of putative Korean pine nut vicilin linear IgE epitopes

Data shown in **Figure 5** were most likely a result of the recognition of linear epitopes by serum IgE as the proteins/protein fragments separated on the SDS gel were denatured. The

pattern of vicilin fragments recognized by the serum samples was used to infer information about the location of the recognition sites within the protein fragment that each of the serum samples recognized. Previously, we determined the N-terminal sequence of each of the natural fragments of Korean pine vicilin (Jin et al., 2008b). The positions of the N-terminal peptides of these fragments are indicated in the amino acid sequence of Korean pine vicilin shown in a monospaced font (**Figure 6**, right panel). The calculated molecular mass of the full length protein is shown above its sequence, and that of each fragment is shown near one end of the fragment. These sequences are placed vertically at the positions proximal to where they would migrate as a band in the SDS gel in the left and middle panels of **Figure 6**. For simplicity, serum 1 and 2 will be used below to represent sera with different recognition patterns. It can be seen from **Figure 6** that besides the full length protein, patient serum 1 and 2 each recognized an additional natural fragment of vicilin. Serum 1 recognized a ~25 kDa band but not the ~30 kDa band bound by serum 2. These data strongly suggested that the linear epitopes recognized by serum 1 lie in the carboxyl terminal half of the protein. Furthermore, this serum did not recognize the ~12 kDa band. As this band contained both the first and the second half of the C-terminal half of the protein, the data indicated that the cleavage site which separated the full length 46.2 kDa protein into a 33.6 kDa fragment and a 12.6 kDa fragment disrupted all the linear epitopes serum 1 recognized. Since linear epitopes are usually 8-10 amino acids (Sathe, Sharma, & Roux, 2008), the linear epitopes recognized can all be tentatively mapped to within a 20-AA peptide centered at the peptidase cleavage site (E344-V363). Whether serum 1 recognizes more than one overlapping linear epitope in this short peptide or only one linear epitope was not the aim of the present study. The exact sequence(s) of the epitope(s) can be determined by synthesizing short overlapping peptides covering the surrounding sequence of this cleavage site

and testing their recognition by serum 1. This may be carried out as part of a future systematic epitope mapping study with scanning overlapping peptides when more patient sera are available. Similarly, Serum 2 recognized the band close to 30/34 kDa, which could be represented by the fragment with a calculated molecular mass of 33.6 kDa, but not the band close to 25 kDa, which could be represented by either of the two peptide fragments with calculated molecular mass of 22.8 and 23.4 kDa, respectively. These data strongly suggested that the linear epitope(s) this serum recognized reside(s) within a short peptide centered at the cleavage site close to the middle of the protein (R251-L270).

Most food allergens came to light after being recognized by patient sera and subsequently identified by sequencing. To date, less than a dozen of protein families contain more than 85% of the known food allergens of plant sources. The exact properties of a protein that make it an allergen is not clear, but it might reasonable to assume that the orthologues of these allergens in other plant food are also likely to be food allergen. In the present case, Korean pine vicilin was predicted as a food allergen. It was previously isolated and sequenced and confirmed to be a bona fide pine nut allergy. The correctness of similar predictions needs to be dealt with case by case. Each time such a prediction is proved, however, it reiterate the need for future studies to understand what makes a protein an allergen.

It is interesting that the naturally occurring fragments of vicilin can be used to map the linear IgE epitopes to the fragment boundaries. As both the full length protein and its fragments exist in pine nut and their relative abundance may be affected by unknown factors, such as environmental conditions, future studies are needed to determine whether epitopes at (or close to) these fragment boundaries are relatively dominant linear IgE epitopes in Korean pine vicilin. Regardless, this might indicate that the flexible loops at the surface of the protein that tends to be

the target of a protease in the seeds might also have a tendency to elicit allergic reactions. It worth noting that in the case of peanut allergen Ara h 3, some of the known dominant linear IgE epitopes was also located in the flexible loops (Jin, Guo, Chen, Howard, & Zhang, 2009).

In short, while full length Korean pine vicilin is present in the harvested pine nuts, most of the protein in its native trimeric structure is cleaved by a peptidase(s) at one or two sites. Denaturing SDS-PAGE separate it into three bands. Native Korean pine vicilin purified from pine nuts and full length recombinant Korean pine vicilin was used to study its recognition by sera of patients with double-blind placebo-controlled food challenges. The results indicated that Korean pine vicilin is a food allergen.

Notes

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

The authors declare no conflict of interest.

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Figure 1. Purification of Korean pine vicilin. Following purification with nickel and Superdex columns, the purified His-tag Korean pine vicilin was reloaded onto the Superdex column to check its purity.

Figure 2. Heat induced transition of recombinant Korean pine vicilin. Purified recombinant Korean pine vicilin was changed into a phosphate buffer (5 mM, pH 7.2) with 100 mM NaCl by repeated concentration and dilution. **A)**, CD spectra of recombinant Korean pine vicilin before (solid line) and after (broken line) thermal scan. **B)**, thermal transition of vicilin monitored by CD at 220 nm. The transition was fitted with a two-state model (solid line).

Figure 3. Urea induced unfolding of recombinant Korean pine vicilin. The folding-unfolding equilibrium was monitored by recording the CD signal at 220 nm. The solid gray line shows the result of a non-linear fitting using a two-state model (see text). The black line shows the linear increase of CD signal as a function of urea concentration before the unfolding of vicilin was induced by urea.

Figure 4. SDS-PAGE analysis of recombinant Korean pine vicilin. Samples of Korean pine vicilin purified from pine nut (N), recombinant Korean pine vicilin (R), and 10 M urea pine nut protein extract (E) were analyzed with 4-20% SDS-PAGE. The molecular masses of the reference bands in the protein marker lane (M) are shown on the left side of the gel image.

Figure 5. Western blot showing recognition of Korean pine vicilin by sera from patients with pine nut allergy. Korean pine vicilin purified from pine nut (N), recombinant Korean pine vicilin (R), and 10 M urea pine nut protein extract (E) were separated with 4-20% SDS-gels and transferred to PVDF membranes. Individual serum (1, 2, 3 in fig. A and 5, 6, 13 in

fig. B as indicated above the respective panels) was used as primary antibody. No serum was used in the control blot under otherwise identical conditions.

Figure 6. Linear IgE epitope mapping. The recognition of native Korean pine vicilin by serum 1 and 2, together with its CBB staining result, is shown to the right. The sequences of Korean pine vicilin and its naturally occurring fragments are shown to the left in a monospaced font aligned vertically to the band positions of the protein or protein fragments in the SDS gel. The N-terminal sequenced residues are shaded. The calculated molecular mass of the peptides are indicated. The deduced positions of the linear epitopes are marked by the ovals.

Table 1. Clinical findings of pine nut allergic patients

Patient Number	Age	SPT ¹ (mm)	Symptoms ²	Diagnostic challenge	Allergy to other food ⁴	Reaction to pine vicilin
1	8	3	U	Dbpcfc ³	cashew	+++
2	10	5	U	dbpcfc	hazelnut	++
3	13	4	A	dbpcfc	cashew	-
4	11	4	GI	dbpcfc	walnut	-
5	14	6	GI	dbpcfc	pistachio	-
6	8	7	U	dbpcfc	Milk, egg	+
7	7	6	TS	dbpcfc	egg	-
8	10	6	GI	dbpcfc	sesame	-
9	13	5	A	dbpcfc	chickpea	-
10	15	5	U	dbpcfc	hazelnut	-
11	6	3	GI	dbpcfc	walnut	-
12	9	3	GI	dbpcfc	egg	-
13	11	4	R	dbpcfc	milk	++++
14	12	4	R	dbpcfc	cashew	-

¹SPT = skin prick testing; ²Abbreviations for symptoms: A, asthma; GI, gastrointestinal symptoms; R, rhinitis; TS, tongue swelling; U, urticaria. ³dbpcfc = double-blind, placebo-controlled food challenge. ⁴Other allergies were diagnosed by dbpcfc.

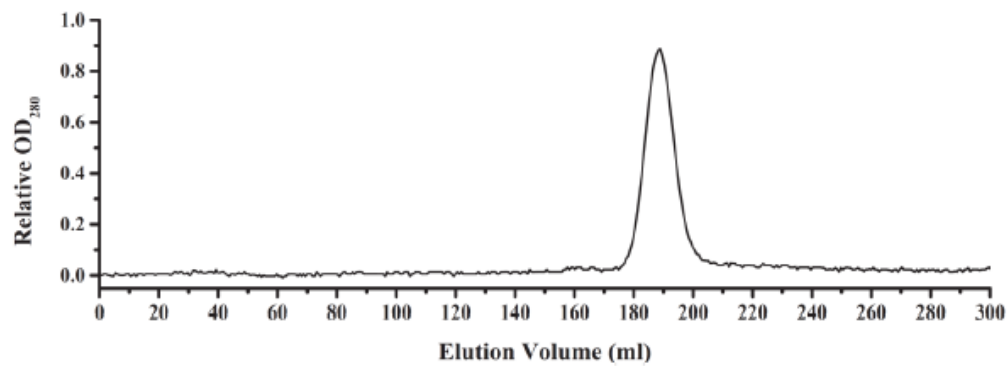


Figure 1

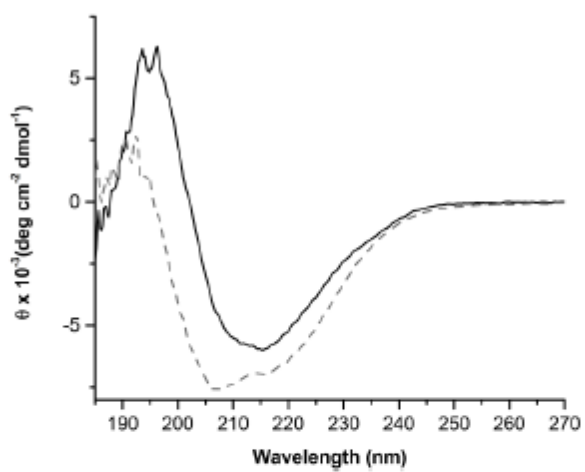


Figure 2A

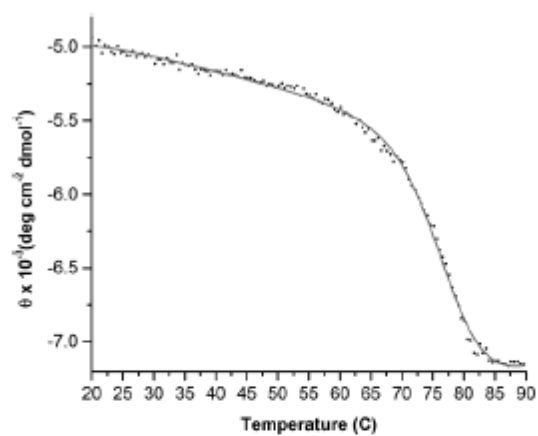


Figure 2B

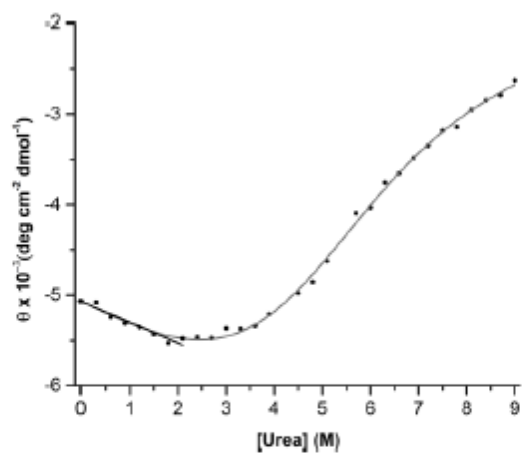


Figure 3

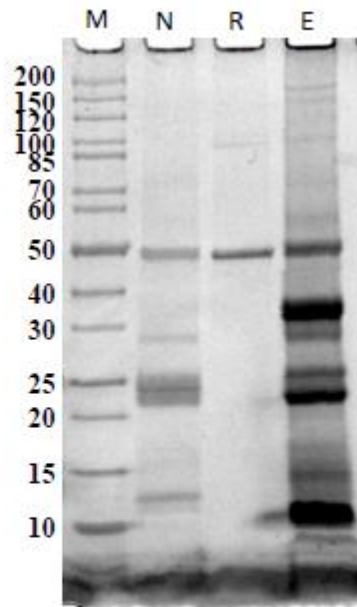


Figure 4

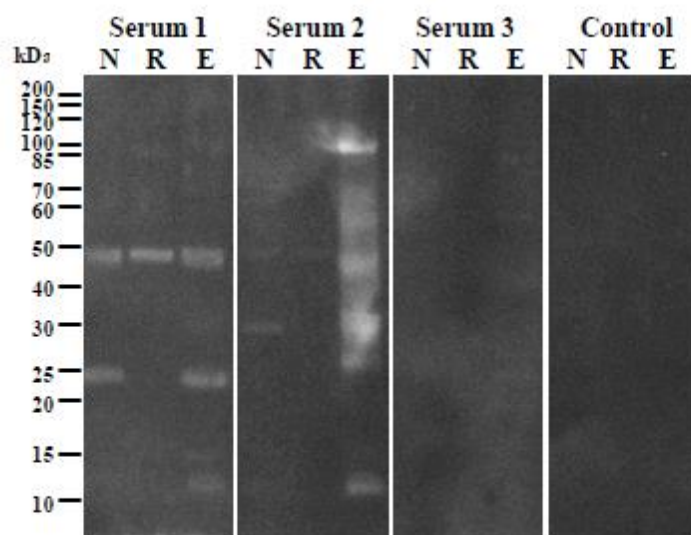


Figure 5A

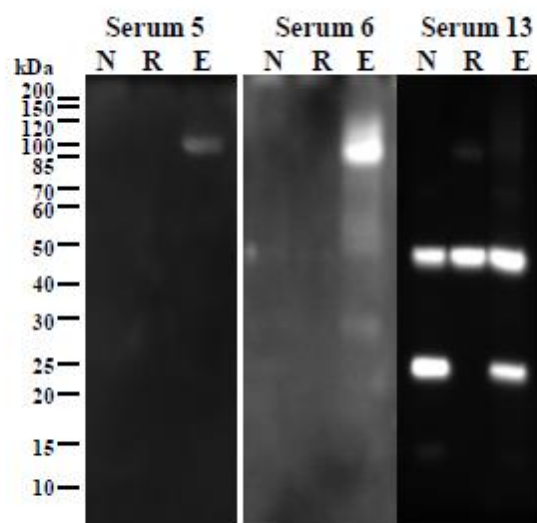
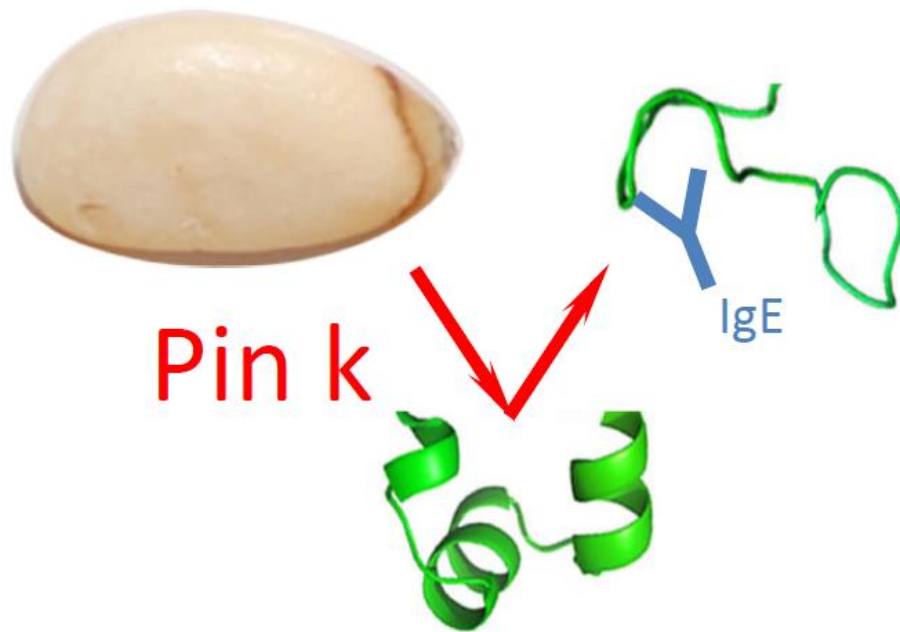


Figure 6

Graphical abstract



Highlights

- Korean pine vicilin was expressed and purified as a 121 kDa trimeric protein.
- Thermal and chemical stabilities of recombinant Korean pine vicilin were studied.
- IgE specific to Korean pine vicilin were detected in 29% of the patient sera.
- Korean pine vicilin was predicted and identified as food allergen.