

Characterization of a 7 kDa pollen allergen belonging to the gibberellin-regulated protein family from three Cupressaceae species

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Short title:

Pollen GRP allergens from three Cupressaceae species

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Manuscript word count (Introduction, Methods, Results, Discussion): 4249

Number of figures: 6

Number of tables: 0

Supporting information: 5 tables

Funding: This work was in part self-funded by Thermo Fisher Scientific and in part supported by APHM, Assistance Publique Hôpitaux de Marseille and by the French Society of Allergology (Société Française d'Allergologie) through a research grant (SFA 2017).

Author contributions: AEE, LM and HL purified and analysed proteins biochemically, immunologically and bioinformatically. JÖ cloned and expressed recombinant proteins. JL conceived and supervised the study. CK and JV performed and supervised the collection of patient samples used in the study. JL, AEE, LM and JV wrote the paper. All authors read and approved the final manuscript.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/cea.13675](https://doi.org/10.1111/cea.13675)

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Conflict of interest statements: AEE, JÖ, HL, LM and JL are employees of Thermo Fisher Scientific. JV has received speaker and consultancy fees from Meda Pharma, Mylan, Sanofi, Thermo Fisher and research grants from the French Society of Allergology. The other authors declare no conflict of interest.

Keywords: Cupressaceae; cypress pollinosis; gibberellin-regulated protein; GRP; IgE; peach allergy; peamaclein; pollen-food allergy syndrome; Pru p 7; recombinant allergen

Abbreviations: CD, Circular dichroism; CIEX, cation exchange chromatography; ETD, electron-transfer dissociation; GRP, gibberellin-regulated protein; HCD, Higher-energy collisional dissociation; HRAM, High-resolution, accurate-mass; MS, mass spectrometry; nsLTP, non-specific lipid transfer proteins; OAS, oral allergy syndrome; PFAS, pollen-food allergy syndrome; SEC, size exclusion chromatography

ABSTRACT

Background: Severe allergy to fruits mediated by a 7 kDa allergen belonging to the gibberellin-regulated protein (GRP) family is known to be associated with Cupressaceae pollinosis.

Objective: To identify and characterize Cupressaceae pollen allergens involved in GRP-related fruit allergy.

Methods: Pru p 7 related proteins from pollen of *Cupressus sempervirens*, *Juniperus ashei* and *Cryptomeria japonica* were identified using a rabbit anti-Pru p 7 antiserum, purified chromatographically and sequenced by mass spectrometry and bioinformatic comparisons. The *C. sempervirens* protein was produced as a recombinant allergen in *Pichia pastoris*. IgE antibody binding to pollen GRP proteins was analyzed in a peach allergic (n=54) and a cypress pollen allergic (n=88) patient population from southern France using ImmunoCAP.

Results: In each of the three Cupressaceae species studied, a 7 kDa pollen protein related to Pru p 7 was identified and found to comprise amino acid sequences of 63 residues in length, 94-98% identical to each other and 67-68% identical to Pru p 7. The *C. sempervirens*, *J. ashei* and *C. japonica* GRP allergens have been officially recognized by the WHO/IUIS Allergen Nomenclature Sub-Committee and named Cup s 7, Jun a 7 and Cry j 7, respectively. Recombinant Cup s 7 showed IgE antibody binding capacity comparable to that of the purified natural allergen. Among 51 peach allergic subjects sensitized to Pru p 7, substantially higher levels of IgE to Cup s 7 than to Pru p 7 were found. Further, the pollen protein was able to completely outcompete IgE binding to Pru p 7 while the reverse competition effect was modest, consistent with primary sensitization by the pollen allergen.

Conclusion & Clinical Relevance: Pru p 7 related pollen allergens from three Cupressaceae species have been characterized and may become useful for the identification of pollinosis patients at risk of developing severe fruit allergy.

INTRODUCTION

Cupressus sempervirens (Mediterranean or Italian cypress) is a major cause of winter pollinosis in Mediterranean regions. The prevalence of sensitisation to cypress pollen has increased dramatically in the past few decades, in certain locations reaching as high as 42% among atopic individuals.^{1, 2} The Mediterranean cypress is closely related to Arizona cypress (*Cupressus arizonica*) and somewhat more distantly to mountain juniper (*Juniperus ashei*), both native to North America, and to Japanese cedar (*Cryptomeria japonica*) and Japanese cypress (*Chamaecyparis obtusa*).³ The highly dominant major allergens in these species (called Cup s 1, Cup a 1, Jun a 1, Cry j 1 and Cha o 1, respectively), to which more than 90% of Cupressaceae pollen allergic patients are sensitized, belong to the pectate lyase protein family. These allergens are highly cross-reactive with each other and share 70%-95% sequence identity.^{4, 5}

Sensitization to pollen is often associated with allergy to different plant foods due to cross-reactivity between homologous pollen and food proteins. Pollen-related food allergy is believed to be driven predominantly or entirely by pollen sensitization. It typically causes mild and local oral symptoms and is referred to as the oral allergy syndrome (OAS). A well-known example of pollen-related food allergy involves sensitization to PR-10 allergens in pollen of Fagales species, most notably birch, and their cross-reactive homologues in a variety of fruits, vegetables, tree nuts and legume seeds. Other examples include the mugwort-spice allergy syndrome and profilin-mediated plant food allergy associated with grass, weed or tree pollen sensitization.⁶

A more severe form of food allergy to peach and certain other fruits has recently been recognized as a pollen-related food allergy. The first molecular evidence of this food allergy emerged with the discovery of a novel, 7 kDa, cysteine-rich peach allergen named Pru p 7 by Tuppo et al.⁷ Pru p 7 belongs to the gibberellin-regulated protein (GRP) family and is similar in size and charge to peach allergen Pru p 3, a non-specific lipid transfer protein (nsLTP), which may previously have caused Pru p 7 to be overlooked. Allergens homologous to Pru p 7 have thereafter been described also in other fruits such as apricot, pomegranate, orange and cherry.⁸⁻¹¹ A strong association between GRP-related food allergy and Cupressaceae pollinosis sensitization has been noted in Japan and Europe,^{10, 12-16} thus defining a novel pollen-food allergy syndrome (PFAS).

Less information is available regarding the pollen part of the Cupressaceae-related PFAS. A 14 kDa allergen isolated from *C. sempervirens* pollen, referred to as BP14, has been shown to share a 13 amino acid stretch of sequence similarity with Pru p 7 and was implicated as a cypress pollen homologue of food GRPs.¹⁵⁻¹⁷ More recently, a *C. sempervirens* pollen protein with an apparent molecular weight of 9.5 kDa in

SDS-PAGE but a mass of 6821.88 Da by mass spectrometry (MS) was isolated and shown to contain two 16-amino acid sequence segments related to Pru p 7,¹⁸ partially overlapping with the previously reported sequence segment of BP14 from *C. sempervirens*.

In this study, we set out to isolate and fully characterize pollen homologues of food GRPs from three representative Cupressaceae species and to investigate the allergenic relationship between the pollen and peach GRP allergens.

METHODS

Patients

Sera from 54 cypress pollen sensitized subjects (41% males, median age 25 years, range 5-65 years), diagnosed with peach allergy as previously described¹³ (Table S1), and 88 consecutive cypress pollinosis subjects, unselected with respect to peach allergy, (52% males, median age 36 years, range 5-78 years) from southern France were included in the study. In both study groups, sensitization to peach and cypress pollen was assessed by SPT (Stallergenes, Antony, France), performed according to the consensus recommendation,¹⁹ and by specific IgE (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden). The study was conducted under approval 2018-003 of the local ethics committee of IHU Méditerranée Infection, Marseille.

Purification of natural Pru p 7 and Pru p 7 related pollen proteins

Pru p 7 was purified from homogenized canned peaches by extraction in 130 mM sodium acetate pH 4.5, clarification by centrifugation and filtration, followed by cation exchange chromatography (CIEX) on a 5 mL SP Sepharose FF HiTrap column and size exclusion chromatography (SEC) on a 120 mL Superdex 75 preparation grade (pg) column. In order to remove any residual amount of Pru p 3, which has a similar size and isoelectric point as Pru p 7, a biospecific affinity adsorption step was applied using an in-house anti-Pru p 3 monoclonal antibody coupled to a 5 mL NHS-activated Sepharose HP HiTrap column, followed by a final step of reversed phase chromatography on a 2 mL Source 15 RPC column. Purity, homogeneity and identity of the nPru p 7 preparation was verified biochemically and immunologically as described.¹³ A polyclonal antiserum against Pru p 7 was generated by immunization of a rabbit (Agrisera, Umeå, Sweden) with the purified protein. Pollen of *C. sempervirens*, *J. ashei* and *C. japonica* (Allergon, Vålinge, Sweden) was extracted in 50 mM sodium acetate pH 4.5 containing 1 M NaCl, clarified by centrifugation and filtration and desalted to 50 mM sodium acetate pH 4.5. The extracts were subjected to CIEX on a 5 mL SP Sepharose FF HiTrap column and selected fractions were assayed for rabbit anti-Pru p 7 IgG binding activity by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden) and analysed by SDS-PAGE of reduced samples. Fractions with the highest binding of anti-Pru p 7 IgG were pooled, concentrated and further purified by SEC on a 120 mL Superdex 30 pg column. Selected fractions were again analysed as above and those with the highest binding of anti-Pru p 7 IgG and absence of other protein bands were pooled as a final preparation for further analyses. All chromatography media, columns and equipment were obtained from GE Healthcare Life Sciences (Uppsala, Sweden).

Expression and purification of recombinant allergens

Synthetic genes designed to encode Pru p 7 and Cup s 7 were cloned between the Eco RI and Sal I sites of expression vector pPICZ α A, such that the recombinant proteins would carry two vector-derived amino acid residues (EF) on the N-terminus following *in vivo* processing by Kex2 and Ste13 and no addition on the C-terminus. The constructs were transformed into *Pichia pastoris* strain X-33 and the resulting strains grown and induced to produce recombinant protein in a 3-litre bioreactor (Belach Bioteknik, Skogås, Sweden). Recombinant Cup s 7 was purified from clarified culture medium by CIEEX on a 24 mL SP Sepharose FF column, followed by SEC on a 120 mL Superdex 30 pg column. Recombinant Pru p 7 was purified by essentially the same procedure, using a 28 mL SP Sepharose FF column and a 505 mL Superdex 75 column. Purity and identity of the recombinant allergen preparations were verified by SDS-PAGE and mass spectrometry.

Mass spectrometry, circular dichroism spectroscopy and N-terminal sequencing

High-resolution, accurate-mass (HRAM) spectrometry analysis was performed using an Orbitrap Fusion Tribrid (Thermo Fisher Scientific, San Jose, CA). Prior to the MS analysis, the protein was reduced by DTT, alkylated with acrylamide and enzymatically cleaved with either trypsin, chymotrypsin or Lys-C. MS data analysis was made on a combination of the spectra obtained from the three digests. Higher-energy collisional dissociation (HCD) and electron-transfer dissociation (ETD) fragmentation spectra were analyzed with PEAKS Studio software (Bioinformatics Solutions Inc., Ontario, Canada). MS analysis of uncleaved, reduced and alkylated protein was also performed in order to determine its molecular mass. Secondary structure content of natural and recombinant GRP was assessed by circular dichroism (CD) spectroscopy using a JASCO J-810 spectropolarimeter (JASCO International Co. Ltd., Tokyo, Japan), with spectra recorded essentially as described²⁰ and presented as mean molar ellipticity per residue. N-terminal sequencing by Edman degradation was performed as previously described.²¹

Antibody measurements and IgE competition experiments

Proteins were immobilized to ImmunoCAP solid phase as described previously.²² Specific IgE antibody measurements were performed on the ImmunoCAP assay platform (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions. Specific IgE values ≥ 0.10 kU_A/L were considered

positive. Rabbit IgG antibodies were detected with the same assay platform, using an in-house goat anti-rabbit IgG/ β -galactosidase conjugate. IgE cross-reactivity between Pru p 7 and Cup s 7 was studied by preincubating four Pru p 7 reactive serum samples for 2 hours at room temperature either with the purified natural or recombinant pollen protein, or with rPru p 7, at a final concentration of 20 μ g/mL, or with sample diluent (Thermo Fisher Scientific, Uppsala, Sweden) serving as a negative control. The samples were then analyzed for IgE binding to rPru p 7 or rCup s 7 immobilized on ImmunoCAP solid phase. The level of inhibition of IgE binding to the solid phase protein by the fluid phase inhibitor was calculated as percentage of the dilution buffer control. Paired IgE values were statistically compared using the Wilcoxon signed-rank test.

RESULTS

Isolation and IgE binding activity of GRP allergens from pollen of three Cupressaceae species

With the aim of purifying a protein homologous to Pru p 7, extracts of *C. sempervirens*, *J. ashei* and *C. japonica* pollen were fractionated by CIEX and SEC. After each step, selected fractions were screened for recognition by a polyclonal rabbit anti-Pru p 7 IgG and by SDS-PAGE. Figure 1 shows the outcome for the *C. sempervirens* as an example. In the CIEX step, a complex elution profile was obtained with a clear rabbit IgG binding activity occurring in fractions from the late part of the salt gradient (Figure 1a). In the SDS-PAGE analysis, the same fractions were found to contain a prominent protein band about 7 kDa in size. Based on the rabbit IgG binding results and electrophoretic appearance, fractions indicated by a horizontal bar in Figure 1a were pooled for further purification by SEC (Figure 1c). Again, selected fractions were screened for antibody binding which clearly identified the central major peak as containing the protein of interest. Fractions containing this peak were pooled as indicated by a horizontal bar and represented the final preparation used for biochemical and immunological analysis.

The same procedures were used for purification of the corresponding protein from *J. ashei* and *C. japonica* pollen, with principally equivalent outcome. The yield of the three pollen allergens was 35-125 µg per gram of pollen. Figure 1d shows an SDS-PAGE analysis of the final preparations from *C. sempervirens*, *J. ashei* and *C. japonica*, together with rPru p 7 for comparison. The three pollen GRP allergens have been officially recognized by the WHO/IUIS Allergen Nomenclature Committee and named Cup s 7, Jun a 7 and Cry j 7, respectively.

IgE binding activity of the three purified Cupressaceae pollen proteins was assessed using experimental ImmunoCAP tests and sera from 16 peach allergic subjects sensitized to cypress pollen. Levels of IgE to Cup s 7 of up to 80 kU_A/L was observed among the sera analysed (median level 32 kU_A/L) and the levels were highly correlated ($r=0.98$) to those against Jun a 7, with a trend towards slightly higher binding to nCup s 7 (Figure 2a). IgE binding to nCup a 7 and nCry j 7 was also strongly correlated ($r=0.85$) but with noticeably lower levels to Cry j 7 (Figure 2b).

Sequence determination of Cup s 7, Jun a 7 and Cry j 7 by mass spectrometry

To establish the amino acid sequences of Cup s 7, Jun a 7 and Cry j 7, proteolytic digests of the purified proteins were subjected to MS/MS analysis and the resulting spectra were matched against a Cupressaceae EST database. For Cup s 7, the best although imperfect match in this database was an EST sequence from male strobilus of *C. japonica* (Acc No BY878079). Peptides aligning with this translated EST

sequence are shown in Figure 3a. It is notable that EST sequence BY878079 contained a stop codon at nucleotide position 302-304, corresponding to position 87 in the translated amino acid sequence, while Cup s 7 derived peptides aligned on either side of this position. This observation raised the possibility that the stop codon was the result of a sequencing error. Indeed, if the A in that TGA stop codon was changed to either a T or a C, it would instead encode a cysteine residue, matching Pru p 7 as well as Cup s 7 peptides at the corresponding position. By further analysis of *de novo* peptides, a 63-residue amino acid sequence of Cup s 7 could be assembled (Figure 3b). Peptides having a highly significant match to the MS/MS spectra could be identified along the entire 63 amino acid sequence (Table S2). The MS/MS analysis further identified polymorphisms at two positions: position 18 (Ala/Leu) and 52 (Asn/His) (Figure 3b, Table S3). N-terminal sequencing by Edman degradation of purified Cup s 7 identified the first four amino acid residues of the protein as Ala-Gln-Ile-Asp, consistent with the N-terminal part of Cup s 7 peptide 1 (Table S2) identified by MS/MS. No peptide match to the first 54 amino acid residues of the translated BY878079 sequence was found. The initial 24 residues were predicted by SignalP (www.cbs.dtu.dk/services/SignalP/) to form a signal peptide while the following 30 residues may represent a propeptide that is removed during protein maturation.

Further corroboration of the Cup s 7 amino acid sequence was obtained by MS analysis of uncleaved protein, performed after reduction and alkylation of the sample, to determine its molecular mass. The analysis revealed a dominant peak at $m/z = 7682.43$, corresponding to a mass of 6828.98 Da of the unmodified protein with all cysteine residues reduced. This is in exact agreement with the monoisotopic molecular mass calculated for the main sequence shown in Figure 3b. The Cup s 7 sequence displayed 68 % identity to the sequence of Pru p 7 and an alignment of them is shown in Figure 3c.

MS/MS analysis of Jun a 7 followed the same path as that for Cup s 7. Again, the best but imperfect match of MS/MS data was to EST sequence BY878079 and the amended version of that sequence, with the erroneous stop codon removed, was used for the analysis of MS spectra. Further analysis of *de novo* peptides, a 63-residue amino acid sequence of Jun a 7 could be assembled. Peptides having a highly significant match to the MS/MS spectra were identified along the entire 63 amino acid sequence (Table S4). No sequence polymorphisms were identified in Jun a 7. MS analysis of uncleaved protein, performed as described above, revealed a molecular mass of 6828.03 Da which is in exact agreement with the monoisotopic mass calculated for the Jun a 7 sequence with all cysteine residues reduced. The Jun a 7 sequence was 98% identical to that of Cup s 7 sequence and 67% identical to that of Pru p 7.

The MS/MS analysis of purified Cry j 7 resulted in a perfect match with a predicted open reading frame of *C. japonica* EST sequence BY900480, with peptides having a highly significant match to the MS/MS spectra

along the entire 63 amino acid sequence (Table S5). No sequence polymorphisms were identified in Cry j 7. MS analysis of uncleaved protein, performed as described above, revealed a molecular mass of 6894.95 Da, in exact agreement with the monoisotopic mass calculated for the Jun a 7 sequence with all cysteine residues reduced. The sequence was 94% identical to Cup s 7 and 68% identical to Pru p 7. An alignment of the three Cupressaceae sequences is shown in Figure 3d.

The Cup s 7, Jun a 7 and Cry j 7 sequences reported in this paper will appear in the UniProt Knowledgebase under the accession numbers C0HLQ2, C0HLQ0 and C0HLQ1, respectively.

Recombinant production and characterisation of Cup s 7

To enable studies of greater numbers of subjects, Cup s 7 was produced as a recombinant protein using a synthetic gene encoding the newly determined main sequence (with Ala at position 18 and Asn at position 52) of the protein. Expression in *Pichia pastoris* resulted in a soluble protein secreted into the medium from which it was purified by CIEEX and SEC. The yield of rCup s 7 was 77 mg per litre of culture medium. Recombinant Cup s 7 (rCup s 7), nCup s 7 and rPru p 7 demonstrated similar electrophoretic appearance on SDS PAGE (Figure 4a), with an apparent molecular weight of 7 kDa, and similar gel filtration behaviour (Figure 4b). CD spectroscopic analysis of natural and recombinant Cup s 7 gave rise to similar spectra (Figure 4c), with minima at 208 and 222 nm characteristic of predominantly α -helical structures, indicating similar secondary structure content and folding of the two proteins.

To assess the immunological activity and authenticity of the rCup s 7 produced, its IgE binding capacity was compared to that of natural Cup s 7 using sera from 47 of the peach allergic subjects (Figure 4d). The comparison revealed highly correlated ($r=0.96$) and comparable levels of IgE binding to the natural and recombinant proteins (median levels 17.5 and 16.5 kU_A/L, respectively).

Cross-reactivity between Cup s 7 and Pru p 7

The immunological relationship between Cup s 7 and Pru p 7 with respect to IgE antibody binding was investigated using sera from the 54 peach allergic subjects (Figure 5a), 51 of which (94%) were sensitized to Pru p 7. The levels of IgE binding to rCup s 7 and rPru p 7 among these sera were clearly correlated ($r=0.60$) but significantly higher to nCup s 7 ($p < 0.0001$, median level 14.6 kU_A/L vs 6.1 kU_A/L).

The representation of epitopes for IgE antibodies on Cup s 7 and Pru p 7 was studied in a reciprocal IgE inhibition experiment using sera from four peach allergic subjects. Blockage of IgE binding to immobilized

rPru p 7 or rCup s 7 by serum preincubation with either natural or recombinant Cup s 7 or with rPru p 7 was measured by ImmunoCAP and the results are shown in Figure 5b as percentage inhibition in relation to serum samples preincubated with buffer alone. IgE binding to rPru p 7 was essentially outcompeted by both natural and recombinant Cup s 7 in all four sera while rPru p 7 only caused modest inhibition of IgE binding to rCup s 7. In a separate control experiment, it was demonstrated that nCup s 7 exerted no inhibitory effect on IgE binding to the unrelated birch pollen allergen Bet v 1 (not shown).

Cup s 7 sensitization among subjects with cypress pollinosis

The prevalence and magnitude of IgE antibody binding to Cup s 7 among sera of 88 cypress pollen sensitized subjects was analysed by ImmunoCAP (Figure 6). Of these sera, 28 (32%) had a detectable IgE response (≥ 0.10 kU_A/L) to nCup s 7. In thirteen (15%) of the sera, similar levels of IgE to nCup s 7 and cypress pollen extract was observed, indicating a dominant role of Cup s 7 in cypress pollen sensitization in this minor subset of subjects.

DISCUSSION

Food allergy is a major health concern across the world. While primary food allergy is considered to carry the highest risk of severe reactions, a significant proportion of food allergies are due to pollen sensitization.⁶ In PFAS, primary sensitization to pollen proteins elicits IgE antibodies that cross-react with similar proteins present in plant-derived food items, potentially causing symptoms on exposure to such foods.⁶ Whereas several examples of PFAS are well-recognized, a relatively newly described food allergy, characterized by sensitization to peach allergen Pru p 7⁷ and homologous GRPs in other foods,^{8, 10-12} has only recently been shown to be strongly and unidirectionally associated with sensitization to Cupressaceae pollen.¹³ In this particular kind of PFAS, symptoms to peach and other fruits are sometimes more severe than in other pollen associated food allergies.

While an allergen homologous and cross-reactive with fruit GRPs would be predicted to exist in Cupressaceae pollen, only fragmentary and in part conflicting published information is so far available. In this study we set out to purify and characterize such pollen GRPs from three different Cupressaceae species and report here the purification and complete sequence determination by mass spectrometry of pollen counterparts of Pru p 7 from Mediterranean cypress (*Cupressus sempervirens*), mountain juniper (*Juniperus ashei*) and Japanese cedar (*Cryptomeria japonica*). The molecular weight obtained through mass spectrometry was in exact agreement with that inferred from the sequence. Like Pru p 7, all three pollen GRPs studied comprised a length of 63 amino acid residues including 12 conserved cysteine residues. Engagement of these in six disulfide bridges provides the basis for a compact and robust hairpin-like structure of members of this protein family such as Pru p 7.²³ Further, the pollen GRPs were highly similar to each other (94-98% sequence identity), had predicted monoisotopic weights and isoelectric points of 6828-6895 Da and 8.3-8.6, respectively. Their level of sequence identity to Pru p 7 was significantly lower, 67-68%, than between each other. Partial peptide sequences of GRP-related cypress pollen proteins have previously been reported. A 13-amino acid sequence from a 14 kDa protein referred to as BP14¹⁶ matches exactly positions 21-33 of our Cup s 7 sequence, and two recently reported 16-amino acid sequences from cypress pollen protein Cypmaclein¹⁸ match exactly positions 1-16 and 25-40 of our sequence. The reported molecular mass of Cypmaclein, 6821.88 Da, is close but not identical to the mass of nCup s 7 (6828.98 Da) that we determined by MS and deduced from the amino acid sequence.

The three purified pollen proteins also appeared immunologically similar, as expected, in regard to IgE antibody binding capacity even if Cry j 7 showed somewhat lower levels in comparison to the Cup s 7 and Jun a 7 in this study. The latter observation may reflect a lack of primary sensitization to *C. japonica* pollen among the patients whose sera were used in the analysis.

To formally verify the correct molecular identification and rule out any influence on the immunological data by other pollen allergen(s) potentially contaminating the GRP preparations, Cup s 7 was generated as a recombinant protein for comparison with the purified natural protein. Recombinant and natural Cup s 7 appeared essentially indistinguishable in SDS-PAGE and analytical gel filtration and showed similar CD spectra. The two forms of the protein also displayed very similar IgE binding capacity, both as solid phase reagents and as fluid phase inhibitors of IgE binding to Pru p 7. Taken together, the data provide evidence for structural and immunological authenticity of the recombinant Cup s 7 generated in this study.

Cross-reactivity between Pru p 7 and cypress pollen allergens has been previously investigated. In one study, Senechal and coworkers demonstrated by an immunoblot experiment with one patient serum inhibition of IgE binding to a 14 kDa band referred to as BP14 in cypress pollen extract by recombinant Pru p 7.²⁴ In a more recent study, Tuppo and coworkers used a microarray assay to show correlation in IgE binding between Pru p 7 and purified Cypmaclein (Cup s 7) among 31 patients sera and cross-reactivity by comparative inhibition of IgE binding to cypress pollen extract and Pru p 7 with the same proteins as inhibitors in five patient sera.¹⁸ Interestingly, when we compared the IgE binding capacity of rCup s 7 and rPru p 7 in sera of our peach allergic population using the ImmunoCAP assay platform, substantially higher binding to the pollen allergen was observed. Preferential IgE binding to Cup s 7 was even more conspicuous in the reciprocal inhibition experiment where Cup s 7 completely outcompeted IgE binding to Pru p 7 while only modest inhibition of IgE binding to Cup s 7 by Pru p 7 occurred, demonstrating unique IgE recognition of determinants displayed by the pollen allergen but not by Pru p 7 among the patient sera used in this experiment. While one could *a priori* not exclude the possibility that peach (or other foods containing an allergen similar to Pru p 7) could act as a primary sensitizer, inducing IgE antibodies cross-reacting with homologous pollen allergens, the relative IgE binding intensity of Cup s 7 and Pru p 7 and their relative efficiency in competing for IgE antibody binding reported here and previously are not consistent with that directionality of sensitization. Instead, the results suggest that Cupressaceae pollen acts as the predominant primary sensitizer, eliciting an IgE response directed to both pollen and food GRP allergens, including Pru p 7.

A molecular analysis of sensitization among 88 cypress pollinosis patients, unselected with respect to peach allergy, showed that only one third of these individuals had detectable IgE to Cup s 7 while a two thirds majority lacked sensitization to this allergen. This finding reveals that Cup s 7 is a minor allergen in cypress pollen and suggests that an identifiable subgroup of cypress sensitized patients will be at risk of allergic reactions to peach or other GRP-containing foods. Considering the substantial absolute number of individuals comprising this subgroup in areas of high Cupressaceae pollen exposure and the potential

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severity of GRP-mediated food allergic reactions, identification of those with GRP sensitization would be a valuable step of risk reduction in the management of this patient group. To this end, generation of a fully immunoreactive recombinant Cup s 7 suitable as a reagent for *in vitro* diagnostic use is a first important step.

In summary, we describe in this report the isolation and molecular characterization of GRP allergens from pollen of three Cupressaceae tree species, the generation of a fully immunoreactive recombinant Cup s 7 allergen suitable for in vitro diagnostic use and present immunological evidence that Cupressaceae pollen exposure is a likely predominant driver of sensitization leading to GRP-mediated food allergy.

ACKNOWLEDGEMENTS

The authors wish to thank Ms Colette Mavier for logistic support and Ms Isabelle Cleach for technical assistance.

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FIGURE LEGENDS

Figure 1. Purification of Pru p 7 related protein from *C. sempervirens* by CIEEX (a-b) and SEC (c). Each chromatogram shows absorbance at 280 nm (solid line) and level of anti-Pru p 7 rabbit IgG binding by selected fractions (hatched line). Horizontal bars indicate fractions pooled for further purification (a) or characterization (c). SDS-PAGE of the selected fractions analysed for anti-Pru p 7 IgG binding activity (b). In panel d, SDS-PAGE of the pooled fractions indicated in panel c (lane 1), the corresponding preparations from *J. ashei* (lane 2) and *C. japonica* (lane 3) pollen and rPru p 7 (lane 4) are shown. Molecular weights of marker proteins (lane M) are indicated to the right in panels b and d.

Figure 2. Pairwise comparison of IgE antibody binding to nJun a 7 and nCup s 7 (a) and nCry j 7 and nCup s 7 (b) among 16 peach allergic subjects sensitized to cypress pollen. Hatched diagonal lines indicate 1:1 slope.

Figure 3. Amino acid sequence determination of nCup s 7 by MS/MS (a-b) and comparisons with Pru p 7 (c), Jun a 7 and Cry j 7 (d). In panel a, four peptides (Pep 1-4) identified by MS/MS are aligned with an unedited translation of EST record BY878079. A predicted signal peptide is underlined and stop codons are represented by asterisks. In panel b, the amino acid sequence of Cup s 7 determined by MS/MS is shown, with amino acids deviating from the sequence encoded by BY878079 underlined and alternative amino acids identified at two polymorphic sites indicated above the sequence. In panel c, an alignment of the amino acid sequences of Cup s 7 and Pru p 7 is shown. Vertical lines, colons and periods indicate identical, conserved and semiconserved positions, respectively. Panel d shows an alignment of the amino acid sequences of Cup s 7, Jun a 7 and Cry j 7. Below the sequences, a consensus line is shown with asterisks indicating invariant amino acid residues.

Figure 4. Comparison of natural and recombinant Cup s 7. SDS-PAGE of recombinant (lane 1) and natural (lane 2) Cup s 7 are shown alongside recombinant Pru p 7 (lane 3), with molecular weights of marker proteins (lane M) indicated to the right (a). Analytical gel filtration of natural (solid line) and recombinant (hatched line) Cup s 7 (b). CD spectroscopy spectra of natural (solid line) and recombinant (hatched line) Cup s 7 (d). IgE antibody binding by natural (x-axis) and recombinant (y-axis) Cup s 7 among 47 peach allergic subjects sensitized to cypress pollen, expressed as IgE concentration in kU_A/L. Hatched diagonal line indicates 1:1 slope.

Figure 5. Comparison of IgE antibody binding to nCup s 7 and rPru p 7 among sera of 54 peach allergic subjects sensitized to cypress pollen (a) and analysis of cross-reactivity by IgE inhibition (b). In (a), the 1:1 slope is indicated by a hatched diagonal line. In (b), inhibition of IgE binding to rPru p 7 or rCup s 7 in four patient sera (subjects #17, 42, 45 and 49 in Table S1) by natural and recombinant Cup s 7 or by rPru p 7, respectively, shown as percent of a buffer control.

Figure 6. Prevalence and level of IgE antibodies to nCup s 7 and cypress pollen extract among 88 subjects sensitized to cypress pollen. Hatched vertical and horizontal lines indicate the 0.1 kU_A/L level and a hatched diagonal line indicates the 1:1 slope. To visualize all observations, all values below 0.1 kU_A/L were set to 0.03 kU_A/L.

Figure 1

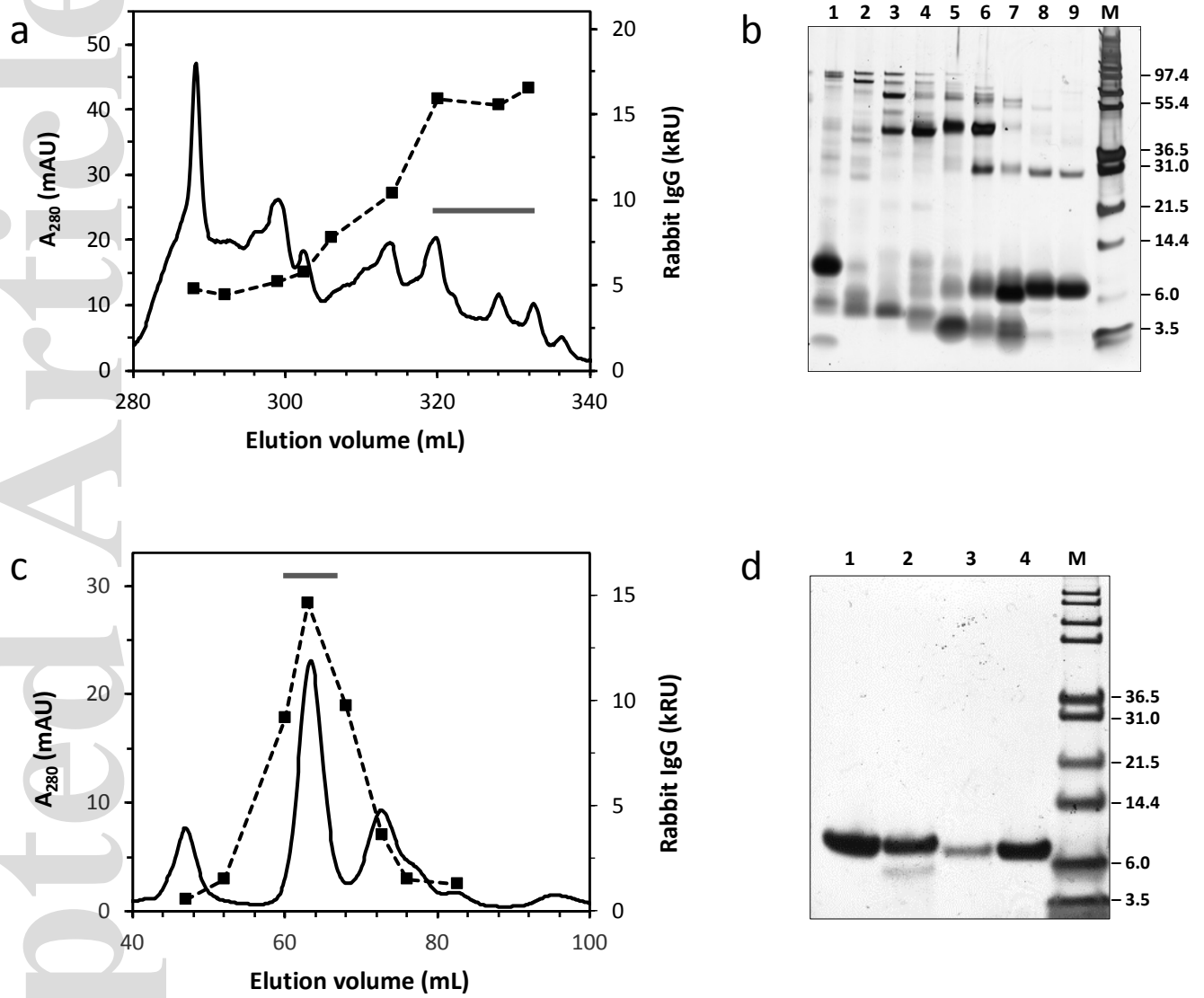


Figure 2

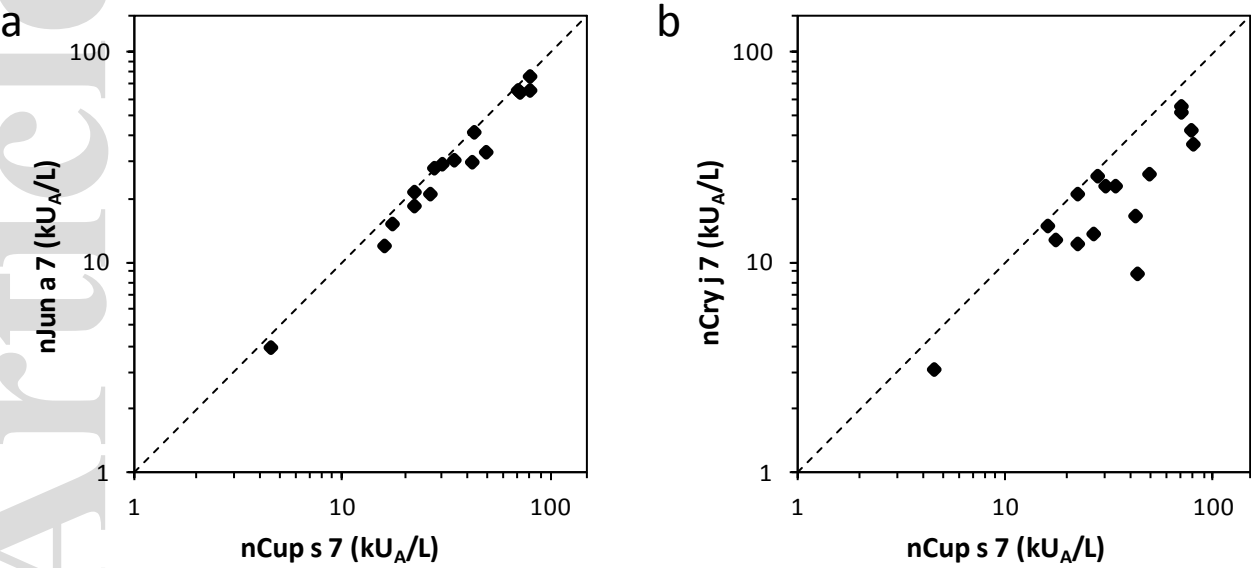


Figure 3



Figure 4

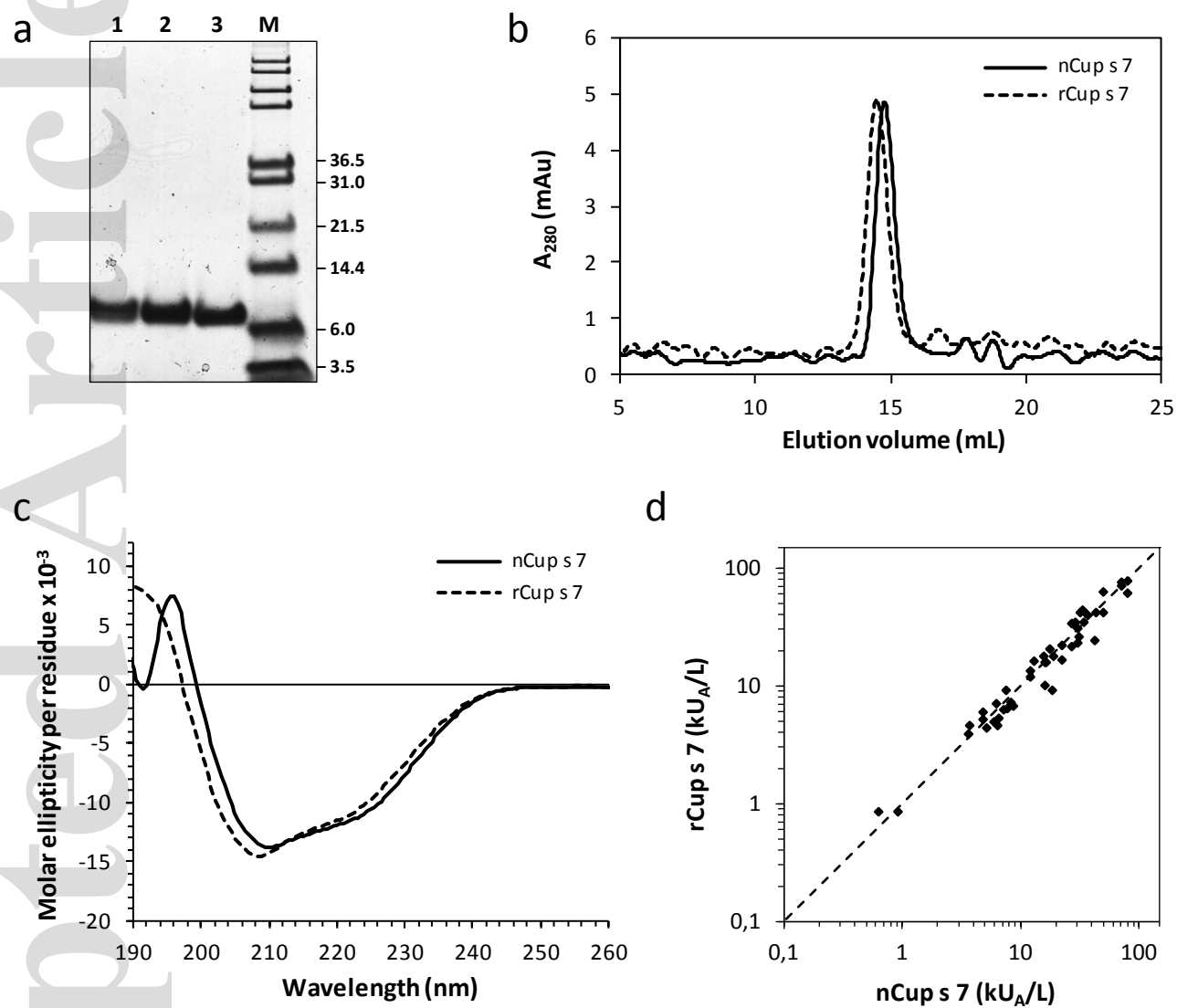


Figure 5

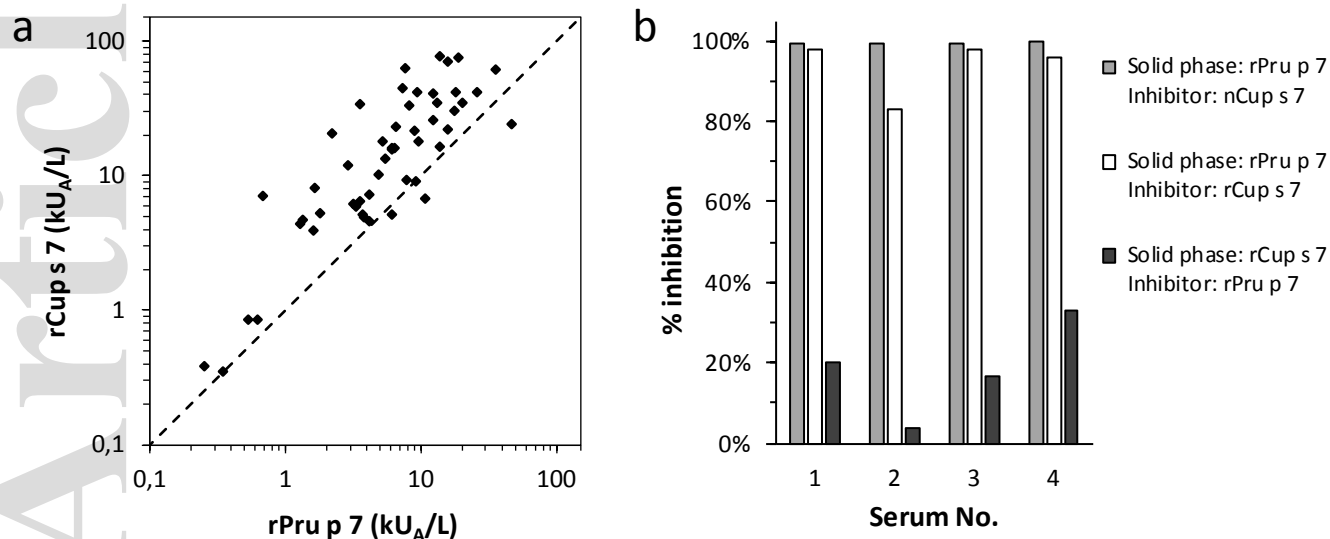
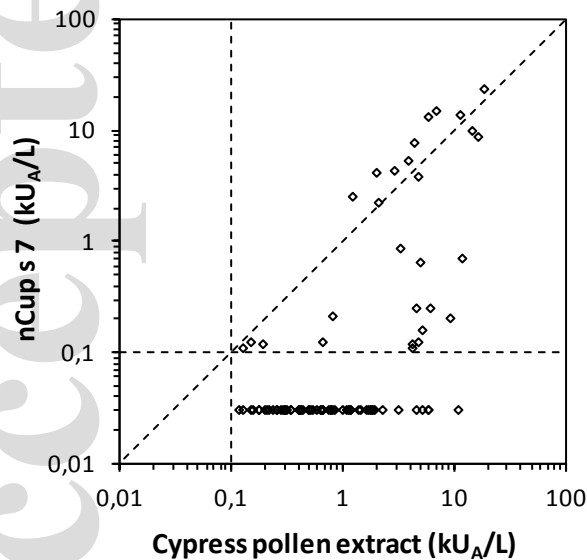


Figure 6



Characterization of a 7 kDa pollen allergen belonging to the gibberellin-regulated protein family from three Cupressaceae species

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Online Supporting Information: Tables S1-S5

Table S1. Peach allergic patients: demographic, clinical and sensitization data.

Subject No.	Sex	Age	Symptom severity grade ¹	Specific IgE ²										
				Cypress pollen	Peach	rPru p 1	rPru p 3	rPru p 4	rPru p 7	CCD (MUXF3)	nCup s 7	rCup s 7	nJun a 7	nCry j 7
1	F	16	3	11,7	0,56	<0,10	<0,10	<0,10	4,96	<0,10	16,0	10,1	nd	nd
2	M	27	3	5,48	0,44	<0,10	<0,10	<0,10	1,83	<0,10	6,57	5,31	nd	nd
3	M	43	3	12,0	3,75	<0,10	<0,10	<0,10	9,59	0,37	18,9	17,8	nd	nd
4	M	35	3	15,2	3,88	43,7	<0,10	<0,10	6,19	<0,10	nd	5,09	nd	nd
5	F	61	3	10,3	1,35	<0,10	<0,10	<0,10	2,90	<0,10	12,1	11,8	nd	nd
6	F	23	2	2,81	5,56	23,2	0,21	1,70	<0,10	<0,10	nd	<0,10	nd	nd
7	F	32	1	18,1	2,31	<0,10	<0,10	<0,10	3,56	<0,10	26,6	33,6	nd	nd
8	M	29	2	7,79	3,66	9,90	<0,10	<0,10	<0,10	<0,10	nd	<0,10	nd	nd
9	F	15	3	20,7	0,11	<0,10	<0,10	<0,10	2,25	<0,10	17,5	20,6	15,3	12,8
10	M	15	3	21,3	0,82	<0,10	<0,10	5,68	7,36	<0,10	33,6	44,3	nd	nd
11	F	5	1	5,27	12,2	0,26	4,86	<0,10	10,8	1,12	8,63	6,78	nd	nd
12	F	21	3	3,18	0,43	<0,10	<0,10	<0,10	3,34	<0,10	4,84	5,93	3,90	3,07
13	M	11	1	17,5	52,4	9,23	56,6	2,98	0,25	2,71	nd	0,38	nd	nd
14	M	12	3	20,6	1,23	<0,10	<0,10	0,10	6,67	0,13	30,5	23,0	nd	nd
15	M	38	2	5,28	0,23	<0,10	<0,10	<0,10	3,23	<0,10	7,12	6,21	nd	nd
16	M	30	3	14,6	3,38	2,96	<0,10	<0,10	7,92	<0,10	18,5	9,19	nd	nd
17	M	13	3	25,4	1,49	<0,10	<0,10	<0,10	12,5	<0,10	31,2	26,1	nd	nd
18	M	41	1	20,4	1,84	<0,10	<0,10	<0,10	13,2	<0,10	29,1	34,9	nd	nd
19	F	14	1	38,3	8,27	62,0	<0,10	<0,10	7,71	<0,10	49,9	63,2	nd	nd
20	F	21	3	14,8	1,35	<0,10	0,23	<0,10	5,55	0,11	12,2	13,4	nd	nd
21	M	11	1, 2	24,9	43,7	41,6	23,7	0,23	0,35	1,76	nd	0,35	nd	nd
22	F	12	2	5,09	3,64	<0,10	8,46	<0,10	<0,10	<0,10	nd	<0,10	nd	nd
23	F	51	3	9,04	1,43	<0,10	<0,10	<0,10	9,33	<0,10	7,55	9,07	nd	nd
24	M	20	3	4,04	0,19	<0,10	<0,10	<0,10	1,62	<0,10	3,65	3,91	nd	nd
25	F	49	3	3,80	<0,10	<0,10	<0,10	<0,10	1,31	<0,10	5,13	4,43	nd	nd
26	F	39	3	4,76	0,64	<0,10	<0,10	<0,10	6,12	<0,10	12,9	16,2	nd	nd
27	F	40	2	2,68	0,69	<0,10	<0,10	<0,10	1,36	<0,10	3,74	4,64	nd	nd
28	F	52	1, 2, 3	4,52	0,33	<0,10	0,12	<0,10	3,73	<0,10	4,81	5,17	nd	nd
29	F	21	3	31,6	5,39	<0,10	1,11	0,97	12,5	0,34	35,8	40,6	nd	nd
30	F	41	3	3,17	0,24	<0,10	<0,10	<0,10	4,24	<0,10	8,34	7,21	nd	nd
31	F	30	3	10,5	4,60	<0,10	<0,10	<0,10	6,38	<0,10	16,4	16,0	nd	nd
32	M	11	1	31,0	24,5	16,6	0,28	<0,10	26,1	1,40	31,8	41,3	nd	nd
33	M	51	3	46,0	3,78	<0,10	<0,10	<0,10	8,21	<0,10	28,0	33,3	27,9	25,9
34	F	9	1	0,94	15,6	1,75	20,2	0,30	0,54	0,39	0,62	0,85	nd	nd
35	M	38	3	9,74	1,36	<0,10	<0,10	1,68	1,66	0,30	nd	8,17	nd	nd
36	F	19	3	6,96	0,43	<0,10	<0,10	<0,10	3,57	<0,10	7,75	6,39	nd	nd
37	F	18	3	12,8	<0,10	<0,10	<0,10	<0,10	5,30	<0,10	15,7	17,8	nd	nd
38	F	47	2	1,20	0,21	<0,10	<0,10	<0,10	0,63	<0,10	0,93	0,86	nd	nd
39	M	14	1, 2	5,77	0,62	<0,10	<0,10	0,25	4,19	0,14	6,44	4,55	nd	nd
40	F	36	3	4,13	0,43	<0,10	<0,10	<0,10	3,81	<0,10	6,00	4,90	nd	nd
41	F	45	3	4,13	<0,10	<0,10	<0,10	<0,10	0,70	<0,10	6,16	7,08	nd	nd
42	M	38	3	23,4	0,46	<0,10	<0,10	<0,10	13,9	<0,10	22,2	16,5	18,6	12,2
43	F	16	1	56,6	0,46	<0,10	<0,10	<0,10	14,1	0,23	79,3	77,4	76,3	42,3
44	M	38	3	49,8	1,38	<0,10	<0,10	<0,10	35,9	<0,10	80,2	60,9	65,0	36,6
45	F	41	2, 3	43,1	0,87	<0,10	<0,10	<0,10	18,2	<0,10	49,3	41,3	33,2	26,5
46	F	20	3	20,8	1,25	<0,10	0,42	0,31	16,1	0,14	22,2	22,1	21,8	21,1
47	F	65	1, 2, 3	32,1	0,85	<0,10	<0,10	<0,10	20,4	<0,10	34,3	34,5	30,5	23,2
48	F	10	2	29,0	1,45	<0,10	<0,10	<0,10	9,54	<0,10	42,9	41,4	41,0	8,87
49	M	43	2	20,8	0,85	<0,10	<0,10	<0,10	9,01	0,30	26,6	21,6	21,0	13,5
50	F	14	3	26,9	0,26	<0,10	<0,10	<0,10	17,8	<0,10	30,6	30,5	29,0	23,0
51	M	10	1, 2	32,4	3,74	0,15	0,27	0,24	47,4	1,35	42,1	24,0	30,1	16,4
52	F	32	3	17,8	0,73	<0,10	<0,10	0,21	6,13	<0,10	16,1	15,8	11,8	14,9
53	M	15	2	81,9	0,48	<0,10	<0,10	<0,10	16,1	<0,10	70,3	70,2	65,9	51,5
54	F	14	1, 2	51,9	10,4	0,12	0,37	<0,10	19,2	<0,10	71,0	76,2	63,9	55,1

¹ Severity of symptoms to peach according to a recently proposed classification of food allergic reactions.^{1,2} Grade 1: local reaction at the site of allergen contact: redness, itching, swelling; Grade 2: mild to moderate systemic reactions involving distant skin locations, upper airways and/or gastrointestinal symptoms; Grade 3: severe systemic reactions (anaphylaxis) with with cardiovascular and/or bronchial and laryngeal involvement: wheezing, stridor, objective dyspnoea, tachycardia, lowered blood pressure and shock

² Specific IgE concentrations expressed in kUA/L; nd: not determined

Table S2. List of nCup 7 peptide sequences identified by MS/MS analysis.

Peptide	Start	End	-10lgP	Experimental mass *	Experimental mass - PTM §	Calculated peptide mass	PTM #
AQIDCDKECNRRCSKA	1	16	96	2051,9299	1838,8185	1838,8179	3 propionamide
AQIDCDKECNRRCSKASAH DR	1	21	116	2618,1860	2405,0746	2405,0740	3 propionamide
AQIDCDKECNRRCSKASAH DRCLKY	1	25	119	3196,4746	2912,3261	2912,3256	4 propionamide
ASAHDRCLKYCGICCEK	16	32	200	2182,9744	1898,8259	1898,8253	4 propionamide
SAHDRCLKYCGICCEKC	17	33	83	2285,9836	1930,7980	1930,7974	5 propionamide
CGICCEKCHCVPPGTAGNED VPCPYANL	26	53	112	3395,3994	2898,1396	2898,1389	7 propionamide
CGICCEKCHCVPPGTAGNED VPCPYANLKNSKGGHKCP	26	63	84	4502,9600	3934,6630	3934,6626	8 propionamide
HCVPPGTAGNEDVPCPYANLKNSKGGHKCP	34	63	96	3379,5317	3095,3832	3095,3827	4 propionamide
CVPPGTAGNEDVPCPYANLKNSKGGHKCP	35	63	107	3242,4729	2958,3244	2958,3238	4 propionamide
DVPCPYANLKNSKGGHKCP	45	63	107	2246,0396	2032,9282	2032,9275	3 propionamide

* Monoisotopic mass (M/Z-1.00794) with PTM (biological or experimentally introduced modifications)

All cysteine residues were modified to propionamide residues prior to MS/MS analysis

§ Monoisotopic mass without PTM

71.03712 Da mass gain per propionamide adduct, 0.984 Da mass loss per amidation

Table S3. List of nCup s 7 isoform variant peptide sequences identified by MS/MS analysis.

Peptide - Amino acid pos 18 L	Start	End	-10lgP	Experimental mass *	Experimental mass - PTM §	Calculated peptide mass ¶	PTM #
SKASLHDRCLKYCGICCE	14	31	112,49	2311,0693	2027,9048	2027,9043	4 propionamide 1 amidation
KASLHDRCLKY	15	25	89,99	1403,7344	1332,6973	1332,6966	1 propionamide
Peptide - Amino acid pos 52 H	Start	End	-10lgP	Experimental mass *	Experimental mass - PTM §	Calculated peptide mass ¶	PTM #
CHCVPPGTAGNEDVPCPYAHLK	33	54	114,11	2597,1284	2312,9799	2312,9792	4 propionamide
DVPCPYAHLK	45	54	112,95	1289,5896	1147,5154	1147,5148	2 propionamide
CVPPGTAGNEDVPCPYAHLKNSKGGHKCP	35	63	93,12	3265,4890	2981,3405	2981,3398	4 propionamide

* Monoisotopic mass (M/Z-1.00794) with PTM (biological or experimentally introduced modifications)

All cysteine residues were modified to propionamide residues prior to MS/MS analysis

§ Monoisotopic mass without PTM

71.03712 Da mass gain per propionamide adduct, 0.984 Da mass loss per amidation

Table S4. List of nJun a 7 peptide sequences identified by MS/MS analysis.

Peptide	Start	End	-10lgP	Experimental mass *	Experimental mass - PTM §	Calculated peptide mass ¶	PTM #
AQIDCDKECNRR	1	12	126	1591,7195	1449,6453	1449,6441	2 propionamide
AQIDCDKECNRRCSKASAH DRCLKY	1	25	100	3196,4746	2912,3261	2912,3251	4 propionamide
SAHDRCLKY	17	25	91	1162,5553	1091,5182	1091,5181	1 propionamide
YCGLCCKK	25	32	66	1129,5083	916,3969	916,3961	3 propionamide
CGLCCKKCHCVPP	26	38	75	1744,7704	1389,5848	1389,5841	5 propionamide
KCHCVPPGTAGNEDVCPCY ANLK	32	54	123	2702,2073	2418,0588	2418,0581	4 propionamide
CHCVPPGTAGNE	33	44	107	1325,5493	1183,4751	1183,4741	2 propionamide
GNEDVCPCYANLK	42	54	129	1566,6807	1424,6065	1424,6061	2 propionamide
DVCPCYANLKNKSGGHKCP	45	63	95	2246,0396	2032,9282	2032,9271	3 propionamide
ANLKNKSGGHKCP	51	63	87	1423,7354	1352,6983	1352,6981	1 propionamide

* Monoisotopic mass (M/Z-1.00794) with PTM (biological or experimentally introduced modifications)

All cysteine residues were modified to propionamide residues prior to MS/MS analysis

§ Monoisotopic mass without PTM

71.03712 Da mass gain per propionamide adduct, 0.984 Da mass loss per amidation

Table S5. List of nCry j 7 peptide sequences identified by MS/MS analysis.

Peptide	Start	End	-10lgP	Experimental mass *	Experimental mass - PTM §	Calculated peptide mass ¶	PTM #
AHIDCDKECNRR	1	11	200	1444,6188	1302,5446	1302,5439	2 propionamide
AHIDCDKECNRRCSKASAH DRCLKY	1	25	83	3205,4751	2921,3266	2921,32586	4 propionamide
ECNRRCSK	8	15	62	1136,5179	994,4437	994,44296	2 propionamide
ASAHDRCLK	16	24	88	1070,5291	999,4920	999,49126	1 propionamide
YCGICCEK	25	32	61	1130,4559	917,3445	917,34386	3 propionamide
CGICCEKCNCVPP	26	38	84	1722,7020	1367,5164	1367,51576	5 propionamide
CNCVPPGTYG	33	42	95	1151,4740	1009,3998	1009,39906	2 propionamide
CNCVPPGTYGNEDESCPCYA NL	33	53	60	2502,9912	2218,8427	2218,84216	4 propionamide
GNEDSCPCYANL	42	53	94	1426,5493	1284,4751	1284,47436	2 propionamide
GNEDSCPCYANLK	42	54	120	1554,6443	1412,5701	1412,56936	2 propionamide
GNEDSCPCYANLKNKSGGH KCP	42	63	40	2534,1101	2320,9987	2320,99807	3 propionamide
DSCPCYANLK	45	54	95	1254,5372	1112,4630	1112,46236	2 propionamide
ANLKNKSGGHKCP	51	63	81	1423,7354	1352,6983	1352,69756	1 propionamide

* Monoisotopic mass (M/Z-1.00794) with PTM (biological or experimentally introduced modifications)

All cysteine residues were modified to propionamide residues prior to MS/MS analysis

§ Monoisotopic mass without PTM

71.03712 Da mass gain per propionamide adduct, 0.984 Da mass loss per amidation

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