SUPPLEMENTARY MATERIAL

METHODS

Purification of cashew nut and hazelnut allergens

Cashew nut (Ana o 1-3) [1] and hazelnut allergens (Cor a 9, 11, and 14) [2, 3] were purified as previously described. Recombinant 2S albumin rAna o 3 was obtained from Indoor Biotechnologies Ltd, Cardiff, UK and rCor a 14 was produced in *Pichia pastoris* using the pPICZαA vector (ThermoFisher Scientific, Waltham, MA, USA) and purified following previously established protocols [4].

Protein identification by Nano-LC ESI Orbitrap MS/MS

Identification of the purified allergens was performed by Nano-LC-ESI Orbitrap MS/MS. Briefly, 5 μg of each protein sample was reduced with 50 mM DTT, alkylated with 200 mM iodoacetamide and digested using Trypsin/LysC Mix (Promega). Peptides (200 ng) were loaded onto a nano-HPLC Ultimate 3000 RSLC system (Dionex). The sample was loaded using 2% acetonitrile in ultra-pure H_2O with 0.05% trifluoroacetic acid as a mobile phase with a flow rate of 5 ml/min. Peptide separation was performed on a 25 cm Acclaim PepMap C18 column. For MS/MS analysis, a high-resolution Q Exactive HF Orbitrap mass spectrometer, which was directly connected to the LC was used. MS1 spectra were collected in the range of 350-2000 m/z for 50 ms. The top 10 intense ions were subjected to Orbitrap for further fragmentation. MS2 spectra were collected in the range 200-2000 m/z for 50 ms. Ions with charge state +1, +7, +8 and >+8 were excluded. Precursor ions were dynamically excluded from reselection for 30 s. Spectra were searched in UniProt/Trembl (downloaded from the publicly available servers (http://www.uniprot.org)).

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Dose-dependent IgE inhibition ELISA assay

Dose-dependent IgE inhibition ELISA assays with the three cashew nut allergens as well as with hazelnut allergens were performed as described previously [5]. The hazelnut allergens Cor a 9, 11, and 14 were used as control allergens for inhibition of IgE binding to cashew nut allergens when using sera of cashew nut allergic patients, and cashew nut allergens Ana o 1-3 were used as control allergens for inhibition of IgE binding to hazelnut allergens when using sera form hazelnut allergic patients. All inhibitions and control inhibitions with Cor a 9 and Ana o 3 were performed at concentrations of 0.01-10 µg/ml. Other control inhibitions were performed with 1 and 10 µg/ml of allergen. IgE inhibitions with rAna o 3, rCor a 14, and rBet v 1 were performed at a concentration of 10 µg/ml.

Reduction and alkylation of Ana o 3, Ana o 2 and Cor a 9

Allergens comprising disulfide bridges Ana o 3, Ana o 2 and Cor a 9 were reduced and alkylated to destroy conformational IgE epitopes. The allergens were incubated with 70 mM dithiothreitol (DTT) at 60°C for 2 hours, followed by 200 mM iodoacetamide (IAA) at room temperature overnight. To inactivate unbound IAA, 50 mM DTT was added, and the samples were incubated for 2 h. Alkylated allergens were dialyzed against PBS, followed by determination of protein concentration.

IgE immunoblot and inhibitions

Samples of cashew nut extract (10 µg) or purified allergens (2 µg) were mixed with SDS sample buffer with (75 mM DTT) or without reducing agent, heated for 5 min at 95 °C and separated by 15% SDS-PAGE. For immunoblotting, the separated proteins were blotted to a nitrocellulose membrane (0.2 µm pore size). After blocking with 5% BSA (w/v) in TBST, membrane strips were incubated over night with pooled sera from cashew nut allergic patients. For inhibition of IgE binding, sera were pre-incubated with 10 µg/ml Ana o 1, Ana o 2, or Ana o 3 before adding them to the membrane. In addition, horseradish peroxidase (HRP)

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was added to the sera at a final concentration of 50 µg/ml to block IgE antibodies directed to cross-reactive carbohydrate determinants (CCDs). As a negative control, normal human serum (NHS) from one donor was used.

Furthermore, to test the purity and cross-reactivity of Ana o 1, Ana o 2, and Ana o 3 an anti-Ana o 3 human IgE monoclonal antibody derived from a patient allergic to cashew was used (Indoor Biotechnologies Ltd, Cardiff, UK). The specificity of the antibody was further tested using the hazelnut allergens (Cora 11, Cor a 9, and Cor a 14) as well as rBet v 1. The 15% SDS-PAGE of cashew and hazelnut allergens (2 µg/line) was performed under reducing and non-reducing conditions and immunoblotting was performed with 10 ng/ml of anti-Ana o 3. For inhibitions of anti-Ana o 3, 1 µg of rAna o 3, Ana o 2, Cor a 9, or Bet v 1 was used.

Sequence comparison

Pairwise percent identity comparisons of the mature amino acid sequences of cashew nut allergens Ana o 1.0101 (UniProt: Q8L5L5, residues 27-538), Ana o 2.0101 (UniProt: Q8GZP6, residues 15-457), Ana o 3.0101 (UniProt: Q8H2B8, residues 21-138), and hazelnut allergens Cor a 9.0101 (UniProt: Q8W1C2, residues 23-515), Cor a 11.0101 (UniProt: Q8S4P9, residues 25-448), Cor a 14.0101 (UniProt: D0PWG2, residues 23-147) were estimated with EMBOSS Needle.

In order to identify sequence similarities confined to short peptides between the non-homologous allergens, the following algorithm was implemented: Each eight-residue peptide of sequence 1 was aligned with each peptide of sequence 2 in an ungapped alignment. Alignments were considered significant if their alignment score exceeded the mean score of all alignments by more than five standard deviations. Finally, these significant alignments were extended to both sides as far as the extension resulted in an increased score.

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