

Received Date : 20-Dec-2015

Revised Date : 12-Feb-2016

Accepted Date : 13-Feb-2016

Article type : Letter to the Editor

IgE to novel citrus seed allergens among cashew allergic children

Josef Brandström^{1,2}, Gunnar Lilja^{1,2}, Caroline Nilsson^{1,2}, Niklas Ingemarsson³, Magnus P Borres^{3, 4},
Peter Brostedt³, Hillevi Englund³

¹Sachs's Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden

²Department of Clinical Science and Education, Karolinska Institutet, Stockholm, Sweden

³R&D, ImmunoDiagnostics, Thermo Fisher Scientific, Uppsala, Sweden

⁴Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden

Running title: Cashew citrus seed cross reactions

Corresponding author: Josef Brandström, Sachs's Children and Youth Hospital, Södersjukhuset,
Stockholm, Sweden, josef.brandstrom@sodersjukhuset.se

Key words: 2S albumin, 7S globulin, 11S globulin, cashew, citrus seeds, food allergy, IgE, storage proteins

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/pai.12553

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Electronic repository: Contains a more detailed material and methods section with a word count of 406.

Abstract

Background: Allergic reactions to lemon and orange seeds have been described among cashew allergic individuals. Here, the objective was to purify and characterize novel potential allergens from citrus seeds and investigate the IgE-reactivity to these allergens. Sera from four cashew allergic cases who had presented with allergic reactions suspected to be caused by citrus seeds were analyzed.

Methods: Proteins from lemon seeds were purified and characterized. Serum samples from four cases of cashew allergic children (2-17 years) who had developed allergic reactions to citrus seeds or foods containing citrus seeds were analyzed by ImmunoCAP for IgE reactivity to the purified lemon seed proteins, cashew and Ana o 3 (2S albumin from cashew). The patients were also subjected to prick-to-prick tests with citrus seeds.

Results: Lemon seeds were demonstrated to contain proteins from the 11S globulin, 7S globulin and 2S albumin families. The children were all sensitized to the three lemon seed storage proteins and had positive skin reactions in prick-to-prick tests with citrus seeds.

Conclusion: The storage proteins 11S globulin, 7S globulin and 2S albumin are “novel” allergens demonstrated in lemon seeds and homologous to the storage proteins in cashew. The suspected serological cross-reactivity between lemon seeds and cashew can probably explain allergic reactions to lemon seeds among cashew allergic individuals.

To the Editor: Allergy to cashew (*Anacardium occidentale*) is increasing and severe reactions are often reported (1, 2). Considering the risk of systemic allergic reactions in cashew allergic individuals, it is of importance to consider cross-reacting allergens from other foods capable to elicit the same

symptoms as well. The *Rutaceae* family (e.g. lemon, tangerine, orange) is botanically closely related to the *Anacardiace* family to which cashew belongs, and cases of cashew allergic individuals reacting to lemon and orange seeds have been described (3, 4). Serological cross-reactivity between lemon seed and cashew has been demonstrated among Greek children (5). In this case series proteins from lemon seeds were purified and characterized biochemically. Identified allergens were utilized for IgE-analysis in four Swedish cashew allergic children who had presented with objective allergic symptoms suspected to be caused by citrus seeds.

Lemon seeds were dried and peeled before seed proteins were extracted into a neutral pH phosphate buffer. Proteins were purified using a two-step approach. First, proteins were separated by gel filtration and then further purified by ion exchange chromatography. Fractions with purified proteins were analyzed with analytical gel filtration and with SDS-PAGE under reducing conditions. Identity of purified proteins was determined using mass spectroscopy (Orbitrap Fusion mass spectrometer, Thermo Scientific, Waltham, Mass., US). For methodological details, see Online Repository.

Three protein fractions from the lemon seed extract were characterized with analytical gel filtration, SDS-PAGE (Fig 1A, B) and mass spectrometry. The protein fraction associated with an 11S globulin profile was in agreement with accession no. gi|567854241, the 7S fraction with accession no. gi|567903090, and the 2S albumin fraction with accession no. gi|568842547. Altogether, mass spectrometry, analytical gel filtration and SDS-PAGE data revealed sizes and protein characteristics corresponding to 2S albumin, 7S globulin and 11S globulin for proteins from lemon seeds.

The four cases in this report (Table 1) were all allergic to cashew and had reactions to citrus seeds (case 2 and 3) or foods contaminated with citrus seeds (case 1 and 4). Apart from their reactions to citrus fruits described, they've all been eating citrus fruits including lemon, both before and after their reactions, without developing any allergic symptoms and were thus considered tolerant to citrus fruit pulp. Informed consent was obtained from patients and caregivers.

Case 1: A 17 year old boy with asthma and allergy to several nuts reacted at age 10 with an anaphylaxis when eating pesto containing cashew. At age 15 he reacted with anaphylaxis with repetitive vomiting, flush and asthma after having a drink containing freshly squeezed carrot, pineapple and lemon juice, symptoms starting within minutes after consumption. He was treated with epinephrine autoinjector, antihistamine and corticosteroids and was admitted to hospital for observation over one night. IgE level to pineapple was 0.1 kU/l and to carrot 0.2 kU/l. The boy eats both fresh pineapple and raw carrots and he has also been eating/drinking lemon/lemon juice both prior to, and after this allergic reaction without having any allergic symptoms. Skin prick test (prick-to-prick test) with lemon seed gave a wheel diameter of 9.5 mm.

Case 2: A 16 year old girl with birch pollen induced asthma and allergic rhino-conjunctivitis had a systemic allergic reaction to cashew at 2 years of age and at age 15 she reacted with anaphylaxis when eating pesto containing cashew. As the patient started to avoid more and more foods due to anxiety of allergic reactions, a more thorough diagnostic work-up was performed; including food challenges to soy and peas which she tolerated well. Also a food challenge to lemon seeds were performed, and even though she's been eating citrus fruits regularly without experiencing any symptoms she reacted at a low dose of 10 mg of lemon seed with swollen lips and itching in her mouth. Prick-to-prick test with seed from lemon gave a wheel diameter of 3 mm.

Case 3: A six year old boy suffering from eczema and allergy to cashew had a reaction with sneezing, urticaria and generalized pruritus after eating cashew at the age of 2 years. At age 6 he reacted with swollen lips and itching in his mouth while chewing on a tangerine seed. A prick-to-prick test with seeds from tangerine seeds was performed and gave a wheel diameter of 10 mm.

Case 4: A two year old girl with recurrent wheeze and eczema reacted with swollen lips and urticaria when eating cashew. Weeks later she reacted with similar symptoms after eating fish with homemade lemon dressing, the dressing was made in a blender and lemon seeds were not removed from the juice prior to blending. She is tolerant to fish. Lemon seed prick to prick test gave a wheel diameter of 3.5 mm.

To investigate the molecular basis behind the citrus/cashew cross-reactions observed in these four cases, sera were tested against cashew and lemon seeds, including the three purified allergens. For quantitative IgE-analysis, ImmunoCAP analyses were made with commercially available tests for cashew, lemon, orange, Ana o 3 and Pru p 3 (lipid transfer protein, LTP, from peach) according to the manufacturer's instructions (Thermo Fisher Scientific, Uppsala, Sweden). Experimental ImmunoCAP with purified lemon seed proteins were developed in house (Thermo Fisher Scientific, Uppsala, Sweden) as described previously (6). All cases were sensitized to cashew and Ana o 3, as well as to lemon seed, orange seed and 2S albumin from lemon (Table 1). Although a limited number of observations, the correlation between IgE reactivity to cashew and lemon seeds, as well as Ana o 3 and 2S albumin from lemon (Figure 1C), was calculated using the non-parametric Spearman's rank correlation. There was a strong correlation between IgE-levels to Ana o 3 and lemon 2S albumin, although not statistically significant (Spearman's $r = 1.00$, $p = 0.08$). Prick-to-prick skin tests with lemon seeds (case 3 tangerine seed), together with the clinical history, confirmed the clinical relevance of the IgE sensitization as all four had a positive reaction (Table 1). All four cases displayed considerably lower IgE-levels to lemon and orange pulp compared to lemon and orange seeds (Table 1).

11S globulin, 7S globulin and 2S albumin storage proteins were isolated from lemon seeds and described as allergens for the first time. The serological cross-reactions between homologous proteins from cashew and citrus seeds were established. These four cases, which had reacted with symptoms to citrus seeds, were IgE positive to lemon seeds and had a positive prick-to-prick skin test reaction.

These findings are in line with earlier observations (3-5). Also, the notion that our cases were clinically tolerant to, and less sensitized to, lemon and orange fruit than the respective seeds (Table 1), stresses the importance of seeds in the cross-reactivity to nuts, as suggested by others (3-5, 7).

Therefore, it might be of importance not only to consider the, perhaps, more obvious risk of cross-reactivity between homologous proteins from different nuts (8, 9) when diagnosing and informing nut allergic patients, but also to consider including potentially cross-reacting fruit seeds. Especially since seed proteins can be hidden allergens that may leak from seeds during food processing but not be

visible in the food ingested. This has previously been demonstrated for seed proteins from apple (4), and in this study by the seed contaminated fruit juice ingested by Case 1. The clinical relevance of these findings is so far hard to judge and it will be a delicate matter for the care physician to give advice to these patients. Citrus fruits are staple foods, and generally considered important ingredients of a balanced diet. Also, among these cases, the fruits themselves don't seem to be a problem for cashew allergic individuals, as long as the seeds are avoided. Future studies addressing tree nut-citrus fruit seed cross-reactivity in different types of citrus fruits and in different populations are needed.

Josef Brandström^{1,2}, MD

Gunnar Lilja^{1,2}, MD, PhD

Caroline Nilsson^{1,2}, MD, PhD

Niklas Ingemarsson³, MSc

Magnus P Borres^{3,4}, MD, PhD

Peter Brostedt³, PhD

Hillevi Englund³, PhD

¹Sach's Children and Youth Hospital, Södersjukhuset, Stockholm, Sweden

²Department of Clinical Science and Education, Karolinska Institutet, Stockholm, Sweden

³R&D, ImmunoDiagnostic Division, Thermo Fisher Scientific, Uppsala, Sweden

⁴Department of Women's and Children's Health, Uppsala University, Uppsala, Sweden

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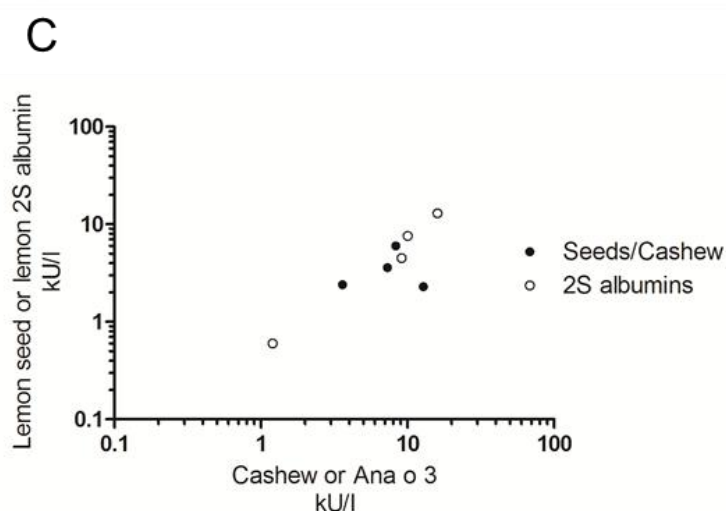
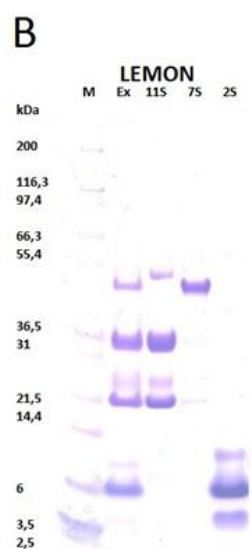
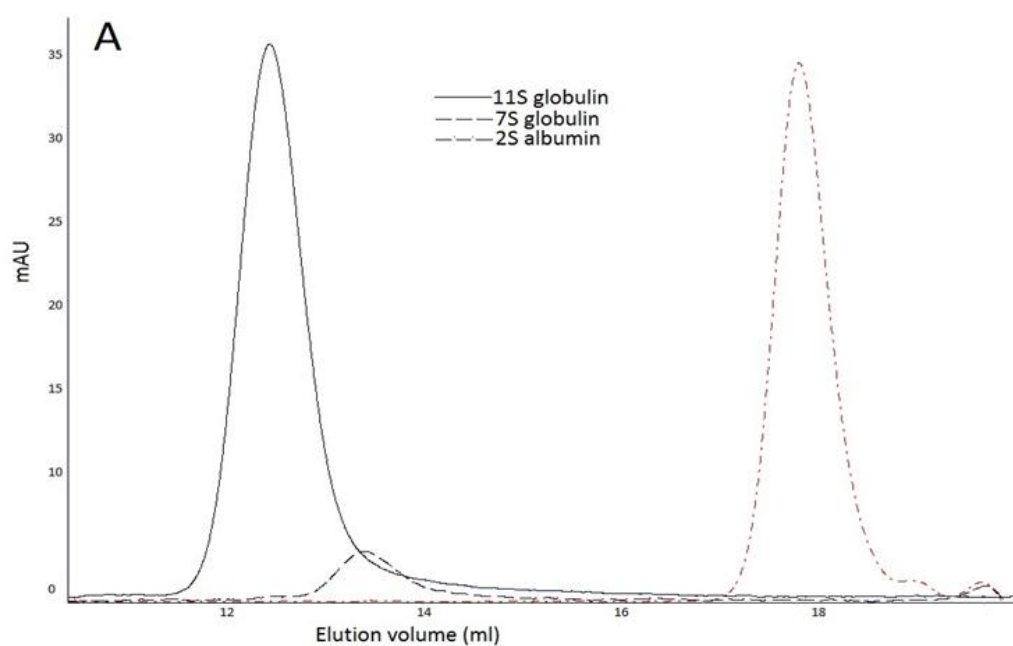
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Table 1. Description of the four cases.

	Patient			
	Case 1	Case 2	Case 3	Case 4
Gender	M	F	M	F
Age	17	16	6	2
Food causing reaction	Drink with lemon juice	Lemon seed	Tangerine seed	Fish with lemon dressing
Clinical symptoms	Anaphylaxis	Local edema, itching	Local edema, itching	Urticaria, itching
Prick-to-prick test lemon/tangerine seed (mm)	Lemon	Lemon	Tangerine	Lemon
	9,5	3	10	3,5
SPT positive control (histamine) (mm)	5	10	5	5
IgE-analyses, kU/l				
Cashew	8,3	7,3	12,8	3,6
Ana o 3	16	10	9,1	1,2
Orange seed	15	8,0	4,4	3,0
Lemon seed	6	3,6	2,3	2,4
Lemon 2S albumin	13	7,6	4,5	0,6
Lemon 7S globulin	1,2	0,3	0,6	0,7
Lemon 11S globulin	1,8	0,8	1,0	1,9
Orange	0,2	0,1	0,0	0,5
Lemon	2,0	1,2	0,8	0,9
Pru p 3 (LTP)	0,2	0	0	4,6

Figure 1

Proteins purified from lemon seeds were characterized by analytical gel filtration (A) and SDS-PAGE (B). M=molecular weight marker, Ex= lemon seed extract. The correlation between IgE-binding to cashew and lemon seeds, as well as Ana o 3 and lemon 2S albumin (Spearman's $r=1.00$, $p=0.08$) is seen in C.



Electronic Repository

Material and methods

Lemon seed extraction: Seeds from ripe lemons were isolated, washed, dried and peeled before grinding. Proteins were extracted 1/10 (w/w) in a phosphate buffer at neutral pH over-night at +4°C followed by centrifugation and filtration.

Protein purification: Proteins were purified using a two-step approach. First, extracts were separated by gel filtration using a Superdex 75 pg XK 50/100 column (GE Healthcare) and a 20mM sodium phosphate buffer pH 7.4, containing 0.15 M NaCl. Fractions with high molecular weight material were further separated with anion exchange chromatography using a HiScreen Capto Q ImpRes column (GE Healthcare). The bound proteins were eluted with a linear salt gradient from 0 to 1 M NaCl in 20 mM sodium phosphate buffer, pH 8.0. Fractions with low molecular weight material were further purified with cation exchange chromatography using a HiScreen Capto SP ImpRes column (GE Healthcare). The bound proteins were eluted with a linear salt gradient from 0 to 1 M NaCl in 130 mM sodium acetate buffer, pH 4.5. Purity of obtained proteins was estimated by SDS-PAGE, analytical gel filtration and mass spectroscopy.

Analytical gel filtration: Purified proteins were analyzed with analytical gel filtration using a calibrated Superdex 200 HR 10/30 column (GE Healthcare). Isocratic elution was performed using 20mM sodium phosphate buffer pH 7.4, containing 0.15M NaCl.

Electrophoresis: SDS-PAGE was performed on Bolt 4-12% Bis-Tris Plus gels in Bolt MES running buffer (Novex by Life Technologies, Carlsbad, CA, US). Samples were prepared at reducing conditions with NuPAGE LDS Sample buffer (Novex by Life Technologies) and heated at 70°C for 10 minutes. Molecular weight calibration was achieved using Mark 12 Unstained Standard (Novex by Life Technologies). Gels were stained by Coomassie Brilliant Blue using a Hoefer Processor (GE Healthcare).

Mass spectroscopy: Proteins of interest were identified using an EASY-nLC 1000 interfaced by a Nanospray Flex to an Orbitrap Fusion mass spectrometer (Thermo Scientific, Waltham, Mass., US). The proteins were reduced, alkylated and digested with trypsin. Separation of the tryptic peptides was performed on an analytical column (50 μ m x 15 cm of a 2 μ m, 100 Å, PepMap RSLC C18) (Thermo Scientific) using a gradient of increasing acetonitrile from water, both containing 0.1% formic acid (Thermo Scientific). Peptides were detected in the Orbitrap Fusion in the range of 350 to 2000 m/z at a resolution of 60,000. The raw MS/MS data files were extracted, sequenced, and searched against the NCBI database using the Peaks Studio 7.0 program.

Fig 1.

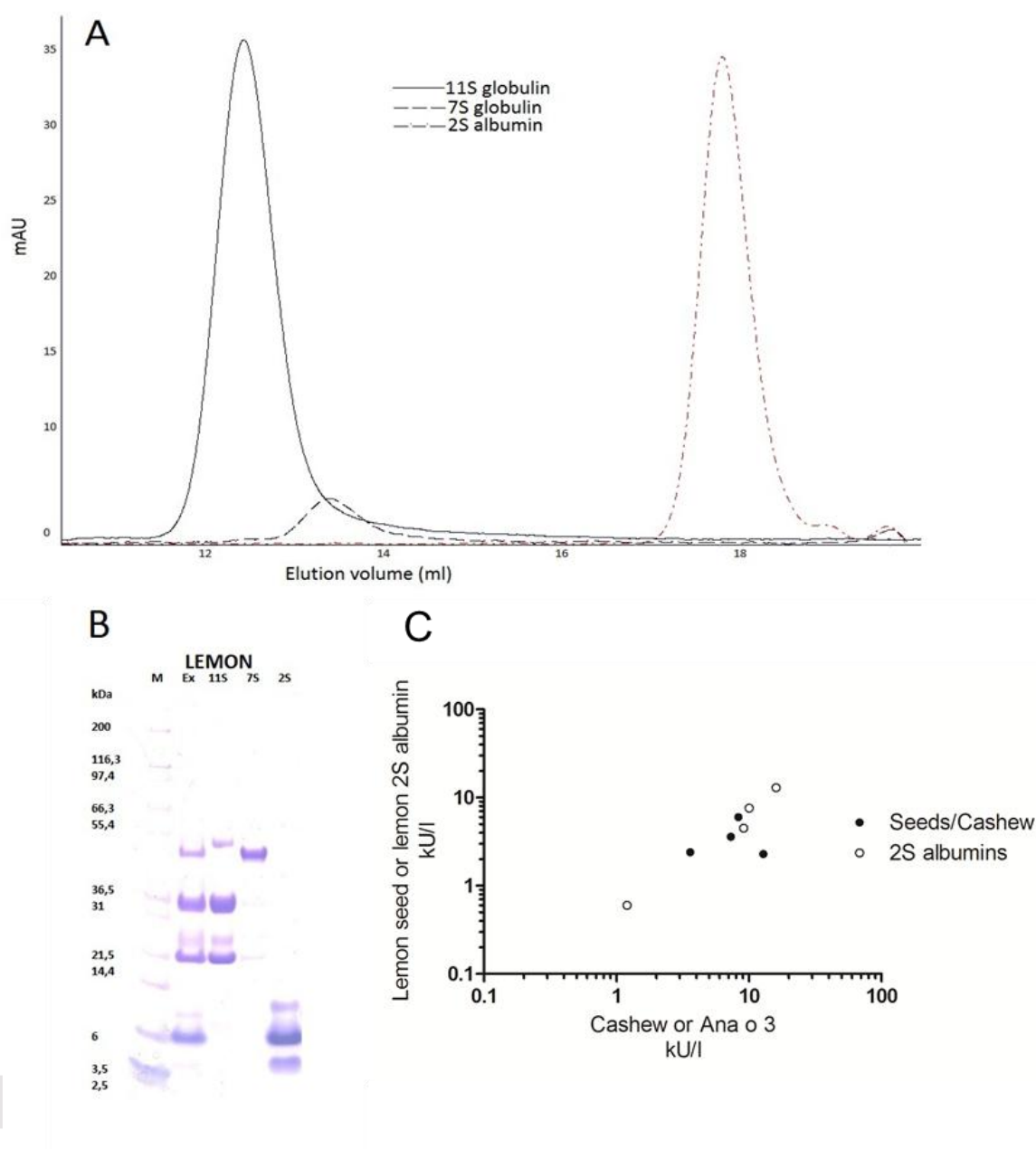


Figure Legend

Fig 1. Proteins purified from lemon seeds were characterized by analytical gel filtration (A) and SDS-PAGE (B). M=molecular weight marker, Ex= lemon seed extract. The correlation between IgE-binding to cashew and lemon seeds, as well as Ana o 3 and lemon 2S albumin (Spearman's $r=1.00$, $p=0.08$) is seen in C.