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Identification and Characterization of IgE-Reactive Proteins and a New Allergen (Cic a 1.01) from Chickpea (*Cicer arietinum*)

Andrea Wangorsch, Anuja Kulkarni, Annette Jamin, Jelena Spiric, Julia Bräcker, Jens Brockmeyer, Vera Mahler, Natalia Blanca-López, Marta Ferrer, Miguel Blanca, Maria Torres, Paqui Gomez, Joan Bartra, Alba García-Moral, María J. Goikoetxea, Stefan Vieths, Masako Toda, Gianni Zoccatelli, and Stephan Scheurer*

Scope: Chickpea (*Cicer arietinum*) allergy has frequently been reported particularly in Spain and India. Nevertheless, chickpea allergens are poorly characterized. The authors aim to identify and characterize potential allergens from chickpea.
Methods and Results: Candidate proteins are selected by an in silico approach or immunoglobuline E (IgE)-testing. Potential allergens are prepared as recombinant or natural proteins and characterized for structural integrity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), circular dichroism (CD)-spectroscopy, and mass spectrometry (MS) analysis. IgE-sensitization pattern of Spanish chickpea allergic and German peanut and birch pollen sensitized patients are investigated using chickpea extracts and purified proteins. Chickpea allergic patients show individual and heterogeneous IgE-sensitization profiles with extracts from raw and boiled chickpeas. Chickpea proteins pathogenesis related protein family 10 (PR-10), a late embryogenesis abundant protein (LEA/DC-8), and a vicilin-containing fraction, but not 2S albumin, shows IgE reactivity with sera from chickpea, birch pollen, and peanut sensitized patients. Remarkably, allergenic vicilin, DC-8, and PR-10 are detected in the extract of boiled chickpeas.
Conclusion: Several IgE-reactive chickpea allergens are identified. For the first time a yet not classified DC-8 protein is characterized as minor allergen (Cic a 1). Finally, the data suggest a potential risk for peanut allergic patients by IgE cross-reactivity with homologous chickpea proteins.

1. Introduction

The incidence of allergic diseases, including food allergies (FA), has remarkably increased.[1,2] However, the frequencies vary among different countries. Up to 10% of the population suffers from food allergy,[2] with an overall prevalence of 5% in adults and 8% in children.[3] In Europe self-reported food allergies show a lifetime and point prevalence in 17% and 6% of the population, respectively.[4]

The manifestation of food allergy is influenced by 1) the foods most commonly consumed where increasing consumption of a particular food may lead to increased sensitization among susceptible consumers,[5] and 2) the exposure to allergenic pollen which can lead to secondary food allergy.[6]

Despite intensive research devoted to identify and characterize allergens in legumes, little is known about the identity and allergenic properties of proteins from chickpeas.[7] According to a market survey in 2010, chickpea consumption has increased by 35% over a period of 21 months in the United States.[8]

Dr. A. Wangorsch, Dr. A. Kulkarni, A. Jamin, Dr. J. Spiric, Prof. V. Mahler, Prof. S. Vieths, Prof. M. Toda, Dr. S. Scheurer
Division Allergology and Section Molecular Allergology
Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 2, 63225 Langen, Germany
E-mail: andrea.wangorsch@pei.de

Dr. A. Kulkarni
Amity University
Mumbai, India

J. Bräcker, Prof. J. Brockmeyer
Analytical Food Chemistry
University of Stuttgart
Allmandring 5B, 70569 Stuttgart, Germany

Dr. N. Blanca-López, Dr. M. Blanca
Allergy Service
Hospital Infanta Leonor
Gran Via del Este 80, Madrid 28031, Spain

Dr. M. Ferrer, Dr. M. J. Goikoetxea
Department of Allergy, IdiSNA (Instituto de Investigación Sanitaria de Navarra)
Clinica Universidad de Navarra
Pio XII, Pamplona 3631008, Spain

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Chickpea's possible association with immunoglobuline E (IgE)-mediated hypersensitivity reactions, especially among children, merits serious attention.[9] Allergy to chickpeas or Garbanzo bean (*Cicer arietinum*), is one of the most common food allergy in Spanish children,[9] and has even been reported in the Indian population, with a prevalence of up to 13%.[7,10,11] Specific IgE-binding protein fractions in crude and boiled chickpea extracts were detected,[12] and the effect of thermal processing and hydrolysis on chickpea allergenicity have been examined.[13] So far, chickpea 2S albumin and plant albumin 2 (Pa2) were considered to evoke allergic reactions in chickpea-sensitive individuals.[14] However, chickpea allergens are not included in the WHO/IUIS Allergen Nomenclature database so far.

By our previous in silico analysis[15] several proteins from *C. arietinum*, showing homology with known allergens, were suggested as putative chickpea allergens. However, IgE-reactive proteins from chickpeas have not been identified and the suspected allergens have not been characterized so far. Hence, the present study addressed the molecular characterization of chickpea proteins engaged in the allergic sensitization.

2. Experimental Section

2.1. Patients' Sera

Sera from 38 patients from Spain (12 patients <14 years; 26 patients >14 years) with a history of clinical reactions after isolated chickpea ingestion were included (**Table 1**). Of note, 20/38 Spanish patients reported systemic reactions upon chickpea ingestion, most frequently urticaria (n = 11) and anaphylaxis (n = 9). IgE-sensitization was confirmed by positive skin tests or ELISA/ImmunoCAP testing (f309, Thermo Fisher Scientific, Uppsala, Sweden) in 35/38 patients. 31/36 tested patients reacted with commercial skin prick test solution or by prick-to-prick testing and 22/35 tested patients showed positive ImmunoCAP results (Table 1).

An informed written consent was obtained from each patient. In patients under the age of 18 years the informed consent

Table 1. Spanish chickpea allergic patients and sera characteristics.

Sera	Sex	Age [years]	Symptoms (chickpea)	Skin test	Immuno CAP/ELISA [kU_A L^{-1}]
1	F	17	OAS	pos[a)]	<0.35
2	F	55	Abdominal pain	pos[a)]	5.77
3	F	40	OAS	pos[a)]	2.31
4	M	44	Anaphylaxis	pos[a)]	<0.35
5	M	24	Anaphylaxis	pos[a)]	<0.35
6	M	11	Urticaria	pos[a)]	2.45
7	M	8	OAS	pos[a)]	<0.35
8	M	7	Anaphylaxis	pos[a)]	1.77
9	M	22	OAS (laryngeal edema)	pos[a)]	0.82
10	M	26	Anaphylaxis	pos[a)]	16.9
11	F	4	Anaphylaxis	pos[a)]	1.33
12	M	3	Urticaria	pos[a)]	<0.35
13	M	4	Urticaria / Angioedema	pos[a)]	5.35
14	M	5	Urticaria	pos[a)]	<0.1*
15	M	11	Urticaria	pos[a)]	9.75
16	F	62	Abdominal pain	neg[a)]	1.37
17	F	15	OAS	neg[a)]	<0.35
18	F	33	Abdominal pain / Vomitus	neg[a)]	<0.1*
19	F	7	Abdominal pain / Vomitus	neg[a)]	<0.1*
20	F	36	OAS	neg[a)]	0.46
21	F	34	Anaphylaxis	nd	0.61
22	M	45	Urticaria	nd	0.37
23	M	<2	Abdominal pain	pos[a)]	15.5
24	F	25	Abdominal pain	Pos[b)]	1.30
25	F	6	Abdominal pain	pos[a)]	4.24
26	F	3	OAS	pos[a)]	39.5
27	F	39	Urticaria (exercise induced)	pos[c)]	<0.35
28	F	61	Abdominal pain / Nausea	pos[c)]	<0.35
29	F	27	Anaphylaxis (exercise induced)	pos[c)]	11.0
30	M	59	Abdominal pain / Nausea	pos[c)]	<0.35
31	M	50	Abdominal pain / Nausea	pos[c)]	<0.35
32	F	26	Anaphylaxis (exercise induced)	pos[c)]	<0.35
33	F	39	Abdominal pain / Diarrhea	pos[c)]	<0.35
34	F	40	Urticaria	pos[c)]	0.65
35	F	33	Urticaria (generalized)	pos[c)]	<0.35
36	F	25	Urticaria (facial)	pos[c)]	6.32
37	F	18	OAS / Urticaria	pos[c)]	3.12
38	M	36	Eosinophilic oesophagitis / Anaphylaxis (mild)	pos[c)]	1.90

OAS, Oral Allergy Syndrome; nd, not determined, [a)] prick-to-prick (boiled chickpeas); [b)] native extract derived from a legume mix (Bial-Aristegui, Bilbao, Spain); [c)] chickpea prick test solution (Leti, Madrid, Spain); Chickpea ImmunoCAP.

Dr. M. Blanca, Prof. M. Torres, Dr. P. Gomez
Jefe de Servicio de Alergología
Hospital Civil
Plaza del Hospital Civil s/n, Pabellon 5, sotano, Málaga 29009, Spain
Dr. J. Bartra, Dr. A. García-Moral
Allergy Unit, Pneumology Department
Clinic Hospital
Sant Antoni Maria Claret, 167, Barcelona, Catalunya 08025, Spain
Dr. J. Bartra
Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS)
University of Barcelona
Carrer del Rosselló, 149, Barcelona 08036, Spain
Prof. M. Toda
Laboratory of Food and Biomolecular Science, Graduate School of Agricultural Science
Tohoku University
Aramaki 468-1, Aoba-ku, Sendai-city 980-8572, Japan
Prof. G. Zoccatelli
Department of Biotechnology
University of Verona
Strada le Grazie 15, Verona 37134, Italy

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was obtained from their parents. The study was approved by the respective Local Ethic Committee. Exclusion criteria were pregnancy, usage of any antihistamines supplements for skin prick testing, other significant medical conditions (e.g., liver, gastrointestinal, kidney, cardiovascular, pulmonary disease, or blood disorders), which might interfere with the induction of food reactions, and patients who received steroids, OMALIZUMAB, allergen immunotherapy, pain killers, or were immune compromised.

Furthermore, nine sera from birch pollen allergic patients preselected by high ImmunoCAP values (CAP classes ≥3) to birch pollen extract and birch pollen allergen Bet v 1 (t3 and t215, Thermo Fisher Scientific), and concomitant IgE-sensitization to peanut extract (f13) and/or Bet v 1-homologous peanut allergen Ara h 8 (f352) were included (Local Ethic Committee, Johann Wolfgang Goethe-University, Frankfurt, Germany). Of note, 4/9 Ara h 8-positive birch pollen allergic patients showed IgE-reactivity to chickpea by ImmunoCAP testing (Table S1B, Supporting Information).

Moreover, sera from peanut reactive patients (n = 15) with peanut specific IgE ImmunoCAP classes ≥2 were included (Table S1A, Supporting Information). Eight sera from symptomatic peanut allergic patients (nos. 40–47) were provided by University Hospital of Erlangen-Nuremberg, Department of Dermatology, Erlangen, Germany (Ethical approval number 4234) and seven sera from peanut sensitized patients were obtained from AbBaltis (no. 39, Kent, UK) or Plasmalab (nos. 48–53, Washington, USA). 14/15 sera showed IgE-reactivity to chickpea by ImmunoCAP testing.

2.2. Extraction of Proteins from Raw and Boiled Chickpeas

For extraction of proteins from raw chickpeas, chickpea flour was prepared using a grinder and further incubated in phosphate buffer (20 mM Na_2HPO_4, 2 mM KH_2PO_4, 5.4 mM KCl, 0.5 M NaCl, pH 7.0) at a 1:10 ratio (w/v).[16] The mixture was agitated overnight at 4 °C and centrifuged twice (30 min, 8.000 g). The supernatant was subjected to a series of syringe filters (1.2, 0.8, and 0.45 µm) and stored as aliquots at 4 °C for 3–4 days or −20 °C for long time storage. For extraction of proteins from thermally treated chickpeas, chickpea seeds were boiled at 100 °C for 10 min and then converted to a paste using a blender. Subsequently, proteins were extracted by the same method as described above, and total protein concentration was determined by the Bradford dye-binding assay (Roti®-Quant, Carl Roth, Karlsruhe, Germany). Extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie (GelCode Blue Protein Stain, Fisher Scientific, Schwerte, Germany) or Ponceau S (Sigma-Aldrich, Taufkirchen, Germany) staining, or by subsequent immunoblotting.

2.3. 2D SDS-PAGE, IgE Immunoblotting, and MS Analysis

2D electrophoresis separation was performed as described in the material and methods section of the supporting information. In brief, for 1D, immobilized pH gradient (IPG) strips pH 4–7 (Bio-Rad, Milano, Italy) and protein from boiled chickpea extract solubilized in 7 M urea–2 M thiourea, 4% CHAPS, 20 mM DTT, 0.5% (v/v) Biolyte 3–10 (Bio-Rad) were used for focusing in a Protean IEF Cell (Bio-Rad) until 25 kVh was reached. The second dimension SDS-PAGE was performed using a 16% polyacrylamide gel as previously described. 2D gels were alternatively stained with GelCode Blue Protein Stain Reagent or subjected to immunoblotting as described below. IgE-reactive spots were carefully cut out from 2D Coomassie stained gels and subjected to in-gel trypsin digestion according to Shevchenko et al.[17] with minor modifications. Details are described in the material and methods section of supporting information.

2.4. Preparation of Chickpea Proteins

Cic a pathogenesis related protein family 10 (PR-10) and Cic a 1.01, a late embryogenesis abundant DC-8 protein (LEA/DC-8), were prepared as recombinant proteins. Cloning, expression, and purification was performed as described in the material and method section of supporting information.

To verify the expression of Cic a 1.01 (XP_01 256 9509, derived from etiolated seedlings) in chickpea, chickpea flour was used for total RNA isolation (RNeasy Kit, Qiagen, Hilden, Germany). RNA was transcribed to cDNA (First-Strand cDNA Synthesis Kit, GE Healthcare) using Poly-T as reverse primer. Subsequent 3' rapid amplification of cDNA ends (RACE) was done using Cic a DC-8 (+): ATG GCG TCG AGG AAA GAT TTC AAG GAA GAC AGA and Poly-T as C and N-terminal primer, respectively. For the full-length sequence determination an additional 5'RACE was implemented using the System for Rapid Amplification of cDNA Ends, version 2.0 (Fisher Scientific). Preparation of rCic a 1.01 was done as described for rCic a PR-10, with slight modifications (material and methods section of Supporting Information).

Both chickpea seed storage proteins, the 7S globulin (vicilin) and 2S albumin, were prepared as enriched fractions from chickpea extract, prepared as described above with the only difference that 10 mM Tris-buffer pH 8.0 was used instead of PBS. Extract of raw chickpeas was applied to a two-step anion exchange chromatography (Hi Prep DEAE 16/10 and Hi PrepQ HP, GE Healthcare) using 20 mM Tris-HCl (pH 7.6) as running buffer, and 20 mM Tris-HCl (pH 7.6), 1 M NaCl for elution. Further purification of the target proteins with appropriate sizes (50-55 kDa for vicilin and 14–17 kDa for 2S albumin) was done by size exclusion chromatography (Sephacryl Hi Prep 26_60, GE Healthcare) using 20 mM MOPS, 150 mM NaCl, 1 mM EDTA pH 7.6 as running buffer. The presence of the respective proteins in the fractions was confirmed by MS analysis.

2.5. Physicochemical Characterization of Proteins

For purified proteins circular dichroism (CD) spectra were recorded using a JASCO J-8 spectropolarimeter with a constant N2 flushing at 20 °C, as described elsewhere.[18] The results were expressed as mean residue molar ellipticity [H] MRD. Recombinant Bet v 1 (Acc. No. X15877) was provided from a former study.[19]

For MS-analysis purified Cic a PR-10, was subjected to reduction by 100 mM DTT (1 h, 56 °C), subsequent alkylation

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with 100 mm iodoacetamide (20 min, RT) and trypsin treatment (overnight, 37 °C). 0.5 µL of α-cyano-4-hydroxycinnamic acid matrix (10 mg mL^{-1} in 50% HPLC grade acetonitrile, 0.1% TFA) was spotted on MALDI sample plate followed by 0.5 µL of sample. Analysis was done by a MALDI-TOF-TOF from Bruker Ultraflex-II and MASCOT software. The identity of Cic a 1.01 and protein fractions containing Cic a vicilin and 2S albumin were confirmed by in-gel digestion according to Spiric et al.[20] followed by liquid chromatography mass spectrometry (LC-MS) analysis. Information that is more detailed is given in the material and methods section of supporting information. Characterization of enriched natural chickpea protein fractions via LC-MS was performed as described in the material and methods section of Supporting Information.

2.6. 1D SDS-PAGE, IgE-Immunoblotting, and Immunoblot Inhibition

Total protein extract, purified proteins and intermediate fractions of enriched proteins were analyzed by SDS-PAGE according to the method of Laemmli,[21] using 12%–16% acrylamide gels. For reducing conditions 100 mm DTT was used along with the sample buffer. Proteins in the gels were visualized by staining with GelCode Blue Stain Reagent (Fisher Scientific). For IgE-immunoblotting 30 µg cm^{-1} of extract from raw chickpeas and 0.5 µg cm^{-1} of purified proteins were applied to non-reducing 12%–16% SDS-PAGE and then transferred onto a nitrocellulose membrane (Amersham Protran 0.2 NC, GE Healthcare) by Semi Dry blotting. Protein transfer was visualized using Ponceau S staining, afterwards the membrane was blocked with Tris buffered saline (TBS), 0.3% Tween and incubated overnight with serum (1:10 in TBS 0.05% Tween, 0.1% BSA, RT). Detection of IgE-binding was done using mouse-anti human IgE-AP antibody (1:750 in TBS, 0.05% Tween, BD Biosciences, Heidelberg, Germany), or mouse-anti human IgE-HRP (1:2000 in TBS, 0.05% Tween, Biozol, Eching, Germany) and subjected to NBT/BCIP (AP conjugate substrate kit, Bio Rad, Munich, Germany) staining or enhanced chemiluminescence (ECL) (LumiGlo, Medac, Wedel, Germany) detection, respectively. For competition experiments immunoblots were incubated overnight with serum (1:10) pre-adsorbed with rCic a PR-10 (10 µg and 50 µg).

2.7. Supporting Material

The data that supports the findings of this study are available in the Supporting Information.

3. Results

3.1. IgE-sensitization Pattern of Chickpea Allergic Patients

Protein extraction from 3 g of flour from raw chickpeas yielded in 527 mg protein (17.56% [g protein per g flour]), while 246 mg protein (8.2% [g protein per g flour]) were obtained from boiled chickpeas.

Both protein extracts were probed with sera from Spanish chickpea allergic patients for IgE-binding by immunoblotting, showing individual and heterogeneous sensitization patterns with an apparent molecular mass ranging from 10 to >100 kDa (**Figure 1**). IgE-binding to extracts from raw and boiled chickpeas was observed in 26/38 (68%) and 15/38 (39%) patients, respectively. The majority of patients was sensitized to multiple proteins, but even mono-sensitization was detectable to a protein from raw chickpeas with an apparent molecular mass of 45 kDa (Figure 1A, serum nos. 3, 18, 21, 29, 31, 32, and 34). Ten sera showed IgE-reactivity exclusively with proteins from raw chickpeas, whereas three sera showed enhanced IgE-reactivity with proteins from boiled chickpeas (Figure 1B, nos. 3, 7, and 37). However, in 12/38 (32%) patients IgE-sensitization was neither detected with proteins from raw nor boiled chickpeas. Results were confirmed for eight of those 12 (67%) patients by ImmunoCAP/ELISA testing, although patients clearly showed chickpea-mediated symptoms and the skin test was positive in 7/8 (87.5%) patients (Table 1). In total, reported clinical reactivity to chickpea was confirmed by skin testing in 31/36 (86%) patients. Remarkably, 3/5 (nos. 16–20) patients who did not react with boiled chickpeas (neither by skin testing nor by immunoblotting) were sensitized to heat-labile proteins (Figure 1). Serological assays using extracts from raw chickpeas showed an overall similar sensitivity for 22/38 (58%) sera by ImmunoCAP/ELISA and for 26/38 (68%) sera by immunoblotting, but divergent results for 11/38 (29%) samples.

Systemic reactions reported in 20/38 (53%) patients was not associated with chickpea specific IgE values (min-max; (mean; median)): <0.35–16.9 (3.20; 1.46) kU$_A$ L^{-1} (serum), including urticaria: <0.35–9.75 (2.71; 1.58) kU$_A$ L^{-1} and anaphylaxis: <0.35–11.0 (3.80; 1.33) kU$_A$ L^{-1}; local reactions: <0.35–39.5 (4.03; 0.40) kU$_A$ L^{-1}.

3.2. Identification and Selection of Potential Chickpea Allergens

Candidate chickpea allergens were selected either based on our previous in silico analysis, that is, the chickpea PR-10 protein (Acc. No. Q9SMK8), pro-vicilin (Acc. No. Q304D4),[15] and 2S albumin (XM_0 044 87544), or identified by MS analysis of IgE-reactive proteins upon 2D IgE-immunoblotting of extract from raw (data not shown) and boiled chickpeas. In line with this, an IgE-reactive vicilin-like protein (NCBI: NP_0 012 96635) and late embryonic abundant (LEA) DC-8 protein (NCBI: XP_0 044 97557, XP_0 045 08082, XP_0 044 94123) were identified by MS in the extract from boiled chickpeas (**Figure 2** and **Table 2**).

All selected chickpea proteins, except DC-8, are members of protein families that have already been described as allergens from various legume foods, like peanut and soybean: PR-10-like proteins Ara h 8 and Gly m 4 with 62% and 56% amino acid identity (aa-id) to Cic a PR-10 (Q9SMK8), respectively, vicilin Ara h 1 and Gly m 5 with 36% and 38% aa-id to Cic a vicilin (NP_0 012 96635), respectively, 2S albumin Ara h 2/6/7 and Gly m 8 with 24–32% and 40% aa-id to Cic a 2S albumin (XM_0 044 87544), respectively. DC-8, a 35 kDa protein of 316 aa without N-glycosylation sites and an isoelectric point (pI) of 6 is characterized by an α-helical 3D-structure,

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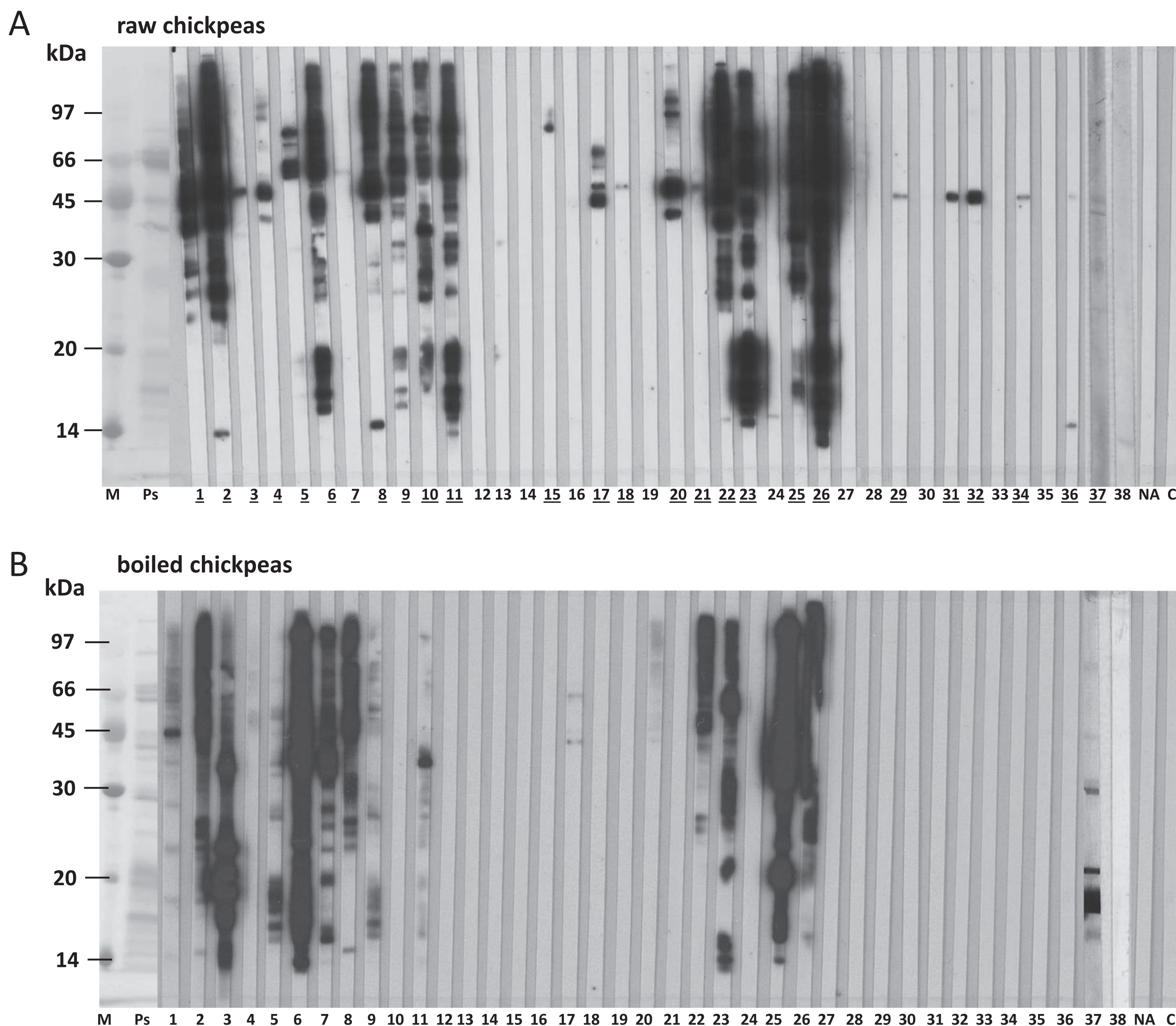

Figure 1. IgE-sensitization pattern of chickpea allergic patients. IgE-reactivity of Spanish chickpea allergic patients ($n = 38$) with protein extracts derived from A) raw and B) boiled chickpeas. M: Molecular marker, Ps: Ponceau S staining; NA: Non-allergic control; C: 2^{ndary} antibody control. Patients with positive reactions are underlined.

which is synthesized late during embryogenesis in plant seed development.[22]

3.3. Chickpea Protein Preparation and Component-Resolved IgE-Reactivity Testing

Selected chickpea proteins were either prepared as recombinant proteins (PR-10 and DC-8) or purified from chickpea extracts (vicilin and 2S albumin). Recombinant Cic a PR-10 was prepared under native conditions with high purity, a concentration of 1.8 mg mL^{-1}, and an apparent molecular mass of around 18 kDa (Figure S1B, Supporting Information). The primary structure of Cic a PR-10 contains the conserved "P-loop" domain, a characteristic feature of members of the PR-10 family, whereas the major Bet v 1 T-cell epitope (Betv1$_{142-156}$) is less conserved (Figure S1A, Supporting Information). A theoretical pI of 5.17 for Cic a PR-10 was calculated using ProtParam ExPASy server.[23] The identity of Cic a PR-10 was confirmed by MALDI-TOF-TOF (Mascot software) showing a match with gi|830 260 057 (Acc. No. AJ275304.1) (data not shown). The sequence of DC-8 was verified by cDNA cloning from chickpea seeds. The newly identified nucleotide sequence (Acc. No. MN276084) is 100% identical to Acc. No. XM_0 044 94123 and was used for cloning and recombinant production of Cic a 1.01. Purified rCic a 1.01 (Figure S2B, Supporting Information) showed a predominant band of 40 kDa and two additional bands between 25 and 28 kDa under reducing conditions, all verified as DC-8 proteins by MS. Both, rCic a PR-10 and rCic a 1.01 showed structural integrity resembling the secondary structure of the major birch pollen allergen Bet v 1, a PR-10

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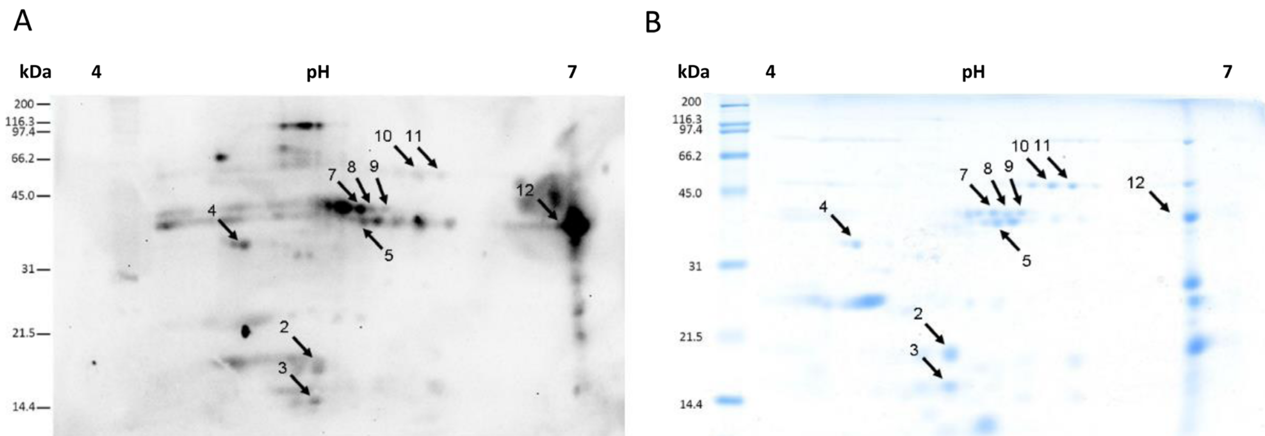

Figure 2. 2-DE (IPG × SDS-PAGE) analysis and IgE-reactivity of proteins derived from boiled chickpeas. A) Immunoblotting using a pooled serum from chickpea allergic patients; B) Coomassie staining. Protein spots, which were subjected to MS analysis, are numbered.

Table 2. MS analysis of IgE-reactive spots (Figure 2B) upon 2D-PAGE immunoblotting of extract from boiled chickpeas.

Protein name	Spot no.	NCBI acc. #	No. peptides identified	Mascot score	Mr. [Da] theor.	pI theor.	Seq. Coverage [%]
Vicilin-like	2	NP_0 012 96635.1	5	172	51 390	6.04	9
Vicilin-like	3	XP_0 012 96635.1	4	187	51 390	6.04	9
Late embryogenesis abundant protein D-34-like	4	NP_0 044 96718.1	2	92	26 522	4.66	4
Alpha-amylase inhibitor	5	Q9SMJ4.1	6	330	56 216	6.20	12
Embryonic protein DC-8-like	7	XP_0 044 97557.1	6	256	31 074	5.35	24
Alpha-amylase inhibitor	7	Q9SMJ4.1	3	82	56 216	6.2	6
Embryonic protein DC-8-like	8	XP_0 044 97557.1	12	457	31 074	5.35	38
Legumin A-like isoform X1	8	XP_0 044 93780.1	5	274	59 306	5.87	11
Alpha-amylase inhibitor	8	Q9SMJ4.1	4	218	56 126	6.2	9
Legumin A-like isoform X1	9	XP_0 044 93780.1	9	411	59 306	5.87	18
Embryonic protein DC-8-like	9	XP_0 044 97557.1	7	294	31 074	5.35	25
Embryonic protein DC-8-like	10	XP_0 045 08082.1	16	605	45 798	5.86	34
Embryonic protein DC-8-like	11	XP_0 045 08082.1	16	658	45 798	5.86	30
Late embryogenesis abundant protein D-34-like	11	XP_01 256 7985.1	5	196	22 155	4.71	25
Embryonic protein DC-8-like	12	XP_0 044 94123.1	14	522	34 486	6.05	34
Alpha-amylase inhibitor	12	Q9SMJ4.1	4	269	56 126	6.2	9

protein (Figure S1C, Supporting Information), and a typical α-helical protein with two minima at 208 and 222 nm in the CD-spectrum of rCic a 1.01 (Figure S2C, Supporting Information), respectively.

Purification of seed storage protein fractions containing Cic a vicilin and Cic a 2S albumin resulted in suitable purity for 2S albumin (Figure S3, Supporting Information, lane 1). MS analysis showed no contamination with other known chickpea proteins. In contrast, the vicilin-containing fraction consisting of two predominant bands (Figure S3, Supporting Information, lane 2), contained glycinin, legumin, and provicilin (data not shown).

IgE-reactivity testing revealed that only 2/35 (5.7%) of chickpea allergic patients were sensitized to rCic a PR-10 protein (**Figure 3**A). Both patients (nos. 6 and 26) showed multiple sensitization to other chickpea proteins (Figure 1), chickpea specific IgE values of 2.09 and 39.5 kU_A L^{-1}, and reported urticaria or OAS in response to ingestion of chickpeas, respectively (Table 1). Moreover, 22% (7/36, nos. 1, 6, 7, 10, 11, 13, 25) were sensitized to rCic a 1.01 (Figure 3B), and 32% (9/28, nos. 7, 10, 12, 14–17, 23, 25) were sensitized to the Cic a vicilin-containing fraction (Figure 3C). Noteworthy, none of the tested chickpea allergic patients was reactive to purified Cic a 2S albumin (not shown). No correlation of IgE-sensitization

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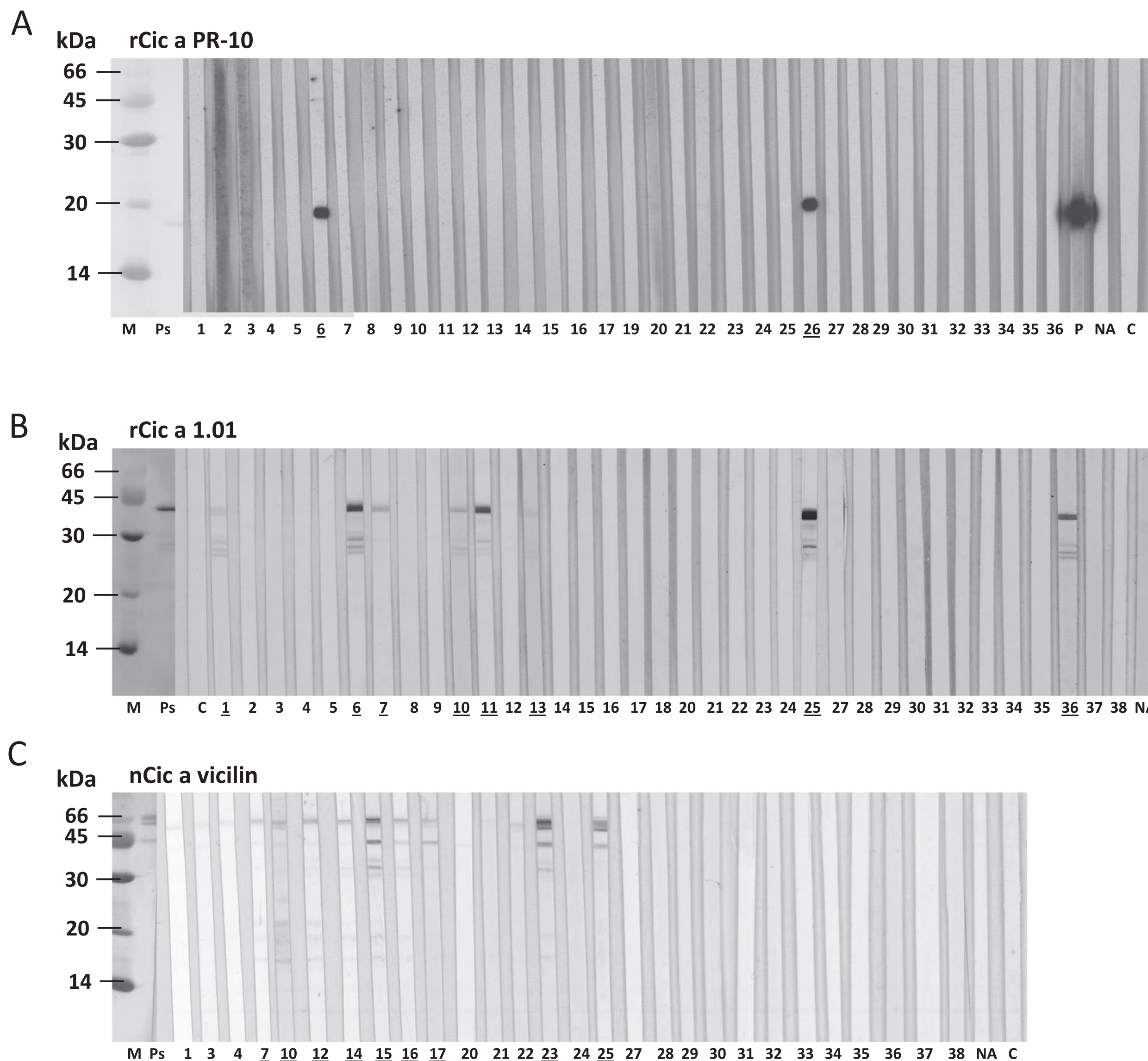

Figure 3. Prevalence of IgE-binding to purified chickpea allergens. Sera from chickpea allergic patients probed with A) rCic a PR-10, B) rCic a 1.01, and C) partially purified Cic a vicilin by IgE-immunoblotting. M: Molecular marker; Ps: Ponceau S staining; P: positive control Bet v 1; NA: Non-allergic control; C: 2^{ndary} antibody control. Patients with positive reactions are underlined.

to certain allergens with severity of symptoms could be determined.

3.4. IgE-Sensitization of Birch Pollen and Peanut Reactive Patients with Chickpea Proteins

Since selected chickpea proteins represent homologous allergens in birch pollen and legume foods, the IgE-sensitization of respective allergic patients was tested.

Notably, 67% (6/9, nos. 2–5, 7, 9) of birch pollen allergic patients with sensitization to homologous allergens Bet v 1 (birch) and Ara h 8 (peanut) showed IgE-reactivity with rCic a PR-10 (**Figure 4**A). Although sera were pre-selected by high Bet v 1-specific ImmunoCAP values and concomitant IgE-sensitization to Ara h 8, Bet v 1, and Ara h 8, specific IgE values cannot be correlated to the reactivity with Cic a PR-10 (data not shown). Accumulation of Bet v 1-like protein in chickpeas and potential IgE-cross-reactivity with Bet v 1 was demonstrated by inhibition of IgE binding to natural Cic a PR-10 upon pre-incubation of Bet v 1-reactive serum (no. 4 in Figure 4A) with rCic a PR-10 (Figure 4B). BSA used as inhibitor (negative control) did not affect IgE-binding to chickpea allergens. However, the IgE-reactivity of Cic a PR-10 was partially retained after thermal treatment of chickpeas (Figure 4C).

Moreover, 80% (12/15, nos. 39–44, 48–53) of sera from peanut sensitized patients showed IgE-reactivity with multiple proteins from boiled chickpeas (**Figure 5**A). Recombinant Cic a PR-10, rCic a 1.01 and the Cic a vicilin containing fraction showed IgE-binding frequencies of 40% (6/15, nos. 39, 41, 45, 46, 48,

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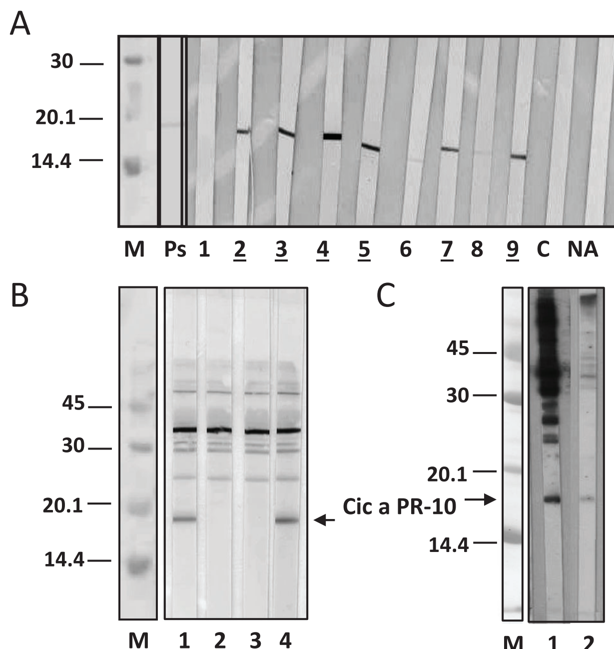

Figure 4. Detection of IgE reactive Bet v 1-homologous Cic a PR-10 protein in chickpeas. A) IgE-binding of Bet v 1-reactive sera from birch pollen allergics (no. 1–9) to rCic a PR-10. M: Molecular marker; Ps: Ponceau S staining; C: 2ndary antibody control; NA: Non-allergic control. Patients with positive reactions are underlined. B) Inhibition of IgE-binding to natural Cic a PR-10 in the extract from raw chickpeas by pre-incubation of a Bet v 1-reactive serum (#4 in (A)) with rCic a PR-10. M: Molecular weight marker; 1: without inhibitor; 2: 10 µg rCic a PR-10; 3: 50 µg rCic a PR-10; 4: 50 µg BSA. C) Detection of IgE-reactive Cic a PR-10 in extracts derived from raw (1) and boiled (2) chickpeas using a Bet v 1-reactive serum (#4 in (A)).

51), 13% (2/15, nos. 42, 51), and 53% (8/15, nos. 39, 41, 43, 49–53), respectively, with sera from peanut reactive patients tested (Figure 5B–D).

4. Discussion

Allergic sensitization of patients to chickpea (*C. arietinum*) is characterized by individual and heterogeneous IgE-sensitization patterns involving numerous proteins. This phenomenon is typical for legume food like pea and peanut, where at least 16 allergens comprising ten protein families are described (www.allergen.org). However, no allergen from the legume chickpea was listed in the database of the WHO/IUIS Allergen Nomenclature Sub-Committee so far.

Since chickpeas are usually consumed after thermal treatment it is noteworthy that 39% of the patients´ sera tested showed IgE-reactivity by immunoblotting with proteins derived from boiled chickpeas, but at the same time 68% and 58% of the patients were IgE-reactive to extracts from raw chickpeas by immunoblotting and ImmunoCAP/ELISA. In line with these observations, a group of patients did not react with boiled chickpeas but showed sensitization to native chickpea proteins. Our data provide evidence that 1) this group of patients is sensitized to native and potential cross-reactive homologous allergens that might be of clinical relevance or 2) allergens derived from boiled chickpeas are underrepresented due to a less efficient extraction process. On the other hand, for a limited number of patients the thermal treatment enhanced the IgE-reactivity to chickpea, which might be due to the generation of neo-epitopes or an enhanced protein solubility by affecting the food matrix after heating. This is in contrast to results of a study published by Gupta et al.,[24] where the glycation of a 26 kDa IgE-binding chickpea protein,[25] belonging to the plant albumin family, is associated with a reduced TH2 immunogenicity in mice. For 8/38 patients IgE sensitization could not be confirmed by any of the serological assays (immunoblotting or ImmunoCAP/ELISA) using aqueous extracts, although all patients reported chickpea mediated symptoms and 7/8 patients showed positive skin testing results. In line with this, the potential clinical significance of lipophilic allergens (and oleosins), which likely are not abundant in aqueous extracts, needs to be considered to explain divergent diagnostic results. The results of ImmunoCAP/ELISA testing and immunoblotting were in accordance in 28/38 samples. Aqueous extracts from raw chickpeas were used in both assays, nevertheless, the divergent results could be explained by different sensitivities of the assays, different chickpea cultivars used, and/or different protein extraction protocols applied. Although skin testing seems to be associated with the highest diagnostic sensitivity, one approach to improve the diagnostic is the application of a component-resolved diagnosis using purified allergen.

The present study investigated for the first time the IgE-sensitization to selected chickpea proteins: PR-10, 2S albumin, a vicilin-containing fraction, and DC-8, a novel chickpea allergen. Only two patients (out of 35 tested) showed a predominant reaction with Cic a PR-10, and only one patient (out of 26 tested) was sensitized to the storage protein Cic a 2S albumin. Remarkably, the vicilin-containing fraction showed an IgE-binding frequency of 32%, but consists of other potential allergens, like legumin and glycinin. As a result of our study only Cic a 1.01 (DC-8) with an IgE-binding frequency of 22% qualifies as minor chickpea allergen. Minor allergens are characterized by an IgE-reactivity of at least 5 patients and a frequency of at least 5% of the respective patient group (www.allergen.org).

Cic a PR-10 belongs to the family of Bet v 1-like allergens identified from legumes in peanut (Ara h 8),[26] soybean (Gly m 4),[27] and mung bean (Vig r 1/Vig r 6).[28] Other Bet v 1-like proteins from legumes are described as Lup a 4 from lupine,[21] Pis a 6 from peas,[29] Pha v 6 from kidney bean,[22] and Vic f 6 from horse bean,[30] however, the allergenic properties of these proteins have not been confirmed. In our previous work,[15] two Bet v 1 homologues chickpea proteins Cic a PR-10 (Q9SMK8 & Q39450, 64% amino acid sequence identity (aa-id)) were identified as putative allergens by in silico analysis. Recombinant Cic a PR-10 (Q9SMK8) showing structural integrity and highest aa-id (49%) to the major birch pollen allergen Bet v 1 (P15494) was characterized in the present study. Although PR-10 proteins are heat-labile proteins, a residual IgE-reactivity to Cic a PR-10 was even detected in the extract from boiled chickpeas. Similar results were obtained for the Bet v 1-like soybean allergen Gly m 4:[27] In regions with exposure to birch pollen, 71% of Bet v 1-sensitized patients were reactive to Gly m 4 provoking soy allergy.[6,27] Oropharyngeal and sometimes severe reactions to Gly m 4 are predominately induced by fresh, but also by processed soy protein containing products in approximately 10% of birch pollen sensitized patients,[27] which could be attributed

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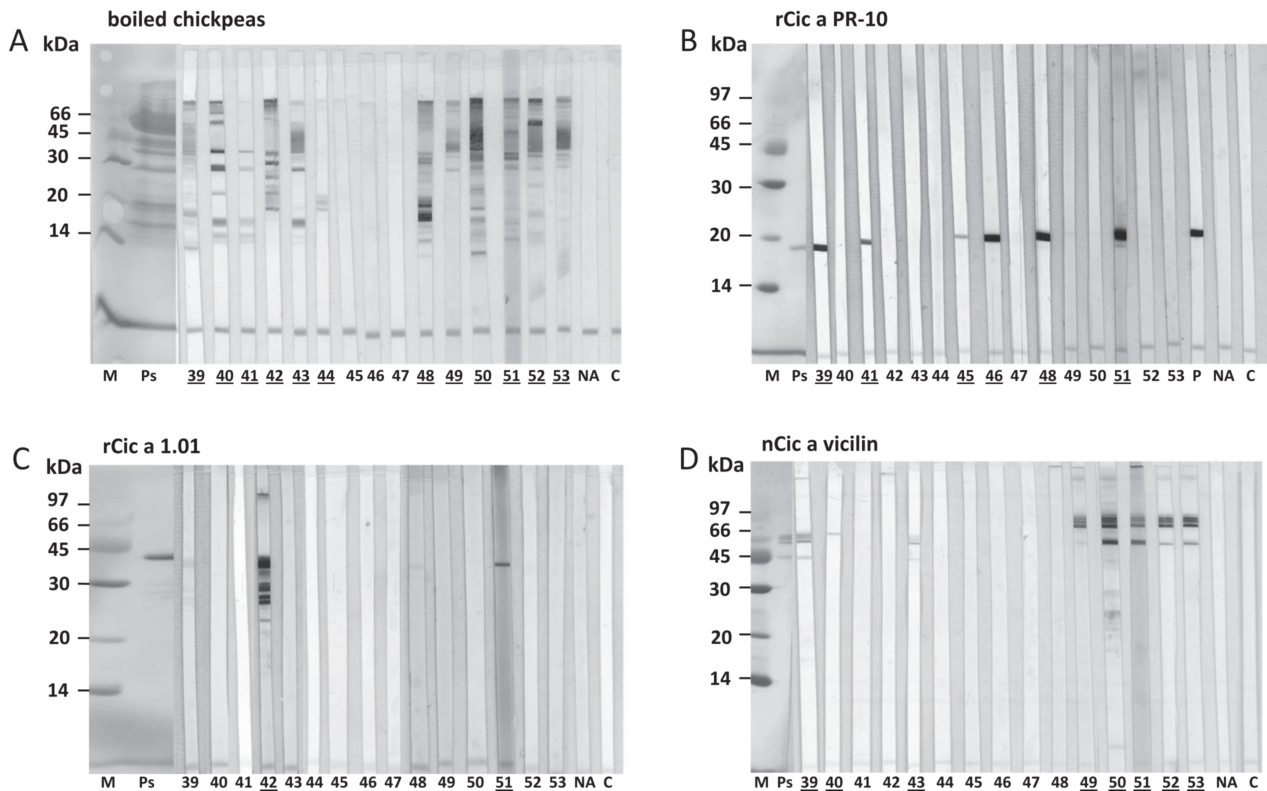

Figure 5. IgE-reactivity of sera from peanut sensitized patients with chickpea proteins. IgE-binding of sera from peanut reactive patients ($n = 15$) with A) extract derived from boiled chickpeas, B) rCic a PR-10, C) rCic a 1.01 and D) a vicilin-containing fraction. M: Molecular weight marker; Ps: Ponceau S staining; NA: Non-allergic control; C: 2ndary antibody control. Patients with positive reactions are underlined.

to matrix effects preserving a denaturation of the allergen, at least partially. However, to conclude a clinical significance of Cic a PR-10 even in a minority of patients is speculative so far.

Moreover, the study provides evidence that Cic a 2S albumin, in contrast to 2S albumin Ara h 2 from peanut, seems not be of clinical relevance. Our data are in agreement with 2S albumin from pea also showing only weak frequency of IgE-sensitization, maybe due to an immune dominant hydroxyproline not expressed in pea and chickpea.[31,32]

An earlier study suggested the basic subunit of legumin and vicilin from chickpea proteins as major allergens.[8] Since vicilin, a stable 7S seed storage globulin, is described as allergen in many legume food, likewise Ara h 1 from peanut, it is tempting to speculate Cic a vicilin plays a role in the manifestation of chickpea allergy. However, it remains unclear which component is mainly engaged in the IgE-reactivity, although 32% of the patients were reactive to the vicilin-containing fraction.

DC-8, a late embryogenic abundant protein (LEA-4), is of particular interest since members of this protein family have not yet been classified as allergens. Interestingly, like other allergenic pathogenesis-related (PR) proteins, they play an important role in abiotic stress response and stress tolerance in plants.[22] Homologous protein sequences of the LEA-4 superfamily are found in lupine, soy and peanut, with aa-identities of 65%, 64%, and 66% to Cic a 1.01, respectively.

A recent study by Gupta et al.[33] described a purified 20 kDa protein from chickpea,[7] showing homology to a fragment of a-dioxygenase (ADF) as IgE-binding protein using sera from chickpea sensitized patients and with TH2 immunogenicity in mice. However, applying an MS approach we did not identify this protein family in our experimental setting.

In order to study the potential risk of allergic reactions in peanut sensitized patients after consuming chickpeas, peanut reactive sera were probed with boiled chickpea extract, Cic a PR-10, Cic a 1.01, and the vicilin-containing fraction. Results showed that patients with IgE-sensitization to peanut also react with chickpea proteins, further supporting the expression of homologous allergens in both food varieties. It is tempting to speculate that the IgE-reactivity to chickpea proteins is mediated by co-sensitization or a genuine sensitization to peanut allergens, for example, Ara h 1 (vicilin), other storage proteins, and a yet not identified homologous DC-8 protein from peanut followed by cross-reactivity. On the other hand, it might be possible that the reactivity to Cic a PR-10 reflects the cross-reactivity to the birch pollen allergen Bet v 1. Nevertheless, ingestion of chickpeas might provide a risk for clinical reactions in a subgroup of peanut allergic patients.

In summary, the IgE-sensitization in chickpea allergic patients from Spain by skin and in vitro testing showed divergent results, whereas skin testing might be a better indicator of chickpea sensitization than serological IgE tests using aqueous protein extracts. The investigated chickpea proteins did not improve the diagnostic sensitivity in comparison to chickpea extracts. Nevertheless, our data suggest that the implementation of Cic a 1.01 and Cic a vicilin might improve the diagnostic value. It is tempting to speculate that further chickpea allergens, like non-specific

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lipid-transfer proteins, α-amylase inhibitor, legumin A-like proteins, or lipophilic and yet unknown proteins are engaged in chickpea mediated food allergy. Finally, the study showed the presence and IgE-reactivity of homologous legume proteins in chickpea. In line with this, consumption of chickpeas might be a risk factor for legume food allergic patients to provoke allergic reactions.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors of this manuscript declare that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Author Contributions

A.W. and A.K. contributed equally to this work and share first authorship. A.W. and A.J. performed most of the experiments, A.K. contributed data on Cic a PR-10, J.B. and J.B. prepared Cic a 2S albumin, J.S. performed MS analysis, clinical partners V.M., N.B.L., M.F., M.B., M.T., P.G., J.B., A.G.M., and M.J.G. recruited patients and provided clinical data, S.V. supported data interpretation, M.T. initiated the study, G.Z. performed 2D-PAGE and MS, S.S. coordinated the study, S.S. and A.W. wrote the manuscript. All authors proofed the manuscript.

Keywords

allergen, chickpea allergy, Cic a 1, *Cicer arietinum*, IgE cross-reactivity

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