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Identification of Rice Proteins Recognized by the IgE Antibodies of Patients with Food Allergies

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ABSTRACT: Similarity among food allergens is a great problem affecting the specificity of diagnosis and treatment of allergic patients. We have observed that 80% of patients with food (including wheat) and pollen allergies have increased IgE antibodies against rice proteins. By immunoblotting, we documented that boiling decreased solubility and IgE reactivity of PBS-extracted rice and wheat proteins, yet in SDS extracts this reactivity was only slightly changed. The sera of patients highly positive on the IgE immunoblot and positive in basophil activation and skin prick test with boiled rice components were used for characterizing the IgE-binding proteins separated by 1D or 2D electrophoresis. Using mass spectrometry, we identified 22 rice SDS soluble proteins. Six of them were new thermostable potential rice allergens: glutelin C precursor, granule-bound starch synthase 1 protein, disulfide isomerase-like 1-1 protein, hypothetical protein OsI_13867, putative acid phosphatase precursor 1, and a protein encoded by locus Os02g0453600. All of the identified rice proteins differed from known wheat allergens, except proteins belonging to the α-amylase/trypsin inhibitor family. Furthermore, we would suggest that in patients with high IgE reactivity to wheat and rice components, the IgE immunoblot and skin prick test with boiled rice proteins could be beneficial before diet recommendation.

KEYWORDS: *food allergy, thermal processing, potential rice allergens, basophil activation test, skin prick test*

INTRODUCTION

Food allergy belongs among the world's most widespread disorders, appearing mainly in early childhood and affecting up to 8% of children and 3−4% of adults in Western countries. In addition, its incidence has continuously risen over the past two decades.[1−3] Wheat (*Triticum aestivum*) and rice (*Oryza sativa*) are important parts of the human diet worldwide. Wheat, however, belongs to the six food allergens, including milk, egg, wheat, peanut, soy, and fish, that account for 90% of food hypersensitivity reactions.[3,4] Wheat proteins could be involved in the three routes of sensitization: inhalation, contact, and ingestion. Inhalation of wheat components may cause baker's asthma,[5] whereas contact with skin may trigger IgE-mediated contact urticaria,[6] and ingestion may cause atopic dermatitis, wheat-dependent exercise-induced anaphylaxis, or gastrointestinal symptoms.[7,8] Diagnostic tests including the estimation of specific IgE antibodies are highly sensitive, but less specific. This low specificity may be due to the insufficient purity of currently used protein extracts instead of isolated allergens but also perhaps because of cross-reactivity among similar proteins in various foods.[9]

In contrast, rice is commonly considered to be hypoallergenic and is frequently recommended as a diet alternative for patients with food allergies. However, several studies describe immediate hypersensitivity reactions to rice. It has been documented that the inhalation of rice flour or vapors from boiling rice may cause bronchial asthma,[10,11] that contact of skin with raw rice may lead to contact urticaria,[12,13] and that ingestion may cause urticaria, bronchial asthma, rhinoconjunctivitis, and anaphylaxis.[14−17]

Rice allergens have already been described, but only two, Ory s 1 (β-expansin) and Ory s 12 (profilin A) (both isolated from rice pollen), were well characterized and have been included in the official allergen database of the IUIS.[18] Analysis of grains from boiled rice revealed in a water/salt-soluble extract the α-amylase/trypsin inhibitor family of proteins of about 14−16 kDa, cross-reacted with other cereal grains, which are recognized by IgE antibodies from the majority of patients with a rice allergy.[19,20] The other rice allergens were identified as a 26 kDa major seed storage protein α-globulin and a 33 kDa plant glyoxalase I.[21,22] Lipid transfer protein (LTP, Ory s 14) was also described as a rice allergen, which cross-reacts with peach/apple LTP.[23] Recently, Satoh et al. have characterized novel IgE-binding rice components such as 52 and 63 kDa globulin-like proteins,[24] which are homologous to cupin superfamily allergens belonging to seed storage proteins. Trcka et al. described a 56 kDa glycoprotein responsible for anaphylaxis after the consumption of food containing boiled rice.[17] All of the rice allergenic components described so far have been water/salt-soluble proteins, but we could not find any information about water/salt-insoluble rice allergens.

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Table 1. Demographic, Clinical, Serological, and Therapy Characterization of Patients and Disease Controls[a]

patient	sex	age	clinical symptoms	RAST value (kIU/L) wheat	RAST value (kIU/L) rice	specific IgE for allergens	restricted diet	therapy
1	F	7	FA, AD	0.75	0.75	eg, mi, so, ha, gl	yes	AH
2	F	28	AR, AB, FA	2.22	0.59	gcp, we, bp, mo, dg, ct	yes	AH
3	M	34	AR, AB	0.65	0.49	gcp, mo, mt, ca, ap	no	AH, ICS
4	M	10	FA, anaphylaxis	0.88	0.64	gcp, mo, ps, ki, nu, ap, gl	no	AH
5	M	3	AD, FA	4.78	1.06	gl, mi, eg, cf, nu	no milk	AH, local CS
6	M	15	AD, FA	1.66	0.86	gr, ha, so, mo, mt, dg, ct	no	AH, Tacrolimus, local CS
7	M	11	AD, FA	1.13	0.68	gr, eg, so, ha, mo, mt, dg, ct	yes	AH, Tacrolimus, local CS
8	M	65	AR, AB	0.71	0.44	gcp, bp, wo, am, po, nu	no	AH, ICS
9	M	32	AR, AB	1.18	0.93	gcp, bp, am, wo, nu, to, ce	no	AH, ICS
10	M	40	AD, AR	1.33	0.62	gcp	no	AH, ICS
11	F	62	FA, AR, AB, CoD	5.27	3.62	ry, gl, gcp	yes	AH, β-mimetics
12	M	51	AC, FA, AB	6.46	0.98	gr, nu, gl, ki, or, la, mt, ct	yes	AH, ICS
13	F	44	AD, FA, AR	5.70	8.37	ry, gcp, nu, ca, po, ce, mt	no	
14	M	62	FA, AB	5.11	3.70	bp, gcp, gl	yes	AH, ICS
15	M	37	AB	0.63	0.67	bp, po, ce, to, ki	no	AH
16	F	39	FA, AB	2.23	0.92	gl, he	yes	AH
17	F	62	FA, AR, AB	2.15	1.33	ry, bp, gr, so, ce, ma, mi, nu, ca, ap, po, to	yes	Nalcrom, Ketotifen, AH, ICS
18	F	51	FA, AB	2.29	1.31	gl, nu, ap, gp	yes	AH, Nalcrom, ALTs
19	F	22	FA, AR, AB	1.43	0.94	gcp	yes	AH, ICS
20	F	56	U, AR, AB	3.83	neg	bp	no	AH, ALTs
21	M	30	AD, AB	0.86	0.22	nu, mt, mo, dg, ct	no	AH, ICS, local CS
22	M	8	FA	1.55	0.25	eg, mi, ry, so, ba	yes	AH
23	F	5	FA, AD	3.60	neg	eg, ha, gl	yes	AH, Tacrolimus, Emolentia
24	F	4	FA, AD	0.47	neg	eg, mi, de	yes	AH, local CS
25	F	58	FA, AB	1.89	neg	ry	yes	AH, β-mimetics
26	M	4	FA, AD	1.12	neg	eg, mi, ha	yes	AH, local CS
27	M	44	FA, AB	1.49	0.19	ry	yes	AH, ICS
28	F	49	FA, AB, WA	30.6	neg	gl, wv	yes	AH, Alutard, ICS
29	F	3	AD, FA	0.73	neg	eg, gl, so, ha, dg	yes	AH
30	M	3	AD, FA	2.85	neg	eg, gl, dg	yes	AH, Tacrolimus, local CS
31	M	2	AD, FA	5.27	0.10	eg, mi, gl, ha, so	no milk	Neocate
32	M	2	AD, FA	0.40	neg	ry, nu, pe, eg, po, dg	yes	AH, Neocate, Nalcrom, ALTs
33	F	2	AD, FA	0.84	neg	eg, mi	yes	AH
34	M	2	AD, FA	10.9	0.10	mi, eg, so, nu, ki, ba, dg	yes	AH, local CS
35	F	24	AD, FA, AB	12.1	0.10	mi, eg, gl, so, ha, dg	yes	AH, IKS, local CS
36	M	23	AD, AR, acne	neg	neg	bp, gr, rp, ct	no	local ATB for acne
37	M	15	AR, AB, CD	neg	0.61	bp, rp, wv, be, fe, mt, dg, hr	no	AH, ICS, local CS
38	M	21	AR	neg	neg	bp, gr, rp, ct	no	AH, NCS
39	M	38	AR	neg	neg	bp, gr, we, rp, wo, fe, mt, mo, ct	no	AH, Cromoglycate
40	F	48	AR	0.22	0.19	bp, gr, wo, wv	no	AH, NCS
41	M	20	AR, AB	0.11	neg.	bp, gr, wo, mt	no	AH
42	F	31	AR	neg	0.13	bp, gr	no	AH
43	M	61	AR	neg	neg	gr, da, dn	no	AH, NCS
44	F	68	AR	neg	neg	bp, wo, gr	no	AH
45	F	65	AR, AB	neg	neg	gr, bp	no	AH, NCS, ICS
46	M	47	AR	neg	neg	gr, bp	no	AH, NCS, ICS
47	F	30	AR	neg	neg	gr, bp	no	AH, NCS
48	F	39	AR	neg	neg	gr, bp	no	AH, NCS, ICS
49	F	27	AR	neg	neg	gr, bp, wo	no	AH
50	F	25	AR	neg	neg	gr, bp, mt	no	AH

[a]Abbreviations: F, female; M, male; AB, asthma; AD, atopic dermatitis; AR, allergic rhinitis; CD, contact dermatitis; CoD, celiac disease; FA, food allergy; U, urticaria; WA, wasp allergy; neg, negative reaction; am, *Ambrosia* spp.; ap, apple; ba, banana; be, bee venom; bp, birch pollen; ca, carrot; ce, celery; cf, cod fish; ct, cat; da, daisy; de, *Dermatophagoides* spp.; dg, dog; dn, dandelion; eg, egg; fe, feather; gcp, grass and cereal pollen; gl, gluten; gp, green pepper; gr, grass pollen; ha, hazelnut; he, herbal; hr, horse; ki, kiwi; la, latex; ma, maize; mi, milk; mo, mold; mt, mites; nu, nuts; or, orange; pe, peanut; po, potato; ps, poppy seed; rp, rye pollen; ry, rye flour; so, soybean; to, tomato; we, weeds; wv, wasp venom; wo, wormwood; AH, antihistamines; ALTs, antileukotriens; ICS, inhalation corticosteroids; CS, corticosteroids; NCS, nasal corticosteroids.

The aim of our study was to characterize and compare potential rice allergens, soluble in phosphate-buffered saline (PBS) and sodium dodecyl sulfate (SDS) buffers extracted from raw or boiled rice using proteomic techniques. IgE-

binding proteins from boiled rice were detected by immunoblotting using sera from patients with food (mainly wheat allergy), food and pollen, or only pollen allergies (disease controls) and from healthy donors (controls). The extracted rice proteins were separated by one-dimensional electrophoresis (1-DE) and two-dimensional electrophoresis (2-DE), and the most frequently recognized IgE-binding proteins were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Moreover, the reactivity to rice proteins was also tested by basophil activation test (BAT) and skin prick test (SPT), and the clinical significance of all tests is discussed herein.

■ MATERIALS AND METHODS

Chemicals. The chemicals used for preparing the PBS and SDS extracts for 1-DE and 2-DE and for the visualization of separated proteins were sourced from Serva (Heidelberg, Germany), Sigma (St. Louis, MO, USA), and Lach-Ner (Neratovice, Czech Republic); protein concentrations in extracts were determined by using a Bicinchoninic Acid Protein Assay Kit from Pierce (Rockford, IL, USA). The other chemicals used for 2-DE were DeStreak and Pharmalyte (pH 3−10 or pH 8−10.5) from Amersham Bioscience (Uppsala, Sweden) and Immobiline DryStrips (18 cm) with a nonlinear pH 3−10 gradient from GE Healthcare (Uppsala, Sweden). For blocking of nitrocellulose membranes (from Serva) was used Tween 20 from Duchefa (Haarlem, The Netherlands) and powdered defatted milk from Promil (Nový Bydžov, Czech Republic). The peroxidase-labeled sheep anti-human IgE antibody was from the Binding Side (Birmingham, UK). Development solution, SuperSignal West Femto Maximum Sensitivity Substrate kit, was from Pierce. For clinical tests were used commercial allergen extracts from Alyostal-Stallergenes (Antony, France).

Characterization of Patients. We characterized IgE reactivity to wheat and rice proteins in the cohort of 50 patients (24 females, 26 males; mean age = 30.8 years; age range, 2−68 years) with food and/or pollen allergies. Thirty-five patients with food allergy (group I), all with a positive reaction to wheat allergens, were further divided into two subgroups according to their IgE reactivity to rice proteins (rice positive, denoted Ia, patients 1−19; and negative or low positive, denoted Ib, patients 20−35). Fifteen patients positive for pollen allergens, but negative for wheat or other food allergens, were used as a disease control (group II, patients 36−50). Sera of 10 healthy donors were used as a healthy control (group III). The clinical symptoms, allergen specificity, and treatment of allergic patients are summarized in Table 1. All sera were stored at −20 °C until use. Written informed consent was obtained from all patients and healthy donors before sampling, and the study was approved by the Ethics Committee of the General Teaching Hospital in Prague (Czech Republic).

Immunoglobulins and Specific IgE. Total IgA and IgG were measured by nephelometry[25] using a Dade-Behring BNII nephelometer (Dade-Behring, Marburg, Germany). The reagents used included OSAR15 N Antiserum to human IgA and OSAS15 N Antiserum to human IgG. The standard laboratory referential ranges are 6.90−14.0 g/L for IgG and 0.70−3.70 g/L for IgA immunoglobulins.

Total and specific IgE antibodies were measured by a chemiluminescent enzyme immunoassay method using an IMMULITE 2000 system (Siemens, Erlangen, Germany). The standard laboratory referential range for total IgE antibodies is 0−100 IU/mL. Allergen specific IgE antibody concentrations above 0.35 kU/L were considered positive. The detection limit was 0.1 kU/L, and functional sensitivity was 0.2 kU/L.

Eosinophilic Cationic Protein. The level of eosinophilic cationic protein (ECP) was measured by a chemiluminescent enzyme immunoassay method using an IMMULITE 2000 system (Siemens). A value within the range of 0−24 ng/mL was considered normal.

Preparation of Extracts from Wheat Flour and Rice Grains. The extracts from wheat flour (Sulamit cultivar) or commercially available long-grain rice (*Oryza sativa* L.) were prepared from raw and boiled forms. Wheat flour and rice grains were boiled for 10 min and dried at 37 °C; the boiled wheat and raw and boiled rice were milled to a fine powder. Proteins from 3 g of wheat or rice were extracted into 10 mL of PBS containing 0.9% NaCl, 0.02% NaH_2PO_4, and 0.05% Na_2HPO_4, pH 7.2; agitated at 37 °C for 2 h; and centrifuged at 3000*g* for 20 min at 20 °C. The PBS-soluble proteins were collected, divided into aliquots, and stored at −20 °C until use. The remaining pellets were resuspended in 10 mL of SDS/2-mercaptoethanol containing buffer, containing 1% SDS, 10% glycerin, 5% 2-mercaptoethanol, and 50 mM TRIS; agitated at 37 °C for 1 h; and centrifuged at 3000*g* for 20 min at 20 °C. The SDS-soluble proteins were collected, divided into aliquots, and stored at −20 °C until use. Protein concentrations in extracts were determined using a Bicinchoninic Acid Protein Assay Kit (Pierce) and slightly modified Bradford method.[26] Final concentration of all samples was adjusted to 2 mg/mL by precipitation with acetone.

Basophil Activation Test. Flow cytometric basophil activation tests (BAT) were performed after the stimulation of blood cells from patients with commercial wheat and rice allergens (Alyostal-Stallergenes), PBS extracts of wheat (Sulamit cultivar), and PBS extracts of raw and boiled rice. Activation of basophiles was measured in whole blood in heparin using the commercially available BasoFlow kit (Exbio, Prague, Czech Republic) following the manufacturer's instruction. The activated basophils expressing CD63 and CD203c were measured by FC500 flow cytometry (Beckman-Coulter, Miami, FL, USA). Samples containing >15% basophils, expressing CD63 (CD203c+, CD63+), were considered positive.

Prick Tests. Skin prick tests were performed with commercial allergens (Alyostal-Stallergenes) and with boiled rice homogenate in PBS. The following food allergens were used for testing: wheat (catalog no. 105), rice (catalog no. 160), boiled rice homogenate in PBS (concentration = 100 μg/mL), negative and positive (histamine) controls. The size of a weal 3 mm or more in diameter was considered as a positive reaction to the tested allergen.

SDS-PAGE and Immunoblotting. Extracted proteins (in a final concentration of 2 mg/mL) were separated by SDS-PAGE as described by Laemmli[27] using a 5−20% polyacrylamide gradient gel under reducing conditions (with 5% 2-mercaptoethanol) and electrophoresis run on a Mini Protean 3 cell (Bio-Rad, Hercules, CA, USA). The separated proteins were stained with Coomassie Brilliant Blue R-250 (CBB) or electrotransferred using a Mini Tran-Blot cell (Bio-Rad) onto nitrocellulose membranes (NC) for 1 h at room temperature. The membranes were blocked with TBS containing 0.1% Tween 20 and 1% milk (TTBS; 0.9% NaCl, 100 mM TRIS, 0.1% Tween 20, and 1% powdered defatted milk), and NC strips were incubated with patient sera, disease, and healthy controls, diluted 1:40 in the blocking buffer (TTBS containing 0.1% milk) overnight at 4 °C. After a washing with TTBS, the strips were incubated with peroxidase-labeled sheep anti-human IgE antibodies diluted 1:5000 with TTBS containing 1% milk for 1 h at room temperature. Development was carried out using a SuperSignal West Femto Maximum Sensitivity Substrate kit, and the exposure times onto the films (Kodak, Rochester, NY, USA) were 30 s, 1 min, and 5 min to characterize and compare IgE reactivity of the individual patient.

SDS-PAGE using longer polyacrylamide gels (18 cm) with a gradient from 5 to 20% was applied for the identification of potential rice allergens, and electrophoresis was run on a Protean II xi cell (Bio-Rad) at a constant current of 5 mA/gel for 1 h, and then 40 mA/gel for 4 h at a temperature of 20 °C. The separated proteins were stained with CBB or by slightly modified silver staining.[28,29] Development and exposure times were the same as described above.

2D Electrophoresis and Immunoblotting. Wheat and rice proteins were precipitated overnight in 20% TCA in acetone containing 0.2% DTT at −20 °C, centrifuged at 3000*g* for 15 min, resuspended in acetone containing 0.2% DTT, centrifuged at 3000*g* for 15 min, and dried. The precipitated samples were dissolved in a rehydration buffer containing 6 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris-base, 0.1% w/v bromophenol blue, 1.2% v/v DeStreak, 1% v/v Pharmalyte (pH 3−10), and 0.5% v/v Pharmalyte

Table 2. Total and Specific IgE, IgA, and IgG Antibodies and ECP (SEM)[a]

group	n	ECP (ng/mL)	total IgE (IU/mL)	total IgA (g/L)	total IgG (g/L)	specific IgE (IU/mL) wheat	specific IgE (IU/mL) rice
I	35	31.54 ± 8.20	1550.4 ± 600.8	1.37 ± 0.17	8.97 ± 0.70	3.57 ± 0.92	0.86 ± 0.26
Ia	19	26.72 ± 4.54	1528.8 ± 491.6	1.72 ± 0.21	10.81 ± 0.55	2.45 ± 0.45	1.52 ± 0.44
Ib	16	45.18 ± 29.96	1576.0 ± 1202.2	0.96 ± 0.24	6.76 ± 1.18	4.91 ± 1.93	0.08 ± 0.03
II	15	34.32 ± 6.79	272.1 ± 58.1	2.1 ± 0.23	11.49 ± 0.55	0.02 ± 0.02	0.06 ± 0.04
III	10	37.85 ± 10.43	40.0 ± 11.4	1.26 ± 0.17	9.79 ± 0.67	neg	neg

[a]Abbreviations: n, number of patients in the tested group; neg, concentration of specific IgE antibodies was under detection limit.

(pH 8−10.5). Eighteen centimeter Immobiline DryStrips with a nonlinear pH 3−10 gradient were used, and the strips were swollen in a total volume of 350 μL with the rehydration buffer containing 200 or 500 μg of protein samples.

Separation in the first dimension (isoelectric focusing) was performed with a Multiphor II unit (Amersham Bioscience). The following running conditions were used: 300 V for 30 min, 600 V for 30 min, 1000 V for 30 min, 2000 V for 30 min, 2500 V for 30 min, 3000 V for 30 min, 3500 V for 3 h, and 3500 V for 18 h; at constant conditions of 6 mA, 15 W, and 20 °C. Proteins on strips were separated in the second dimension on a 12% SDS-PAGE using a Protean II xi cell (Bio-Rad) at a constant current of 5 mA/gel for 1 h and then 40 mA/gel for 4 h, at a temperature of 20 °C.[30]

Proteins were visualized by modified silver staining[28,29] or electrotransferred with TE77XP Semidry Blotters (Hoefer, Holliston, MA, USA) onto NC membranes, and their IgE-binding proteins were visualized with pooled patient serum (patients 71, 113, and 134) or the serum of disease or healthy controls, as described above (1-DE and immunoblotting).

MALDI-TOF MS and Protein Identification. CBB-stained protein bands or spots were excised from the gel, washed, and digested with trypsin as described previously.[31] Mass spectra were acquired on an Ultraflex III MALDI-TOF/TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with LIFT technology for MS/MS analysis. The mass spectra were searched against SwissProt 2012_06 or NCBInr 20120623 database subsets of rice proteins, using an in-house MASCOT search engine. Proteins with a MOWSE score over the threshold of 48 (SwissProt) or 64 (NCBInr), calculated for the utilized settings, were considered as identified. If the score was only slightly higher than the threshold value or the sequence coverage was too low, the identity of the protein candidate was confirmed by MS/MS analysis.

RESULTS AND DISCUSSION

Reactivity of Patient IgE Antibodies with Wheat and Rice Extracts. Because rice is frequently recommended as an alternative diet for patients with food allergies and due to the relatively frequent occurrence of wheat allergy, we focused on testing IgE reactivity to rice in patients having food allergies, which included wheat (group I, $n = 35$), with pollen allergy (group II, disease controls, $n = 15$), and healthy controls for comparison (group III, $n = 10$). As shown in Table 1, patients 1−19 had IgE antibodies specific to rice (subgroup Ia), whereas others (20−35) had very low or no IgE reactivity to rice (subgroup Ib). In the disease controls suffering only from a pollen allergy (group II), IgE reactivity to rice was very low, if at all existent. As shown in Table 2, the mean level of total IgE was increased in patients with food allergies (groups I, Ia, and Ib) and in patients with a pollen allergy (group II), as compared with healthy donors, whereas the mean levels of total IgA, IgG, and ECP were very similar in all three groups.

To characterize IgE reactivity to wheat and rice allergens in more detail, we employed immunoblotting techniques. As both wheat and rice are most often consumed after thermal processing, we prepared PBS and SDS extracts of raw and boiled wheat and rice. The heterogeneity of the extracted proteins was characterized by SDS-PAGE (Figure 1). The spectra of PBS-extracted rice and wheat proteins were decreased after boiling, and in the case of the rice extract, high molecular weight components above 80 kDa newly appeared. The increased temperature slightly affected the profile of SDS-extracted rice proteins and partially changed the heterogeneity of the SDS-extracted wheat proteins. In general, the effect of boiling can alter the solubility of proteins by changing the protein structure and triggering the formation of protein aggregates.[32−34]

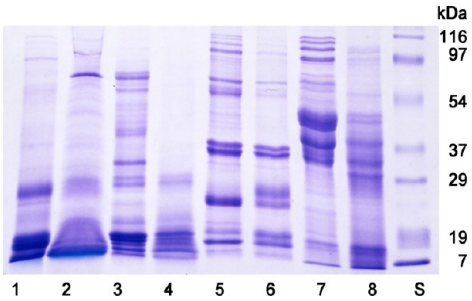

Figure 1. SDS-PAGE protein profiles of PBS and SDS extracts from raw and boiled wheat flour or rice grains. Lanes: 1, PBS extract of raw rice; 2, PBS extract of boiled rice; 3, PBS extract of raw wheat; 4, PBS extract of boiled wheat; 5, SDS extract of raw rice; 6, SDS extract of boiled rice; 7, SDS extract of raw wheat; 8, SDS extract of boiled wheat; S, molecular weight standard. Ten micrograms of protein was loaded per well, and the gel was stained with CBB.

The specific IgE-binding to proteins in raw or boiled wheat flour was demonstrated by immunoblot using sera of the patient group and controls, as described above. An illustrative example of IgE reactivity of five patients to wheat proteins is documented in Figure 2. The individual IgE reactivity with raw wheat components in PBS extract, ranging from 7 to 80 kDa (Figure 2A), was markedly reduced by boiling (Figure 2B). Only the low molecular weight IgE-binding proteins were detected, which corresponds to the lower extraction of high molecular weight wheat proteins in PBS (Figure 1, lane 4). The similar effect of thermal processing (such as baking) on the IgE binding to Tris-HCl-extracted wheat proteins has also been described previously.[35] Our data support the finding that the low molecular weight wheat components of about 14 kDa belonging to the α-amylase/trypsin inhibitor family were identified as major wheat allergens.[4,36] Interestingly, the heterogeneity of IgE reactivity with SDS-extracted proteins from raw and boiled wheat was affected only slightly, which may be explained by the higher thermal stability of these proteins.

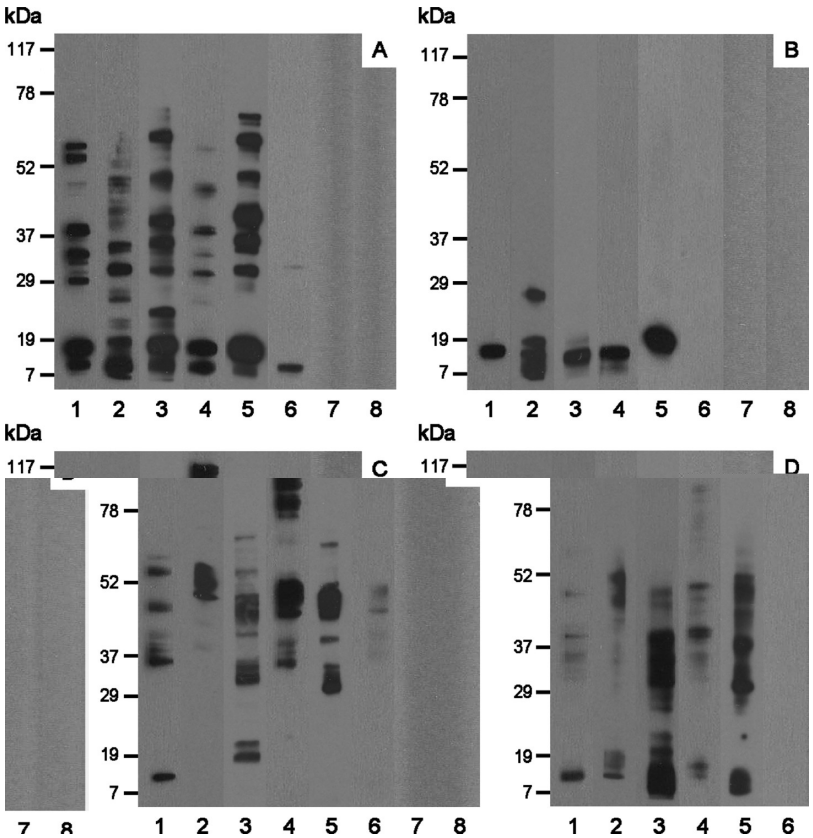

Figure 2. IgE immunoblotting results for wheat protein components in PBS or SDS extracts, from raw and boiled forms of wheat: (A) PBS extract of raw wheat; (B) PBS extract of boiled wheat; (C) SDS extract of raw wheat; (D) SDS extract of boiled wheat. The figure provides an example of the IgE reactivity of five patients with food allergies (lanes 1−5), a disease control with a pollen allergy (lanes 6), a healthy donor (lanes 7), and the secondary anti-human IgE antibody (lanes 8).

We further characterized the heterogeneity of IgE-binding components in PBS and SDS extracts of raw or boiled rice using 19 patients' sera (group Ia) with IgE antibodies specific to rice (Table 1). An illustrative example of IgE reactivity of five patients to rice components on an immunoblot is shown in Figure 3. We detected the whole spectrum of IgE-binding proteins from 9 to 117 kDa in a PBS extract from raw rice (Figure 3A). By evaluation of individual reactivity to separated proteins, we estimated that rice proteins of about 14, 29, 37, 52, and 60−65 kDa are recognized by IgE antibodies, with the highest frequency about 60−70%; proteins of about 19, 24, 33, 45, 78, 98, and 117 kDa were detected with a lower frequency from 30 to 45%. The thermal processing of PBS-extracted rice proteins changed the number of IgE binding PBS-extracted proteins (Figure 3B), as well as the frequency of recognition (from 10 to 30%). The main IgE-binding proteins in the SDS extract from raw rice were of about 37 and 60−65 kDa and were detected with frequencies of 53 and 32%, respectively. Surprisingly, in the SDS extract the number of IgE-binding proteins increased after boiling (Figure 3D), and the frequency of recognition of these proteins with molecular weights of about 14, 19, 24, 29, 37, and 60−65 kDa varied from 35 to 80%.

We can conclude that thermal processing had a different effect on the solubility of wheat and rice PBS- or SDS-extracted proteins and either reduced or increased IgE-binding capacity and the heterogeneity of individual proteins. In general, food proteins exposed to heat can form complexes with fats, sugars, and other food components that could affect the extractability of proteins. One of the most important modifications of food proteins is a nonenzymatic glycation, Maillard reaction.[37,38] All of these processes may uncover so-called cryptic allergenic epitopes or even introduce new structural modifications (including the formation of new antigenic epitopes) and markedly affect the allergenicity of these food proteins.

Reactivity of Patients to Wheat and Rice Extracts in BAT and SPT. To compare IgE-mediated cell response, we used commercial and PBS extracts from wheat and rice. We found a comparable level of basophil activation in the group Ia patients (n = 10) with commercial and PBS wheat extracts (mean values of 49.0 ± 9.5 and 49.9 ± 8.8%, respectively). The similarly small differences in BAT values were obtained using commercial and PBS rice extracts (mean values of 20.8 ± 9.9 and 25.9 ± 9.7%, respectively). However after boiling rice, BAT activation with a PBS extract was decreased to values close to the negative control. The activation of basophils was negative for the disease controls, employing all tested extracts (n = 4).

Patients with high specific IgE serum antibodies and positive in BAT to raw wheat and rice (patients 14 and 17−19) were further tested by the skin prick test (SPT) using wheat and rice extracts. The SPT was performed with commercial extracts of wheat and rice and with a boiled rice homogenate in PBS (100 μg/mL) containing both PBS- and SDS-soluble rice proteins. The data, summarized in Table 3, indicated that three of four patients tested clearly reacted to the boiled rice homogenate. These patients (14, 17, and 18; characterized in Table 1) were IgE positive for more allergens and were on a wheat-free diet.

The lower positivity in BAT compared to SPT could be caused by using different rice proteins in the tests. In BAT, only

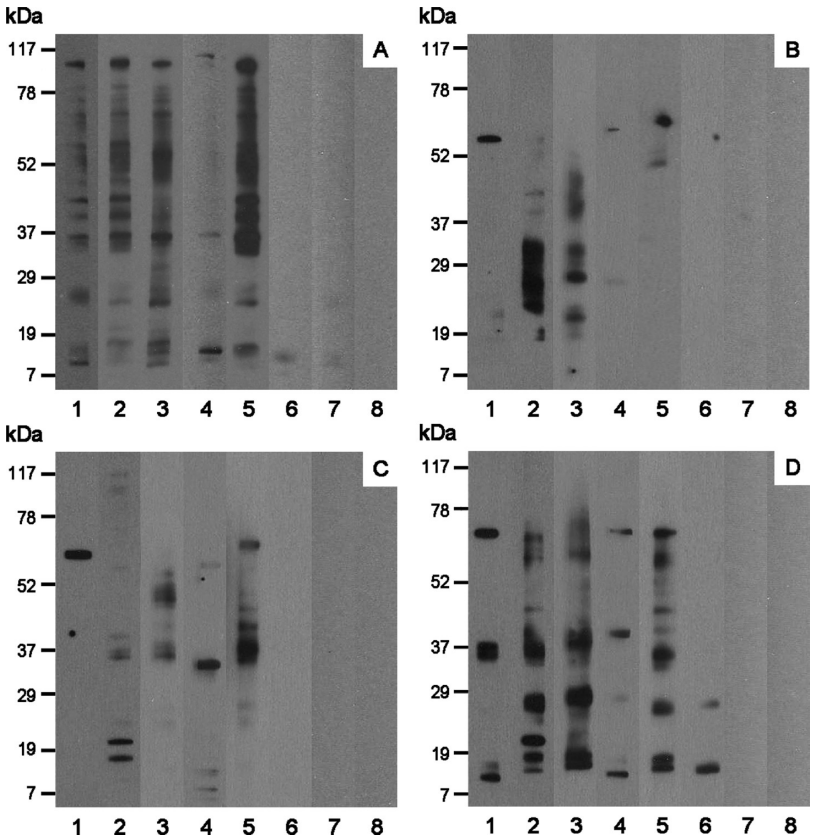

Figure 3. IgE immunoblotting results for rice protein components in PBS and SDS extracts, from raw and boiled forms of rice: (A) PBS extract of raw rice; (B) PBS extract of boiled rice; (C) SDS extract of raw rice; (D) SDS extract of boiled rice. The figure provides an example of the IgE reactivity of five patients with food allergies (lanes 1−5), a disease control with a pollen allergy (lanes 6), a healthy donor (lanes 7), and the secondary anti-human IgE antibody (lanes 8).

Table 3. Skin Prick Test

	diameter of weal on skin (mm)				
patient	wheat[a]	rice[a]	boiled rice homogenate	negative control	positive control[b]
14	5	5	3	0	8/25
17	5	3	3	0	10
18	3	3	3	0	7/25
19	5	0	2	0	7/20

[a]Commercial extract. [b]Assessments of both the weal (swelling and edema) and erythema (flame, redness) reactions were recorded. Patient 17 had only weal reaction.

PBS-soluble proteins from boiled rice could be used, whereas in SPT the whole rice homogenate (containing both PBS- and SDS-soluble proteins present in real foodstuffs) was applied. We showed that rice proteins soluble in SDS maintained IgE-binding capacity, also after boiling, as detected by immunoblotting. These proteins cannot be tested in the BAT assay (using PBS extracts) but could be involved in the SPT reactivity of IgE highly positive patients.

Although rice is often recommended as a diet alternative to wheat-allergic patients, our data suggest that additional testing of a boiled rice homogenate in SPT could be beneficial, especially for patients with high IgE antibodies specific to rice (measured, e.g., by an IMMULITE 2000 system and/or immunoblot), prior to the prescription of a suitable diet.

Characterization and Identification of IgE-Binding Rice Proteins. We identified potential IgE-binding rice proteins in PBS- and SDS-extracted boiled rice. The proteins were separated by either 1-DE or 2-DE, and the IgE-binding proteins were detected using the pooled sera from three selected patients (14, 17, and 18 with IgE antibodies specific to rice and positive reaction to rice in BAT and SPT). The 1-DE-separated proteins recognized by patient IgE antibodies with the highest frequency were identified by MALDI-TOF MS (Figure 4). Rice proteins (one or two) reacting with the pooled sera of healthy donors or with secondary antihuman IgE antibodies were not included in the MS analysis (data not shown). We identified IgE-binding proteins in the SDS (Figure 4, lane 1) and in the PBS extracts (Figure 4, lane 2). A list of all of the identified proteins can be found in Table 4. In the SDS extract, bands 1 and 2 were a mixture of two or three rice seed proteins (RA5, RAG2, and RAG1) belonging to the α-amylase/trypsin inhibitor family. One of them, RAG2, was previously identified in a raw rice PBS extract as an IgE-binding protein.[39] Bands 5 and 8 corresponded to the large family of glutelins, major seed storage proteins encoded as a multigene family. Six major glutelin genes or subunits have been identified in the Asian japonica rice subspecies. These genes have been classified into two subfamilies, GluA and GluB, according to their relatedness in nucleotide sequences.[40] Bands 3 (SDS extract) and 10 (PBS extract) corresponded to a 19 kDa globulin belonging to the α-globulins, previously isolated and identified in a salt extract from rice by Satoh et al.[24] Two separated proteins, glutelin C precursor (band 4) and granule-bound starch synthase 1 chloroplastic/amyloplastic protein (band 9), were identified as new potential rice allergens. Simultaneously

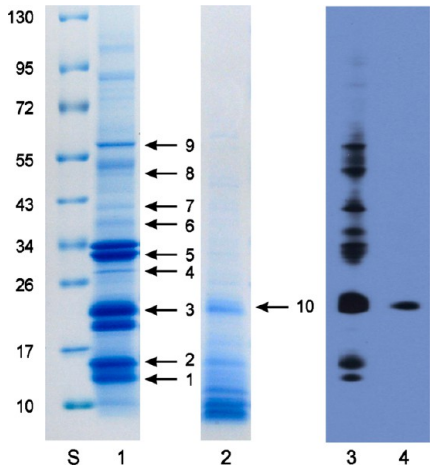

Figure 4. Proteins separated under reducing conditions on a large 1-DE gel from SDS (lane 1) and PBS (lane 2) boiled rice extracts. The gel was stained by CBB, and bands selected for identification were marked by arrows. The bands were selected on the basis of the IgE reactivity of pooled patient sera with SDS (lane 3) and PBS (lane 4) boiled rice extracts on immunoblot; lane S is molecular weight standard. The numbering of the selected protein bands refers to Table 4.

with us, this rice protein was identified as a potential rice allergen by Krishnan and Chen.[41] Band 7 was also a mixture of proteins: aspartate aminotransferase and two rice proteins encoded by gene loci Os08g0545200 and Os01g0905800. In summary, from the 1-DE gel we identified two separated unique IgE-binding rice proteins as new potential rice allergens: glutelin C precursor and granule-bound starch synthase 1 chloroplastic/amyloplastic protein; others were identified in a combined mixture.

Therefore, we utilized 2-DE to further separate proteins far more effectively. We analyzed 16 spots by MS (numbered 101−116, Figure 5A,B) and identified 9 IgE-binding proteins (Table 5). Spot 101 corresponded to disulfide isomerase-like 1-1 protein. Spots 102 and 103 were identified as a hypothetical protein OsI_13867, and spot 108 was identified as a putative acid phosphatase precursor 1. Spots 109 and 110 corresponded to a glutelin type-A 1, a large seed storage protein also identified in the 1-DE gel. Spot 111 was a mixture of two proteins, a glutelin type-A 1 and a rice protein encoded by the gene locus Os02g0453600. A rice protein encoded by the same gene locus was identified in spot 112 together with a glutelin type-B 1. Herein, we could only speculate about the IgE-binding capacity of the protein encoded by locus Os02gO453600, because both spots 111 and 112 were mixed with the already known IgE-binding proteins, glutelin type-A 1 or glutelin type-B 1, respectively.[40] Glutelin type-B 1 was also identified in the separated spot 113. Spots 114, 115, and 116 corresponded to a 19 kDa globulin, the seed allergenic protein RAG2, and a protein encoded by the gene locus

Table 4. MALDI-TOF MS Identification of Potential Rice Allergenic Proteins from SDS-PAGE Experiments

band[a]	extract	protein (potential allergen) name	accession no.	MW (kDa)	peptides matched	sequence coverage (%)	MS/MS confirmation
1	SDS	seed allergenic protein RA5	RA05_ORYSJ	17	6	41	no
		seed allergenic protein RAG2	RAG2_ORYSJ	18	6	34	no
2	SDS	seed allergenic protein RA5	RA05_ORYSJ	17	11	53	ELGAPDVGHPMSEVFR CEAISHMLGGIYR
		seed allergenic protein RAG2	RAG2_ORYSJ	18	9	65	ELGATDVGHPMAEVFPGCR
		seed allergenic protein RAG1	RAG1_ORYSJ	18	5	49	QLAAVDDGWCR GAASAADEQVWQDCCR
3	SDS	19 kDa globulin	GL19_ORYSJ	21	12	22	no
4	SDS	glutelin C precursor[b]	37993736	55	13	21	no
5	SDS	glutelin type-A 1[b]	GLUA1_ORYSJ	56	10	26	LQAFEPIR GLLLPHYTNGASLVYIIQGR
		glutelin type-A 3[b]	GLUA3_ORYSJ	56	5	13	GLLLPHYSNGATLVYVIQGR
		glutelin type-B 1[b]	GLUB1_ORYSJ	56	4	7	no
7	SDS	Os08g0545200	115477633	39	13	40	LPPVGPYDVR FGFSQEDVEEAFEVSAR
		aspartate aminotransferase	AATC_ORYSJ	44	9	34	LIFGADSPAIQENR
		Os01g0905800	297598143	39	8	23	IGPNEPSQLSIDLNAQGLAR
8	SDS	glutelin type-A 1	GLUA1_ORYSJ	56	8	26	VEHGLSLLQPYASLQEQEQGQVQSR
		glutelin type-B 1	GLUB1_ORYSJ	56	8	17	YTNIPGVVYIIQGR
		glutelin type-B 4	GLUB4_ORYSJ	57	6	19	no
9	SDS	granule-bound starch synthase 1 chloroplastic/amyloplastic protein	SSG1_ORYSI	66	15	30	no
10	PBS	19 kDa globulin	GL19_ORYSJ	21	6	20	FQPMFR

[a]Band 6 was not identified by mass spectrometry. [b]Identified as an N-terminal fragment of the intact protein.

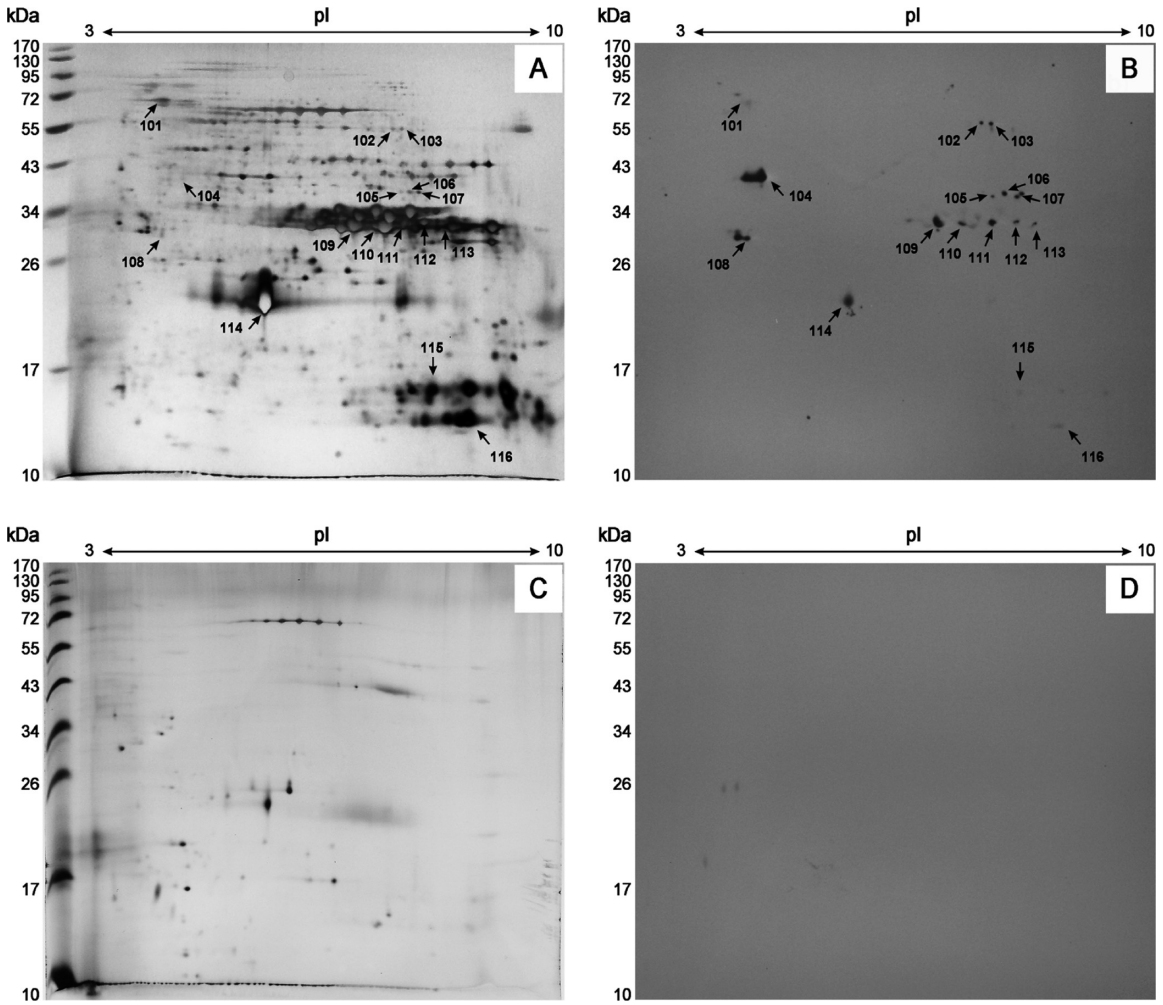

Figure 5. Spectra of proteins in the boiled form of the rice SDS and PBS extracts separated by 2-DE: (A) proteins from the SDS extract visualized by silver staining; (B) proteins recognized by the IgE antibodies of patients with food allergy; (C) proteins from the PBS extract visualized by silver staining; (D) proteins recognized by the IgE antibodies of patients with food allergy. The spots selected for identification are numbered. The numbering refers to Table 5.

Table 5. MALDI-TOF MS Identification of Potential Rice Allergenic Proteins in the SDS Extract from 2-DE Experiments

spot[a]	protein (potential allergen) name	accession no.	MW (kDa)	p*I*	peptides matched	sequence coverage (%)	MS/MS confirmation
101	protein disulfide isomerase -like1-1	PDI11_ORYSJ	57	5.0	11	31	SDYDFGHTLHANHLPR
102	hypothetical protein OsI_13867	218193892	52	7.0	7	20	no
103	hypothetical protein OsI_13867	218193892	52	7.0	4	10	FPDEQVVGAAVGGYR
108	putative acid phosphatase precursor 1	53792717	28	4.9	5	26	LYNELQGLGIHIILLTGR
109	glutelin type-A 1	GLUA1_ORYSJ	56	9.1	10	12	QFQCTGVSVVR LQAFEPIR
110	glutelin type-A 1	GLUA1_ORYSJ	56	9.1	9	13	GLLLPHYTNGASLVYIIQGR
111	Os02g0453600	115445979	57	9.0	5	11	EFFLAGKPR
	glutelin type-A 1	GLUA1_ORYSJ	56	9.1	4	8	LQAFEPIR
112	Os02g0453600	115445979	57	9.0	8	14	QFSFGGSPLQSPR
	glutelin type-B 1	GLUB1_ORYSJ	57	9.3	3	7	no
113	glutelin type-B 1	GLUB1_ORYSJ	57	9.3	7	13	QLFNPSTNPWHSPR
114	19 kDa globulin	GL19_ORYSJ	21	7.5	6	43	QGYYGEGSSEEGYYGEQQQQPGMTR
115	seed allergenic protein RAG2	RAG2_ORYSJ	18	8.1	8	51	QLAAVDDSWCR CQPGMGYPMYSLPR
116	Os07g0216700	115471187	16	7.5	10	68	AGYGGYGDVGEYCR ELAAVPMQCR

[a]Spots 104, 105, 106, and 107 were not identified by MS.

Os07g0216700, respectively. We also tried to characterize rice proteins from the PBS extract (Figure 5C), but no or very low IgE reactivity was detected (Figure 5D). We can suppose that the loss of IgE binding to PBS-extracted proteins, including the

19 kDa globulin identified from 1-DE, may be caused by the damage of IgE epitopes during the treatment of the sample for 2-DE (higher salt concentration and detergent). In summary, from the 2-DE gel we identified nine rice proteins, of which five were already known and four have newly been described as potential rice allergens: disulfide isomerase-like 1-1 protein, hypothetical protein OsI_13867, putative acid phosphatase precursor 1, and protein encoded by locus Os02g0453600. Interestingly, although plant food allergens belong to a rather limited number of proteins, the already known IgE-binding rice proteins and those newly identified by us differ widely from the known IgE-binding wheat proteins (except proteins belonging to the α-amylase/trypsin inhibitor family).[31]

In conclusion, we have demonstrated that patients with food (mainly wheat) and pollen allergies often have increased levels of IgE antibodies specific to rice. More than 80% of these patients reacted to SDS-extracted boiled rice proteins, and those with the highest IgE-binding capacity were positive in a SPT. On the basis of these data, we could suggest that the patients with high IgE antibodies specific to rice, and positive on immunoblot, should be further tested by a SPT with boiled rice components before rice is recommended as a suitable hypoallergenic diet alternative. Using proteomic techniques we analyzed SDS-extracted rice proteins and identified six new potential rice allergens (from 1-DE and 2-DE) that also retain IgE-binding capacity after thermal processing, potentially representing important allergens in rice-containing foodstuffs. However, further experiments are necessary to confirm these proteins as newly defined rice allergens.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; BAT, basophil activation test; CBB, Coomassie Brilliant Blue; ECP, eosinophilic cationic protein; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; NC, nitrocellulose; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate buffer; SDS-PAGE, sodium dodecyl sulfate solution−polyacrylamide gel electrophoresis; SPT, skin prick test; TBS, Tris-buffered saline

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