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Immunoglobulin E-reactivity of wheat-allergic subjects (baker's asthma, food allergy, wheat-dependent, exercise-induced anaphylaxis) to wheat protein fractions with different solubility and digestibility

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Baker's asthma, food allergy to wheat, and wheat-dependent, exercise-induced anaphylaxis (WDEIA) are different clinical forms of wheat allergy. We investigated the correlation of solubility and digestion stability of wheat allergens with the IgE-reactivity patterns of different patient groups. Three wheat protein fractions were extracted according to their solubility: salt-soluble albumins and globulins, ethanol-soluble gliadins, and glutenins soluble only after treatment with detergents and reducing reagents. Sera from subjects with history of each variant of wheat allergy were characterized by CAP FEIA and immunoblotting. There was a high degree of heterogeneity of recognized allergens between the different subject groups as well as within these groups. However, subjects with WDEIA showed similar immunoglobulin E (IgE)-reactivity patterns to gliadins and especially to a 65 kDa protein. Subjects with baker's asthma as well as the food-allergic subjects had the most intense IgE-reactivity to the albumin/globulin fraction. The latter group additionally showed IgE-reactivity to the other fractions. Divergent results of immunoblotting and CAP-FEIA demonstrated that the detection of wheat-specific IgE highly depends on the applied method, thus the diagnostic tool must be carefully chosen. Most wheat allergens were rapidly digested as analyzed by determination of IgE-reactivity on immunoblots to wheat extracts after simulation of gastric and duodenal digestion. However, ethanol-soluble gliadins were stable to gastric enzymes and exhibit low solubility in gastric and duodenal fluids. Therefore, they are likely to be important in food allergy to wheat.

Keywords: Immunoglobulin E / Wheat allergy / Wheat proteins

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1 Introduction

Three clinical forms of immunoglobulin E (IgE)-mediated allergy to wheat flour are known: Baker's asthma and IgE-mediated food allergy, which additionally may occur as wheat-dependent, exercise-induced anaphylaxis. These clinical forms are distinguished from nonallergic food hypersensitivity to wheat, for example, celiac disease, since nonallergic hypersensitivity is not IgE antibody-mediated [1]. Baker's asthma is an occupational disease which is often caused by the inhalation of wheat flour [2 – 5]. Food allergy occurs after ingestion of wheat flour or wheat-containing foods. Subjects suffering from exercise-induced anaphylaxis show an allergic reaction after ingestion of wheat only in connection with physical strain [6].

Wheat proteins are classified into three fractions according to their solubility: (i) salt-soluble albumins and globulins, (ii) salt-insoluble gliadins, and (iii) glutenins [7]. A relationship between protein solubility and the various clinical manifestations of wheat allergy has been suggested [8]. Salt-soluble proteins are resorbed in the respiratory tract and may elicit IgE-mediated allergic reactions [8], whereas salt-insoluble proteins may be resistant to digestion during

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Abbreviations: IgE, immunoglobulin E; **RAST**, radio allergosorbent test; **SDF**, simulated duodenal fluid; **SGF**, simulated gastric fluid; **WDEIA**, wheat-dependent, exercise-induced anaphylaxis

passage through the stomach and intestine. Structurally intact allergens, large enough to cross-link mast cell-bound specific IgE antibodies, may be resorbed and lead to allergen-specific sensitization or trigger allergic symptoms [8].

Sensitized individuals have IgE antibody reactivities to a wide range of wheat proteins [9]. From the albumin/globulin fraction the α-amylase inhibitor subunits with molecular masses from 12 to 18 kDa are considered to be important allergens for subjects with baker's asthma [2, 10–14]. Weiss *et al.* [14] identified an IgE-reactive, highly allergenic 27 kDa protein by immunoblotting. *N*-Terminal amino acid sequencing of proteins identified by 2-D PAGE and blotting showed that several 27 kDa proteins with different isoelectric points and functions exist. Sander *et al*. [15] demonstrated a great variation of IgE-binding patterns of wheat flour proteins in baker's asthma and detected more than 100 IgE-binding proteins by means of 2-D immunoblotting. Palosuo *et al.* [16, 17] studied the wheat-specific IgE-reactivity of subjects with exercise-induced anaphylaxis. All subjects had IgE antibodies against a 65 kDa omega-5 gliadin (Tri a 19) which appeared to be a major allergen for this form of wheat allergy. A 65 kDa gliadin was also described as an allergen by two other authors, in connection with exercise-induced anaphylaxis and dermatitis herpetiformis, respectively [18, 19]. Several major wheat allergens were described by different investigators who preferentially analyzed one of the three forms of wheat allergy: food allergy [20–22], wheat-dependent, exercise-induced anaphylaxis [16], baker's asthma [23, 24], or atopic dermatitis [25, 26]. Tanabe *et al.* [25] and Maruyama *et al.* [26] identified a glutenin containing repeated Gln-Gln-Gln-Pro-Pro motifs as an allergen.

In this study, we analyzed and compared the IgE-reactivity of three wheat protein fractions (albumin/globulin, gliadin, glutenin) using sera of subjects suffering from baker's asthma, food allergy to wheat, or wheat-dependent, exercise-induced anaphylaxis (WDEIA). IgE-reactivities of each group against salt-soluble albumins and globulins (fraction 1), ethanol-soluble gliadins (fraction 2), and glutenins (fraction 3), which are soluble after treatment with detergents and reducing agents, were investigated by immunoblotting. Furthermore, specific IgE against wheat and gluten was determined by a commercially available fluorescent-enzyme immunoassay (CAP FEIA, Pharmacia Diagnostics, Uppsala, Sweden) and the results compared to the results obtained by immunoblotting.

The stability of the allergens against digestion by gastric and duodenal enzymes may be a reason for different IgE-reactivities to wheat proteins in cases of respiratory or food allergy. The influence of digestion and the relationship to solubility of the allergens were investigated by simulated digestion of the three wheat protein fractions. IgE-reactivity was tested after digestion of wheat proteins under simulated gastric and duodenal conditions by immunoblotting.

2 Materials and methods

2.1 Sera

Sera of subjects with symptoms of baker's asthma (n = 15), food allergy against wheat (n = 14; 5 adults, 8 children) or wheat-dependent, exercise-induced anaphylaxis (n = 3) were obtained from the sera collection of the Paul-Ehrlich-Institut, Langen (Germany), the Charité, Berlin (Germany), and the Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin (BGFA), Bochum (Germany). Clinical data of the patients are briefly summarized in Table 1.

2.2 Detection of specific IgE

The IgE-reactivity against wheat flour and gluten was tested by CAP FEIA, a fluorescence enzyme immunoassay (Pharmacia Diagnostics, Uppsala, Sweden) (Tables 1, 2).

2.3 Fractionation of wheat grain proteins

Wheat grain provided by Engelbert's Bioprodukte (Itter, Austria) was milled at 10 000 rpm (3 pulses, 5 s) using an electric mill (Grindomix GM 200; Retsch, Haan, Germany). The fractionation of wheat proteins was performed according to Weiss *et al.* [14, 27]. One gram wheat flour was stirred in 4 mL 50 mM Tris-HCl , pH 8.8, at 4°C for 1 h. The suspension was centrifuged at 20 000 × g for 20 min at 4°C. The supernatant was the albumin/globulin fraction. Each extraction step was followed by washing and centrifugation of the precipitate. First, the precipitate was washed three times with 50 mM Tris-HCl, pH 8.8, and one time with distilled water, and mixed with 4 mL 75% v/v ethanol for 2 h at room temperature. The suspension was centrifuged and the supernatant was the gliadin fraction. The precipitate was washed three times with 75% ethanol and one time with distilled water. Glutenins were extracted with 4 mL 50 mM Tris-HCl, pH 8.8, containing 1% SDS and 0.5% DTT by stirring for 2 h at RT and centrifuged at 20 000 × 6 for 1 h. The supernatants were aliquoted and stored at –80°C. The protein content was measured according to the method of Bradford using a commercial kit (Coomassie Plus Protein Assay Reagent, Pierce, Rockford, IL, USA) [28].

2.4 Electrophoresis and immunoblotting

SDS-PAGE was performed according to Schaegger and von Jagow [29] using a 14%T/2.5%C separating gel and a stack-

Table 1. Characteristics of wheat-allergic subjects tested in this study

Serum	Source	Clinical symptoms after inhalation or ingestion of wheat	CAP wheat flour	IgE-reactivity in immunoblot		
				Fraction 1	Fraction 2	Fraction 3
1	PEI	Nonallergic control	0	–	–	–
Baker's asthma, adults						
2	PEI	Dyspnea	3	+	-	+
3	PEI	Asthma after inhalation of flour dust	3	+	-	+
4	PEI	Itching after contact of skin with wheat and rye; asthma	2	+	+	+
5	PEI	Rhinitis, conjunctivitis, itchiness, asthma after contact with wheat, rye, barley, rice, wheat pollen	4	+	+	+
6	PEI	Baker's asthma	0	–	–	–
7	PEI	Baker's asthma	0	–	–	–
8	BGFA	Baker's asthma	3	+	–	+
9	BGFA	Baker's asthma	3	+	–	+
10	BGFA	Baker's asthma	3	–	–	–
11	BGFA	Baker's asthma	3	–	–	–
12	BGFA	Baker's asthma	3	+	–	–
13	BGFA	Baker's asthma	3	+	–	+
14	BGFA	Baker's asthma	3	–	–	–
15	BGFA	Baker's asthma	3	–	–	–
16	BGFA	Baker's asthma	3	+	–	–
Food allergy, adults						
17	PEI	OAS, stomach ache, gastrointestinal complaints through wheat flour or bread	0	+	–	–
18	PEI	Heartburn through wholemeal products and oat flakes	1	+	–	+
19	PEI	OAS	2	+	–	+
20	PEI	Edema, rhinitis, conjunctivitis, cough, dyspnea	0	-	+	+
21	Charité	Urticaria, dizziness	2	+	+	+
Food allergy, children						
22	Charité	Worsening of atopic eczema	5	+	+	+
23	Charité	worsening of atopic eczema	5	+	+	+
24	Charité	Positive oral provocation: urticaria	4	+	+	+
25	Charité	Positive oral provocation: urticaria	5	+	+	+
26	Charité	Positive oral provocation: impairment of eczema	n.t.	+	–	+
27	Charité	Positive oral provocation: urticaria, vomiting	3	+	+	+
28	Charité	Positive oral provocation: urticaria, edema	0	+	+	+
29	Charité	Positive oral provocation: urticaria, eczema, dyspnea	n.t.	+	+	+
Wheat-dependent exercise-induced anaphylaxis, adults						
30	Charité	WDEIA	0	–	+	+
31	Charité	WDEIA	2	+	+	+
32	Charité	WDEIA	2	–	+	+

n.t., not tested
PEI, Paul-Ehrlich-Institut, Langen, Germany
BGFA, Berufsgenossenschaftliches Forschungsinstitut, Bochum, Germany
OAS, oral allergy syndrome

ing gel of 5%T/2.5%C, respectively. Extracts were diluted with SDS-PAGE sample buffer (50 mM Tris-HCl, pH 8.8, 1% SDS, 0.5% DTT, 20% glycerol and 0.005% bromophenol blue) and boiled for 5 min at 95°C. After cooling to about 60°C, DTT was added to a final concentration of 0.2% to avoid reformation of disulfide bonds. The protein load on the gel was 20 µg/cm. Electrophoresis was performed in a MiniProtean 3 cell (BioRad, Munich, Germany) at 100 V for 30 min followed by 200 V for 45 min (Power Supply PP 2000, Biometra, Göttingen) for gel size $80 \times 66 \times 1$ mm. After electrophoresis the gels were stained with Coomassie Brilliant Blue (Pierce) to evaluate proper protein separation. Proteins were transferred from the separating gel onto nitrocellulose membrane BA-85 with pore size 0.2 µm (Schleicher und Schuell, Dassel, Germany) by semidry blotting using a Novablot apparatus (Amersham Pharmacia Biotech, Freiburg, Germany) at 0.8 mA per cm^2 for 45 min [30]. The nitrocellulose was two times incubated in blocking solution (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, containing 0.3% Tween 20) for

Table 2. IgE-reactivity against gluten in CAP FEIA

Serum	CAP class gluten (gliadin + glutenin)	CAP class wheat flour
Food allergy		
21	3	2
Exercise-induced anaphylaxis		
30	2	0
31	3	2
32	3	2

15 min, air-dried and stored at −20 °C until further usage. To prepare the blotted membrane for immunodetection, it was washed in TBS containing 0.05% Tween 20 for 5 min. The blot was then incubated in patients' sera (1 : 6 dilution) over night at room temperature using a surf blot apparatus (IDEA Scientific, Minneapolis, USA). After washing three times, the blot was incubated with alkaline phosphatase-conjugated mouse-anti-human-IgE (1 : 750, BD Pharmingen, San Diego, CA, USA) at room temperature for 3 h. After washing three times the blot was developed with 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) using a commercial kit (BioRad) [31].

2.5 *In vitro* digestion

The digestion was performed in a two-step procedure. First, a gastric digestion according to Astwood *et al.* [32] and, second, a duodenal digestion according to Vieths *et al.* [33] with modifications of Astwood *et al.* was performed. Gastric and duodenal conditions were simulated using commercial enzyme tablets which are used to substitute individuals with reduced pepsin production or pancreas function. Gastric tablets (Enzynorm forte, Medicopharm, Vienna) which contain 225 – 250 mg porcine stomach extract per tablet were used for simulated gastric fluid (SGF). A tablet was ground with mortar and pestle, dissolved in 100 mL 30 mM NaCl, and the pH was adjusted to 1.2 with HCl. Powdered porcine pancreatin extract containing 3700 FIP units pancreatic lipases, 3000 FIP pancreatic amylases, and 250 FIP pancreatic proteases was used to mimic duodenal conditions (FIP = Fédération Internationale Pharmaceutique, 1 FIP is equivalent to the amount of enzyme which hydrolyses under described conditions 1 µmol of specific substrate per minute). Substrates for proteases are *N*-benzoyl-L-arginine-ethylester (trypsin, papain) or *N*-acetyl-L-tyrosine-ethylester (chymotrypsin) [34, 35]. For simulated duodenal fluid (SDF) 0.25 g of this powder was dissolved in 20 mL TBS, pH 7.4 (1 M Tris, 1 M NaCl, 25 mM $MgCl_2$). Extracted wheat protein fractions containing 40 µg protein or 10 mg milled wheat grain was incubated with SGF to final volume of 200 µL for incubation times from 0 s to 1 h at 37°C. To investigate the combined digestion by both SGF and SDF extracts containing 60 µg protein were incubated in SGF for 4 min (final volume 200 µL) and then 200 µL SDF was added. Samples were incubated for different times from 0 s to 45 min at 37°C. All incubations were carried out under agitation. Buffer controls without enzymes were performed with the same amounts of wheat protein. SDS-PAGE sample buffer was added and the sample was heated to 95°C to stop digestion. SDS-PAGE and immunoblot analysis were performed as described above. Serum pools were used to analyze IgE-reactivity against digested wheat proteins in the patient group baker's asthma (sera 3, 5) and food allergy (sera 21, 25, 27 – 29). To represent the group of patients with exercise-induced anaphylaxis, serum 31 was used.

3 Results

3.1 IgE-reactivity of each patient group to wheat protein fractions

Immunoblot analysis demonstrated different IgE-binding patterns among the subject groups as well as within the groups. There was a high degree of heterogeneity of recognized allergens. No major allergens were found to dominate all groups as shown by the allergograms in Fig. 1. Representative results from selected subjects are shown in Fig. 2. Subjects with baker's asthma did not show any recurring pattern of IgE-reactivity. Nine of 15 tested subjects suffering from baker's asthma showed reactivity to albumins/globulins (fraction 1), seven had IgE antibodies against glutenins (fraction 3) and only two against gliadins (fraction 2) with low avidity. Some individuals showed strong IgE-reactivity to allergens of fraction 1 in the range of 12 – 20 kDa and allergens with higher molecular masses. Only few allergens were detected, and with low avidity, in the glutenin fraction (Figs. 1 and 2).

Sera of six adults and 8 children with food allergy were tested. Food-allergic children showed, in some cases, very intense reaction to many proteins of fractions 1 and 3. Seven childrens' sera contained IgE antibodies against the gliadin fraction. Most gliadin allergens had molecular masses between 30 and 90 kDa. The sera did not react with any similar allergen patterns, although sera with the highest affinities reacted with some proteins common across the group such as the 12, 16, 27, and 75 kDa proteins in fraction 1, and 45 and 75 kDa proteins in fraction 3 (Figs. 1 and 2, for example No. 22, 23). In adults with food allergy, different IgE reactivity patterns were obtained. Four sera contained IgE-antibodies against fraction 1, two against fraction 2, and four against fraction 3. Detection intensity was low except for serum 21 which detected gliadin and glutenin allergens with high intensity and also salt-soluble pro-

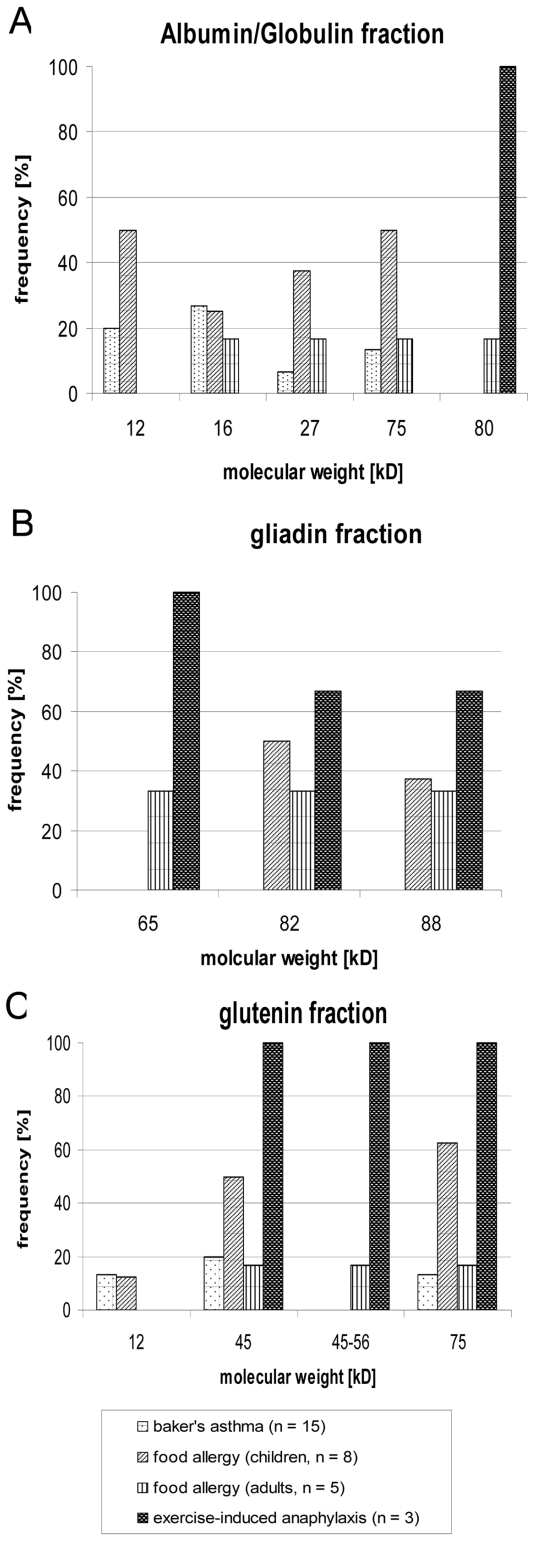

Figure 1. Frequency of IgE reactivity against wheat proteins of subjects suffering from baker's asthma, food allergy against wheat and wheat-dependent, exercise-induced anaphylaxis. (A) fraction 1 (albumins/globulins), (B) fraction 2 (gliadins), (C) fraction 3 (glutenins).

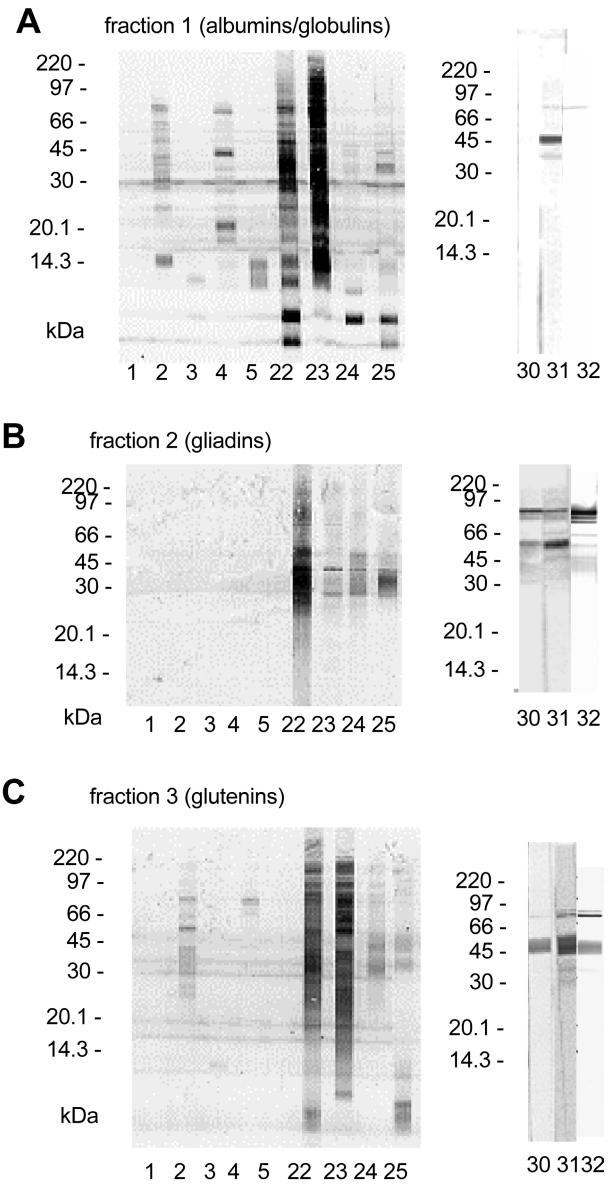

Figure 2. IgE reactivities of subjects with baker's asthma, food allergy and WDEIA to (A) albumin/globulin, (B) gliadin, and (C) glutenin fraction of wheat proteins. 1, Nonallergic subject; 2–5, baker's asthma; 22–25, food allergy; 30–32, WDEIA.

teins, but with less intensity. Only three sera of subjects with WDEIA were available (Nos. 30–32). These patients showed strong IgE-reactivity to gliadins, in the molecular mass range of 65, 82, and 88 kDa, and to glutenins, but very low reactivity to the albumin/globulin fraction (Figs. 1 and 2). A similar protein binding pattern to that of the WDEIA subjects was found in one patient from the group of food-allergic adults (No. 21).

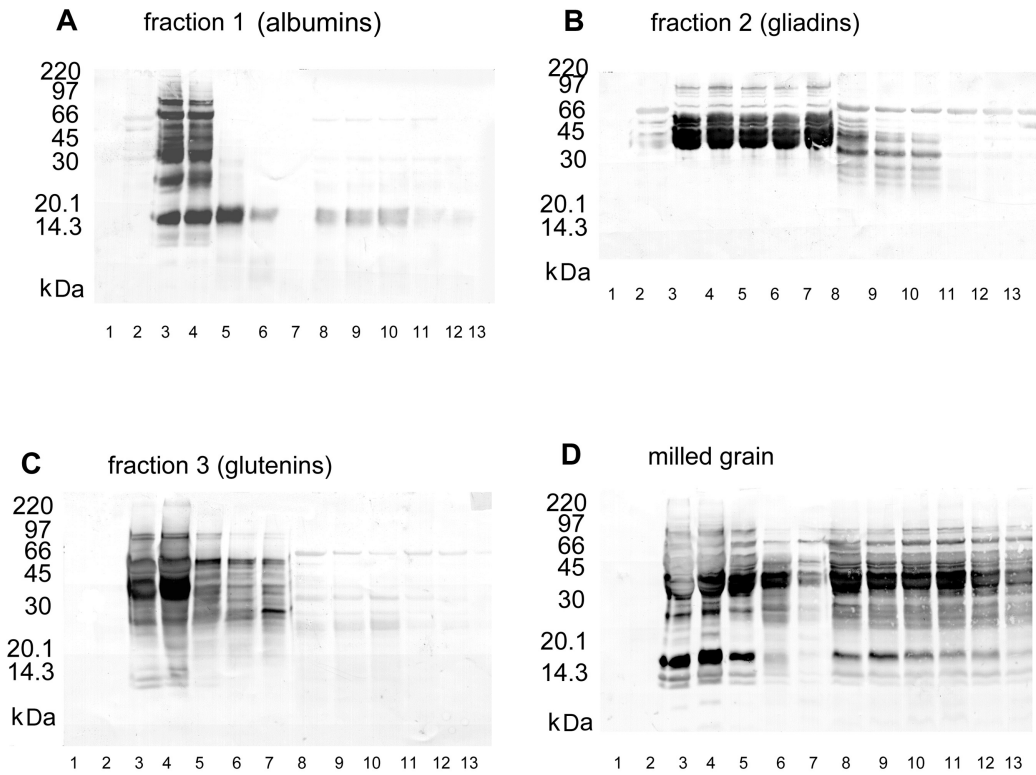

Figure 3. Digestion of proteins (A–C) of wheat fractions 1–3 and (D) milled grain by SGF and SDF and immunoblot analysis using pooled sera of wheat-allergic subjects suffering from food allergy. 1, SGF; 2, SDF; 3, untreated wheat extract; 4–7, incubation with SGF; 4, 0 s; 5, 30 s; 6, 4 min; 7, 1 h; 8–13, combined incubation with SGF and SDF: 4 min incubation in SGF followed by 8, 0 s; 9, 30 s; 10, 1 min; 11, 4 min; 12, 15 min; and 13, 45 min incubation in SDF.

3.2 Comparison of immunoblotting and CAP FEIA

The CAP FEIA is a quantitative antibody fluorescent-enzyme immunoassay that measures the amount of circulating allergen-specific IgE in serum. In immunblotting, extracted proteins are separated according to their molecular weights by SDS-PAGE, and detected with patient's serum after transfer to a nitrocellulose membrane.

In two patients with clear symptoms of baker's asthma (Nos. 6, 7), no IgE-reactivity to wheat could be detected by CAP FEIA and immunoblot. In nine patients, IgE against wheat was detected by both methods. However, in four patients suffering from baker's asthma (Nos. 10, 11, 14, 15), CAP FEIA against wheat was clearly positive, but no IgE-reactivity to any of the three fractions could be seen in the immunoblots. In cases of food-allergic children and adults, generally CAP FEIA and/or immunoblotting correlated with the diagnosis of wheat allergy as IgE-reactivity to wheat could be demonstrated for 72% of the cases in CAP FEIA and for 100% of the cases in immunoblotting (Table 1).

In all three cases of WDEIA (Nos. 30–32) and in the food-allergic subject with WDEIA-like IgE-binding pattern (No. 21), CAP FEIA and immunoblotting (Table 1, Fig. 2) showed contradictory results as levels of wheat-specific IgE were low in CAP FEIA, but very reactive to salt-insoluble wheat proteins (gluten = gliadin and glutenin) by immunoblotting. There was no IgE-reactivity to fraction 1 on immunoblots from patients 30 and 31. Results were confirmed by determination of IgE-reacitivity in commercially available CAP FEIA to gluten which revealed positive results in each of the four patients (Table 2).

3.3 Digestion of protein in SGF and SDF

Extracts were evaluated for stability to SGF and SDF using pharmaceutical enzyme preparations from pork. Digested samples were resolved by SDS-PAGE. Allergens were detected with sera from each group of wheat-allergic subjects (two pooled sera: baker's asthma and food allergy (Fig. 3) and one serum from a subject with WDEIA (Fig. 4)) to include all specific allergens detected by the different groups.

To study the kinetics of these digestions, different incubations between 0 s and 1 h for SGF and, for combined diges-

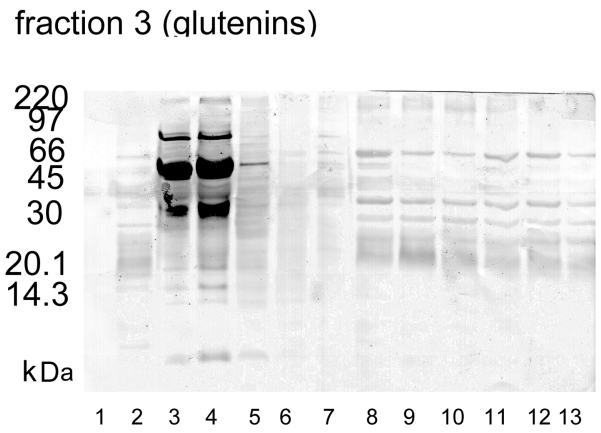

Figure 4. Digestion of proteins of wheat fraction 3 (glutenins) by SGF and SDF and immunoblot analysis using a serum of wheat-allergic subject with exercise-induced anaphylaxis (serum 31). For description of 1 – 13 see Fig. 3.

tion 4 min for SGF, followed by 0 s to 45 min for SDF, were performed. 30 mM NaCl, pH 1.2, and TBS, pH 7.4, with and without extracts were used as controls. Representative results are shown using a serum pool of five food-allergic children for detection (Fig. 3). In patients with baker's asthma, similar results were obtained, but IgE-reactivity was less reactive as was already found with the undigested extracts (results not shown).

Albumins and globulins were degraded rapidly by SGF. Most allergens were undetectable on blots after 30 s of digestion. 16 and 20 kDa allergens detected with high intensity by food-allergic subjects were digested after 1 h (Fig. 3A). There was no reduction of IgE-reactivity to gliadins of subjects with food allergy (Fig. 3B) or WDEIA after digestion in SGF, but they were rapidly digested in SDF. Subjects with baker's asthma generally did not show IgE-reactivity against extracted gliadins (see examples in Fig. 2B) or extracted and digested gliadins (results not shown).

Glutenins were digested more slowly in SGF than albumins and globulins, but faster than gliadins (shown for food-allergic patients in Fig. 3C). Rapid digestion of the 75, 50, and 30 kDa allergens within 30 s was detected using IgE antibodies from the subject with WDEIA (No. 31, Fig. 4). After 1 h digestion, allergenic glutenins were still detectable with sera from all three groups of subjects. However, the intensity of the reaction was strongly reduced. Glutenins were rapidly digested in SDF (Fig. 3C). The milled wheat grain still contained allergens after 4 min of incubation in SGF and 45 min in SDF with little reduction of IgE antibody reactivity (Fig. 3D).

4 Discussion

4.1 IgE-reactivity to wheat in the different patient groups

A great heterogeneity in IgE-reactivity to wheat was found for subjects with baker's asthma or food allergy, but no major wheat allergen could be demonstrated for these patients. However, sera of patients with WDEIA detected in gliadin fraction a 65 kDa allergen which could be the same found by Palosuo *et al.* [16, 17, 36] who described a 65 kDa omega-5 gliadin as a major allergen for subjects with WDEIA. In these patients, we found very similar IgE-binding patterns which have to be verified by investigation of more subjects. Furthermore, they showed similar IgE-binding patterns to the fractions 2 and 3 which may be due to sequence or structural homologies of gliadins and LMW glutenin subunits [22].

Subjects with respiratory allergy to wheat showed intense reactivity to fraction 1. On the other hand, sera of subjects with allergic reactions after ingestion of wheat contained high levels of IgE antibodies against salt-insoluble wheat proteins. These results are in accordance with the suggested relationship between solubility of wheat proteins and clinical manifestation; however, most sera also showed some IgE-reactivity against other fractions of wheat proteins with differences in intensity.

Less than 50% of tested sera from individuals with baker's asthma or food allergy recognized 12, 16, and 27 kDa allergens with high intensity and/or high frequency in albumin/globulin fraction. A 27 kDa allergen was described by Weiss *et al.* [14] and allergens between 12 and 20 kDa in the salt-soluble fraction were found by various investigators [2, 10, 13, 20, 37, 38]. In summary, food-allergic children showed higher frequency and intensity of reaction to fractions 1 and 3 than food-allergic adults and subjects with baker's asthma.

In some patients, IgE-reactivity patterns to fractions 1 and 3 looked very similar (for example, see Fig. 2, Nos. 23, 25). IgE-binding to these fractions could be inhibited by each other (results not shown), which could be due to contamination of fraction 3 with proteins from the albumin/globulin fraction. However, in order to avoid contamination, protein isolation followed extensive washing. Before extraction of each subsequent protein fraction, the precipitate having been washed three times with extraction buffer and one time with distilled water.

4.2 CAP FEIA *versus* immunoblotting

In CAP FEIA, proteins from wheat extract are coupled to the solid phase without denaturing treatment. It is likely

that wheat extracts used for the commercially available wheat CAP contain mainly salt soluble wheat proteins. In preparation for immunoblotting, isolation of extracted wheat proteins from each fraction was performed under reducing and denaturing conditions, as the extract was cooked after addition of SDS and dithiothreitol (DTT), in order to render the proteins negatively charged and to split disulfide bonds. Comparison of results from CAP FEIA and immunoblotting demonstrates that these methods can lead to divergent conclusions.

Sera of food allergic children revealed similar results in CAP FEIA and immunoblotting. However, in subjects with baker's asthma, high IgE-reactivity in CAP FEIA, but almost no IgE-reactivity in immunoblotting, was found. These findings could be due to the route of sensitization *via* the respiratory tract. Since they inhale native allergens, subjects with baker's asthma may have larger quantities of specific IgE antibodies against conformational epitopes than patients suffering from food allergy to wheat, who are likely to have been sensitized *via* the protein-denaturing gastrointestinal tract. Native protein conformation and conformational epitopes of salt-soluble proteins may be less denatured by CAP-FEIA than by immunoblotting in which SDS and reducing agents are used in sample preparation. The different IgE-reactivities described in the literature may therefore partly be explained by the different methods applied (*e.g.*, RAST, ELISA, immunoblotting). Various treatments of antigens in these methods may consequently be responsible for the seemingly inconsistent results obtained.

In patients with WDEIA, no IgE-reactivity to fraction 1, but high IgE-reactivity to salt-insoluble fractions (Fig. 2, Table 2) indicated that only salt-soluble proteins were present in the wheat extracts used for the commercial CAP-FEIA. Therefore, CAP-FEIA may not detect IgE-reactivity of individuals with exercise-induced anaphylaxis and, in some cases, of food-allergic subjects. Similarly, Simonato *et al.* [20] found that IgE-reactivity to the insoluble wheat proteins detected by immunoblot was in contrast to negative results for wheat CAP-FEIA. Therefore, in case of a history suggesting wheat allergy, negative results by wheat CAP should be confirmed by immunoblot or by CAP FEIA for IgE-reactivity against gluten (gliadins + glutenins).

In summary, differences of the patient groups' IgE-responses are likely to affect the outcome of the assays. In turn, the assays depend on the presence of the recognized allergens in the respective probe extracts and their stability to the particular sample treatment applied for each method.

4.3 Stability of wheat allergens against digestion

Stability against digestion is considered an important requirement of allergens for sensitization and eliciting allergic reactions *via* the gastrointestinal route. Intact epitopes on proteins or protein fragments of sufficient size to cross-link receptor-bound IgE antibodies on mast cells seem to be required to cause these effects.

In this study, experimental digestive treatment time of salt-soluble proteins and glutenins was very short by comparison to the 2 h mean passage time of food through the stomach. Rapid digestion of the albumin/globulin fraction *in vitro* was also described by Cole [39]. The results suggested this could be due to the fact that protein extracts were used instead of food and that extracted proteins may be more accessible to enzymes than in whole food. To investigate this, milled wheat grain with identical protein content as contained in extracts was tested.

Milled wheat grain still contained recognizable allergens after 4 min of incubation in SGF and 45 min in SDF with little reduction of IgE-reactivity (Fig. 3D). It is likely that this was caused by the fact that proteins had to be extracted first by the SGF before they could be digested. Especially salt-insoluble proteins are likely extracted slowly and, therefore, digested slowly. Gliadins were the only fraction which showed high stability against gastric digestion. Nevertheless, they were digested in duodenal fluid as rapidly as the other fractions (Fig. 3B). This result is in accordance with Aoki *et al.* [41] who found that the allergenicity of wheat was reinforced after incubation with pepsin, but abolished by further tryptic digestion. Because of their higher stability, salt-insoluble proteins may elicit allergic reactions *via* the gut dissimilarly to salt-soluble allergens.

Our data suggest that in most cases of patients with food allergy to wheat and baker's asthma, respectively, serological response to different allergens, which may exhibit different digestion stability, causes these diseases. Nevertheless, there are subjects with baker's asthma who later suffer from food allergy too [41]. This may be explained by the induction of IgE against stable wheat proteins *via* the respiratory tract. When those stable allergens are ingested they may also elicit allergic reactions *via* the gastrointestinal tract.

Maleki *et al.* [42] showed for the major peanut allergen Ara h 1 that epitopes are protected from proteolytic enzymes by the quaternary structure of this allergen that occurs naturally as highly stable homotrimer. Wheat proteins can also form quaternary complexes, for example the α-amylase-inhibitors in albumin/globulin fraction together with the proteins of the glutenin fraction. Conditions during extraction (pH, time, detergents, reducing reagents) may affect the 3-D structural stability of the proteins and thus modulate their immunological properties. Sample preparation for SDS-PAGE, which includes strong heating, could also have a modulating effect. Baking was shown to increase digestion stability of IgE-binding wheat proteins [43].

As indicated by our study, patients suffering from one of the three clinical variants of IgE-mediated allergic reaction to wheat can be distinguished by their individual patterns of IgE-reactivity to wheat allergen fractions. These patterns reveal a relationship between properties of wheat allergens, such as solubility and digestion stability, and the different routes of sensitization to wheat in each clinical variant of wheat allergy.

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