Component-resolved diagnosis of baker's allergy based on specific IgE to recombinant wheat flour proteins*

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Background: Sensitization to wheat flour plays an important role in the development and diagnosis of baker's asthma.
Objectives: We evaluated wheat allergen components as sensitizers for bakers with work-related complaints, with consideration of cross-reactivity to grass pollen.
Methods: Nineteen recombinant wheat flour proteins and 2 cross-reactive carbohydrate determinants were tested by using CAP-FEIA in sera of 101 bakers with wheat flour allergy (40 German, 37 Dutch, and 24 Spanish) and 29 pollen-sensitized control subjects with wheat-specific IgE but without occupational exposure. IgE binding to the single components was inhibited with wheat flour, rye flour, and grass pollen. The diagnostic efficiencies of IgE tests with single allergens and combinations were evaluated by assessing their ability to discriminate between patients with baker's allergy and control subjects based on receiver operating characteristic analyses.
Results: Eighty percent of bakers had specific IgE levels of 0.35 kU_A/L or greater and 91% had specific IgE levels of 0.1 kU_A/L or greater to at least one of the 21 allergens. The highest frequencies of IgE binding were found for thiol reductase (Tri a 27) and the wheat dimeric α-amylase inhibitor 0.19 (Tri a 28). Cross-reactivity to grass pollen was proved for 9 components, and cross-reactivity to rye flour was proved for 18 components. A combination of IgE tests to 5 components, Tri a 27, Tri a 28, tetrameric α-amylase inhibitor CM2 (Tri a 29.02), serine protease inhibitor–like allergen (Tri a 39), and 1-cys-peroxiredoxin (Tri a 32), produced the maximal area under the curve (AUC = 0.84) in receiver operating characteristic analyses, but this was still lower than the AUC for wheat- or rye flour–specific IgE (AUC = 0.89 or 0.88, respectively).
Conclusions: Component-resolved diagnostics help to distinguish between sensitization caused by occupational flour exposure and wheat seropositivity based on cross-reactivity to grass pollen. For routine diagnosis of baker's allergy, however, allergen-specific IgE tests with whole wheat and rye flour extracts remain mandatory because of superior diagnostic sensitivity. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Baker's asthma,* Triticum aestivum*, recombinant allergens, alpha-amylase inhibitor, cross-reactivity, grass pollen, rye flour, wheat allergy, specific IgE, component-resolved diagnosis*

Wheat *(Triticum aestivum)* is a source of numerous allergens responsible for different manifestations of IgE-mediated allergy, depending on the route of exposure.[1] Ingestion of wheat can induce food allergy or wheat-dependent exercise-induced anaphylaxis; inhalation of wheat and rye flour is the main cause of baker's asthma.

Knowledge of the relevant allergen components might help to improve diagnostics, such as skin prick tests, which have been found to lack sensitivity[2-4]; to standardize *in vitro* IgE antibody assays[5]; or to optimize immunotherapy. In addition, discrimination between different clinical manifestations of wheat allergy or different allergic phenotypes might be supported by analyzing a patient's individual IgE reaction profile to single allergens, as has been proposed previously.[6-8]

In German wheat-sensitized bakers the recombinant allergens most frequently bound by serum IgE were the wheat α-amylase inhibitors Tri a 28 and Tri a 29.01 and a thiol reductase homologue (Tri a 27).[9] In a panel of Spanish bakers with baker's asthma, microarrays with 12 wheat seed allergens purified from natural sources identified the wheat tetrameric α-amylase inhibitor subunit CM16 (WTAI-CM16), the lipid transfer protein Tri a 14, and Tri a 28 as the most frequent allergens.[10] In The Netherlands, where most of the work on wheat flour exposure assessment and influence on sensitization rates has been performed,[11-13] no data on antiwheat reaction profiles in sensitized bakers are available.

It cannot be excluded that the most important sensitizing wheat or rye flour single allergens differ per country with consequences for diagnosis. Therefore we tested the same panel of recombinant wheat flour allergens used earlier for German bakers[9] also in populations of Spanish and Dutch bakers with occupational allergy. The allergen panel was completed with the serine protease inhibitor–like allergen (SPILA),[14] which is now denominated by the Allergen Nomenclature Sub-Committee of the International Union of Immunological Societies as Tri a 39, and the lipid transfer protein isoallergen wheat nonspecific lipid transfer protein (nsLTP) type I subfamily 9.1 (nsLTP 9.1; Tri a 14.0101).[15-17]

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*Allergen names, including isoallergen and variant numbers, used in this study were approved by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee.

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Abbreviations used
AUC: Area under the curve
CCD: Cross-reactive carbohydrate determinant
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
HRP: Horseradish peroxidase
MBP: Maltose-binding protein
MUXF: Carbohydrate part of bromelain
nsLTP: Nonspecific lipid transfer protein
ROC: Receiver operating characteristic
sIgE: Allergen-specific IgE
SPILA: Serine protease inhibitor–like allergen
Tri a 12.0102: Wheat profilin
Tri a 14.0101: Wheat nonspecific lipid transfer protein type I subfamily 9.1 (nsLTP 9.1)
Tri a 14.0201: Wheat non-specific lipid transfer protein type I subfamily 9.7 (nsLTP 9.7)
Tri a 15.0101: Wheat monomeric α-amylase inhibitor 0.28 (WMAI-0.28)
Tri a 19.0101: Wheat ω5-gliadin
Tri a 21.0101: Wheat αβ-gliadin
Tri a 25.0101: Wheat thioredoxin H
Tri a 27.0101: Wheat thiol reductase homologue
Tri a 28.0101: Wheat dimeric α-amylase inhibitor 0.19 (WDAI-0.19)
Tri a 29.0101: Wheat tetrameric α-amylase inhibitor CM1 (WTAI-CM1)
Tri a 29.0201: Wheat tetrameric α-amylase inhibitor CM2 (WTAI-CM2)
Tri a 30.0101: Wheat tetrameric α-amylase inhibitor CM3 (WTAI-CM3)
Tri a 31.0101: Wheat triosephosphate isomerase (TPIS)
Tri a 32.0101: Wheat 1-cys-peroxiredoxin
Tri a 33.0101: Wheat serpin
Tri a 34.0101: Wheat GAPDH
Tri a 35.0101: Wheat dehydrin
Tri a 39.0101: Wheat SPILA

Our ultimate aim is to define which wheat flour allergens should be included in a component-resolved analysis of baker's sensitization. Not only the frequency of allergen-specific IgE (sIgE) in bakers' sera but also sIgE concentrations play a role in an allergen's effects.[18] Because IgE measurements by means of CAP-FEIA have the advantage of producing quantitative IgE values with an analytic sensitivity of 0.1 kU_A/L,[5] this was our method of choice. Furthermore, important single allergens usually constitute a relevant amount in natural extracts. It is not self-evident that allergens produced by using recombinant techniques are expressed under natural conditions in sufficient amounts to be responsible for sensitization. Therefore we investigated whether IgE binding to the detected allergens could be inhibited by flour extracts.

An additional complication in wheat allergy diagnosis is the well-known cross-reactivity of grain flours and grass pollen, although the responsible proteins sharing common epitopes have not been identified, with the exception of profilin.[19-21] Thus a positive IgE reaction to crude wheat extracts in a baker with respiratory symptoms strongly suggests but does not prove a work-related allergic sensitization. Therefore to improve the specificity of IgE antiwheat diagnostics, on the one hand, we studied cross-reactivity of wheat allergen components with grass pollen allergens in IgE inhibition experiments, and on the other hand, we included a control group of grass pollen–sensitized subjects with sIgE to wheat flour but no occupational exposure to wheat flour and evaluated their IgE reactions to the panel of wheat allergen components. This selection of negative control subjects was preferred to bakers without complaints (who might be already occupationally sensitized and become allergic later in life) or control subjects without wheat flour sIgE (which should rarely bind wheat components and would require a high number of control subjects to find any positive results). Thus the negative control group for occupational wheat flour allergy consisted of 29 selected cases from the 3 countries with cosensitization to wheat flour and grass pollen, probably because of IgE cross-reactions. The IgE reactions in the control group were compared with the "true-positive" reactions of occupational allergic sensitization to wheat flour in 101 bakers with occupational disease and specific IgE to wheat flour. IgE tests to all single allergens and several combinations of tests were evaluated by using receiver operating characteristic (ROC) analyses to assess their ability to discriminate between the 2 groups.

METHODS

Patients and sera

The 40 German bakers with work-related complaints were the same as described previously[9] and were selected based on physician-diagnosed occupational disease and wheat allergy. The 24 Spanish bakers had work-related asthma and rhinitis and a positive inhalation challenge test result to wheat or rye flour. All consecutive bakers attending La Paz Hospital in 2005 to 2008 who were given a diagnosis of baker's asthma caused by wheat flour were selected. The 37 Dutch bakers were selected during the validation study of The Netherland's health surveillance system[13] and had work-related asthma, rhinitis, or both. All bakers were sensitized to wheat flour (sIgE ≥0.35 kU_A/L), 81 (80%) had asthma, 95 (95%) had rhinitis, and 93 (92%) were male. Diagnosis of work-related asthma followed the consensus statement of the American College of Chest Physicians.[22] The mean ages of German, Spanish, and Dutch bakers were 40 ± 15, 38 ± 10, and 36 ± 9 years, respectively.

From each country, also control sera from patients with hay fever or known pollen sensitization without occupational exposure but with sIgE to wheat flour (≥0.35 kU_A/L) were obtained (10 German, 10 Dutch, and 9 Spanish control subjects). Twenty-one control subjects were male (72%), 21 (72%) had rhinitis, and 17 (59%) had asthma. The mean ages of the German, Dutch, and Spanish control subjects were 30 ± 15, 36 ± 10, and 33 ± 18 years, respectively.

Cloning of wheat allergens

By using the sequence information for the wheat SPILA published by Constantin et al[14] (GenBank accession no. EU051824), the following 5′ primer, including a restriction site for *Fsp*I (5′-tgc gca ATG AGC CCT GTG GTG AAG AAG CCG-3′), and 3′ primer, including a restriction site for *Hind*III (5′-a agc TTA GCC GAC CCT GGG GAC CTG GGC AAT-3′), were designed. The cDNA of SPILA was obtained from the wheat endosperm phage surface-displayed cultivar Wyuna cDNA library[23] and subcloned and sequenced in the pDrive-vector system (Qiagen, Hilden, Germany). After identification, SPILA was expressed as a maltose-binding protein (MBP)–SPILA hybrid in *Escherichia coli* carrying an 8-amino-acid spacer (ISEFVISA) between its carrier protein MBP and the ATG start codon of the target protein SPILA. The SPILA nucleotide sequence is published in GenBank (accession no. HE972340).

The translated protein sequence of HE972340 in comparison with EU051824 differs by 2 amino acid changes: glycine to arginine (position 11) and glycine to serine (position 16). All other wheat flour allergens, with the exception of Tri a 14.0101 and wheat ω5-gliadin (Tri a 19.0101), which were obtained by Thermo Fisher Scientific (Freiburg, Germany), were produced as described previously.[9]

Specific IgE determination and IgE inhibition

Total IgE and sIgE antibodies to wheat flour (f4), rye flour (f5), grass pollen mixture (gx1), ω5-gliadin Tri a 19.0101 (f416), nsLTP Tri a 14.0101 (f433), horseradish peroxidase (HRP; o400), and the carbohydrate part of bromelain (MUXF; Ro214) on ImmunoCAPs and to single wheat flour proteins bound to streptavidin ImmunoCAPs were measured by using CAP-FEIA (Thermo Fisher Scientific), which had a detection limit of 0.1 kU_A/L. In case of sIgE values of greater than 100 kU_A/L, sera were retested in dilution.

Expression and binding of the wheat flour fusion proteins and the isolated *E coli* carrier MBP to streptavidin ImmunoCAPs were performed as described previously.[9] For IgE inhibition experiments, wheat and rye grain extracts produced as previously described[24] and a grass pollen mixture from *Lolium perenne*, *Poa pratensis*, *Dactylis glomerata*, *Festuca pratensis*, and *Phleum pratense* (all from Allergon, Angelholm, Sweden) were used in protein concentrations of 10 mg/mL. For all wheat flour allergens, 2 positive sera from different countries were diluted to sIgE values of 2 to 3 kU_A/L in case of higher sIgE concentrations. Then 50 μL of these sera was mixed with 10 μL of inhibition solution or PBS before sIgE concentrations were measured in CAP-FEIA. In preliminary quality control experiments with inhibition solutions with a concentration of 5 mg of protein/mL and 20 sera from grass pollen and cereal–cosensitized bakers, the grass pollen mixture inhibited IgE binding to grass pollen gx1 ImmunoCAPs by 95% ± 2%, the wheat flour extract inhibited IgE binding to f4 ImmunoCAPs by 77% ± 9% and the rye flour extract inhibited IgE binding to f5 ImmunoCAPs by 87% ± 6%.

Statistical methods

Statistical analyses of group differences by using Mann-Whitney and Kruskal-Wallis tests, 2-way ANOVA with Bonferroni multiple comparison, and Pearson correlations between the logarithm of sIgE values to different allergens or to wheat flour and the sum of single allergens, as well as ROC analyses of allergens and combinations of test results, were performed with GraphPad Prism software (GraphPad Software, San Diego, Calif). For ROC and correlation analyses, sIgE values of less than the detection limit of 0.1 kU_A/L were set to 0.07.

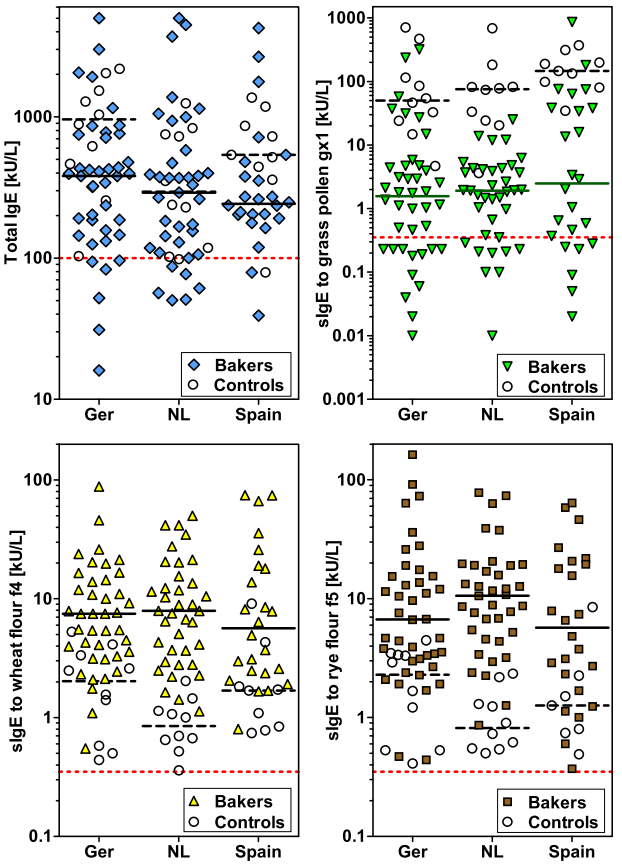

FIG 1. Total IgE and sIgE concentrations to grass pollen (gx1), wheat flour (f4), and rye flour (f5) of the study groups of 101 bakers and 29 control subjects. The median values of bakers *(solid lines)* and control subjects *(dashed lines)* and cutoff values *(dotted lines)* for sIgE (≥0.35 kU_A/L) and total IgE (>100 kU_A/L) are indicated.

RESULTS

Of 101 bakers with work-related asthma, rhinitis and wheat flour sensitization, or both, 86 also had increased total IgE concentrations (>100 kU_A/L), all had sIgE to rye flour, and 75 had sIgE to grass pollen (Fig 1). Bakers from the different countries were not significantly different concerning age or total and sIgE levels to wheat and rye flour or grass pollen. The same was true for control subjects from the different countries. Control subjects had significantly higher sIgE values to grass pollen ($P < .0001$) and lower sIgE values to wheat and rye flour than bakers ($P = .0005$), whereas differences for total IgE levels were not significant. There was a strong and significant correlation in bakers' sera between wheat and rye flour sIgE levels ($r_{log} = 0.90$) and a moderate correlation between wheat flour and total IgE levels ($r_{log} = 0.47$) or grass pollen sIgE levels ($r_{log} = 0.44$).

All sera were tested for sIgE binding to 19 recombinant wheat single allergens and to the 2 cross-reactive carbohydrate determinants (CCDs) and displayed individual binding profiles. All single allergens were recognized by IgE (≥0.35 kU_A/L) from at least 1 of the bakers' sera, whereas IgE from control sera showed reactions at this cutoff level to only 10 of the 21 allergens (Fig 2). Of combined bakers' and control sera, 108 (81% of the bakers and 93% of the control subjects) had sIgE levels of 0.35 kU_A/L or greater to any single allergen. Ninety-two bakers' sera and all control sera had sIgE levels of 0.1 kU_A/L or greater to any allergen. Most bakers' sera reacted to more than 1 allergen, with many significant correlations between allergens (data not shown), especially between the WTAI isoallergens CM1 and CM2 ($r_{log} = 0.92$), the CCDs MUXF and HRP ($r_{log} = 0.87$), WDAI and WMAI ($r_{log} = 0.78$), WTAI CM2 and CM3 ($r_{log} = 0.77$), WTAI CM1 and CM3 ($r_{log} = 0.74$), and the nsLTP isoallergens ($r_{log} = 0.68$).

The sensitization patterns of bakers from the different countries were similar but displayed some differences. However, according to 2-way ANOVA results, country was not a significant determinant of the IgE test result, whereas allergen was significant. Therefore the results from all 101 bakers and 29 control subjects from the different countries were combined for analyses. For all single allergens and more than 100 IgE test combinations of 2 to 10 single allergens, sensitization frequencies in both groups at different cutoff values, mean sIgE levels, and efficiencies to discriminate between bakers and control subjects were calculated. The overview of the results for all single allergens and a number of selected allergen test combinations is presented in Table I. Measure for the relevance as baker-specific allergen and selection criterion for test combinations was the area under the curve (AUC) of ROC analyses with as definition of a positive reaction: at least 1 positive reaction in the test panel.

The allergens eliciting the most positive IgE test results in bakers were thiol reductase (27%, ≥0.35 kU_A/L; 37%, ≥0.1 kU_A/L), WDAI-0.19 (24%, ≥0.35 kU_A/L; 39%, ≥0.1 kU_A/L),

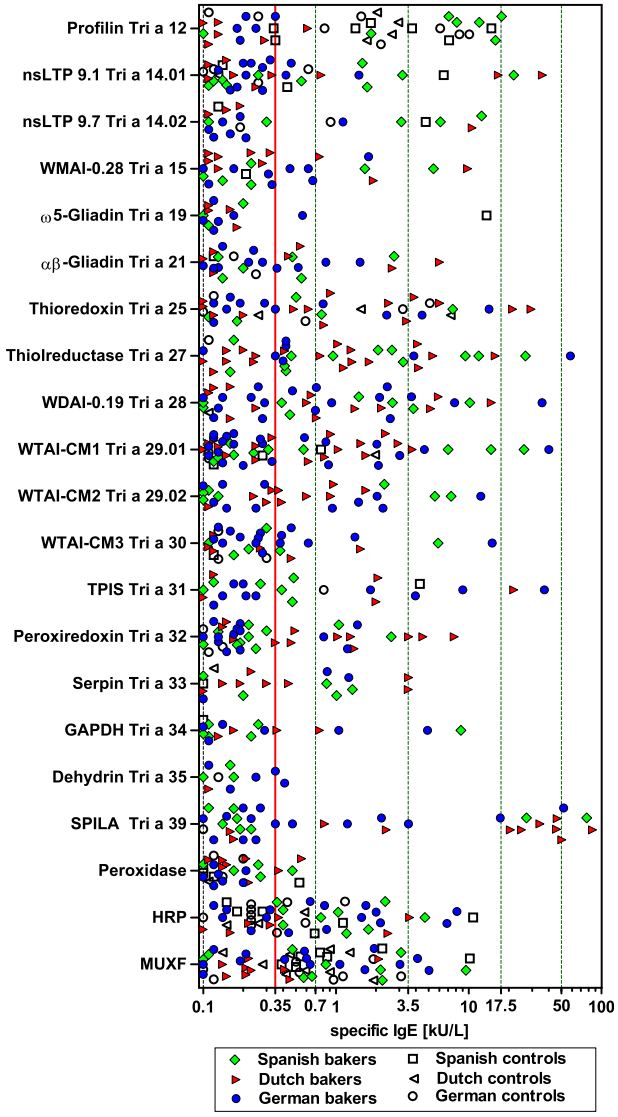

FIG 2. sIgE concentrations of 0.1 kU_A/L or greater in sera of bakers and control subjects to recombinant wheat allergens and the CCDs HRP and MUXF measured by using CAP-FEIA. The *solid line* marks the usual cutoff for positive values, and the *dashed lines* indicate the limits between the different CAP classes (class 1, ≥0.35-0.7 kU_A/L; class 2, 0.7-3.5 kU_A/L; class 3, 3.5-17.5 kU_A/L; class 4, 17.5-50 kU_A/L; and class 5, 50-100 kU_A/L).

WTAI-CM1 (21%, ≥0.35 kU_A/L; 49%, ≥0.1 kU_A/L), SPILA (18%, ≥0.35 kU_A/L; 34%, ≥0.1 kU_A/L), thioredoxin (18%, ≥0.35 kU_A/L; 30%, ≥0.1 kU_A/L), peroxiredoxin (14%, ≥0.35 kU_A/L; 35%, ≥0.1 kU_A/L), and WTAI-CM2 (14%, ≥0.35 kU_A/L; 28%, ≥0.1 kU_A/L). With the exception of thioredoxin, all of these allergens reacted significantly less with IgE from pollen-sensitized control subjects and, as a consequence, produced AUC values in ROC analyses that were significantly greater than 50%. In contrast, control sera showed more often IgE reactions of 0.35 kU_A/L or greater, with the CCD component MUXF (69%), profilin (55%), and HRP (28%) resulting in significantly decreased AUC values of less than 50%.

Combinations of the 5 allergens thiol reductase, WDAI-0.19, WTAI-CM2, peroxiredoxin, and SPILA produced the highest value in ROC analyses, with an AUC of 84% (Table I) and with a cutoff point of 0.12 kU_A/L or greater, the highest performance measures concerning discrimination between bakers with wheat allergy and pollen-sensitized control subjects (Table II). ROC analysis of sIgE tests to wheat flour produced an AUC of 89% with an optimal cutoff value of 5.33 kU_A/L or greater and sIgE tests to rye flour produced an AUC of 88% with an optimal cutoff value of 4.52 kU_A/L or greater. However, performance measures at optimal cutoff points of IgE antiwheat and antirye flour tests were lower than those of the best test combinations of components (Table II).

Wheat flour inhibited binding to all single-allergen CAPs by 25% to 89% (Fig 3). The lowest inhibition rate was obtained for profilin, and the highest inhibition rate was for thiol reductase and the dimeric α-amylase inhibitor WDAI-0.19. Rye flour inhibited binding to all single allergens with the exception of profilin, nsLTP 9.1, and αβ-gliadin by 24% to 84%, thus in a similar range as wheat flour. Grass pollen produced a significant inhibition of greater than 20% only for profilin, both nsLTPs, ω5-gliadin, thioredoxin, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the CCDs. Inhibition rates with wheat and rye flour were significantly correlated ($r_{log} = 0.45$), whereas inhibition rates with grass pollen were not correlated with those of the cereals.

For each serum, we compared the sum of sIgE values to the single allergens with sIgE to the wheat flour CAP. For bakers' sera, a significant strong correlation ($r_{log} = 0.69$) was observed, and for the control subjects, a moderate correlation ($r_{log} = 0.49$) was observed. For bakers, in most cases the wheat flour CAP value was higher than the sum of IgE to single allergens, whereas for control subjects, the sum was higher (Fig 4).

DISCUSSION

Several single wheat flour allergens relevant to baker's asthma have been identified in the last 30 years, and a number of them have been characterized on a molecular level. The current allergen nomenclature list of the World Health Organization/International Union of Immunological Societies comprises 20 wheat allergens (www.allergen.org). The *Triticum aestivum* allergen numbers start with Tri a 12 (profilin) and end with Tri a 39 (SPILA), leaving a place for wheat pollen allergens homologous to characterized grass pollen allergens or seed allergens from related cereals. In addition to 3 recently published food allergens (the high-molecular-weight glutenin Tri a 26,[25] the low-molecular-weight glutenin Tri a 36,[26,27] and the α-purothionin Tri a 37[28]), only the wheat germ agglutinin Tri a 18[29] is missing in our study. However, in a recent study with 81 bakers with work-related symptoms, none reacted to the native Tri a 18.[8] All other 16 denominated allergens were part of our test panel, and for Tri a 14 and Tri a 29, 2 isoallergens were used, respectively. In addition to these 18 denominated single allergens, in the present study among 101 bakers we included 2 CCDs and the recombinant wheat peroxidase. The latter component did not react with German bakers' sera in our previous study[9] and therefore has no allergen number, but its native counterpart was described as a prominent allergen for Spanish bakers.[30] In agreement with previous study results, each baker showed an individual IgE-binding profile with great interindividual variation,[31] and none of the single allergens reached the status of a major allergen.[7-9]

TABLE I. Overview of sIgE data with frequencies, sensitivities, and specificities obtained with a cutoff of 0.35 kU_A/L or greater

Allergen name		German bakers, n = 40	Dutch bakers, n = 37	Spanish bakers, n = 24	All bakers n = 101	All bakers Mean IgE (kU_A/L)	All control subjects n = 29	All control subjects Mean IgE (kU_A/L)	Sensitivity	Specificity	AUC	*P* value
Profilin	Tri a 12.0102	1 (3%)	1 (3%)	6 (25%)	8 (8%)	0.66	16 (55%)	2.38	0.08	0.45	**0.24**	**<.0001**
nsLTP 9.1	Tri a 14.0101	3 (8%)	3 (8%)	5 (21%)	11 (11%)	0.91	3 (11%)‡	0.32	0.11	0.89	0.52	.72
nsLTP 9.7	Tri a 14.0201	1 (3%)	1 (3%)	3 (13%)	5 (5%)	0.37	2 (7%)	0.23	0.05	0.93	0.51	.88
WMAI-0.28	Tri a 15.0101	4 (10%)	3 (8%)	2 (8%)	9 (9%)	0.27	0 (0%)	0.03	0.09	1	0.61	.08
ω5-Gliadin	Tri a 19.0101	1 (3%)	0 (0%)	0 (0%)	1 (1%)	0.03	1 (3%)	0.50	0.01	0.97	0.54	.46
αβ-Gliadin	Tri a 21.0101	4 (10%)	4 (11%)	3 (13%)	11 (11%)	0.21	0 (0%)	0.05	0.11	1	0.57	.24
Thioredoxin	Tri a 25.0101	5 (13%)	10 (27%)	3 (13%)	18 (18%)	1.02	5 (17%)	0.65	0.18	0.83	0.51	.88
Thiol reductase	Tri a 27.0101	6 (16%)*	11 (30%)	10 (42%)	27 (27%)*	1.64	0 (0%)	0.03	0.27	1	**0.68**	**.004**
WDAI-0.19	Tri a 28.0101	10 (25%)	8 (22%)	6 (25%)	24 (24%)	1.11	0 (0%)	0.03	0.24	1	**0.68**	**.003**
WTAI-CM1	Tri a 29.0101	8 (20%)	9 (24%)	4 (17%)	21 (21%)	1.24	2 (7%)	0.15	0.21	0.93	**0.65**	**.01**
WTAI-CM2	Tri a 29.0201	5 (13%)	6 (16%)	3 (13%)	14 (14%)	0.43	0 (0%)	0.03	0.14	1	**0.64**	**.02**
WTAI-CM3	Tri a 30.0101	6 (15%)	2 (5%)	2 (8%)	10 (10%)	0.32	0 (0%)	0.06	0.10	1	0.57	.23
TPIS	Tri a 31.0101	4 (10%)	3 (8%)	3 (13%)	10 (10%)	0.81	2 (7%)	0.20	0.10	0.93	0.57	.28
1-cys-Peroxiredoxin	Tri a 32.0101	3 (8%)	9 (24%)	2 (8%)	14 (14%)	0.33	0 (0%)‡	0.04	0.14	1	**0.63**	**.03**
Serpin	Tri a 33.0101	2 (5%)	3 (8%)	3 (13%)	8 (8%)	0.16	0 (0%)	0.03	0.08	1	0.55	.43
GAPDH	Tri a 34.0101	2 (5%)	2 (5%)	1 (4%)	5 (5%)	0.19	0 (0%)	0.02	0.05	1	0.56	.33
Dehydrin	Tri a 35.0101	2 (5%)*	0 (0%)	0 (0%)	2 (2%)*	0.03	0 (0%)	0.03	0.02	1	0.53	.64
SPILA	Tri a 39.0101	7 (19%)†	9 (24%)	2 (9%)	18 (18%)†	5.12	0 (0%)	0.03	0.18	1	**0.66**	**.01**
Peroxidase		0 (0%)	2 (5%)	1 (4%)	3 (3%)	0.06	1 (3%)	0.08	0.03	0.97	0.47	.64
HRP		10 (25%)	3 (8%)	9 (38%)	22 (22)%	0.48	8 (28%)	0.64	0.22	0.72	**0.37**	**.03**
MUXF		10 (25%)	3 (8%)	8 (33%)	21 (21%)	0.44	20 (69%)	1.07	0.21	0.31	**0.24**	**<.0001**
≥1 Allergen		32 (80%)	29 (78%)	20 (83%)	81 (80%)	0.75	27 (93%)	0.32	0.80	0.07	0.54	.55
≥1 Recombinant wheat allergen		27 (68%)	27 (73%)	17 (71%)	71 (70%)	0.78	22 (76%)	0.26	0.70	0.24	0.51	.87
≥1 α-Amylase inhibitor		15 (38%)	14 (38%)	7 (29%)	36 (36%)	0.67	2 (7%)	0.06	0.36	0.93	**0.69**	**.002**
≥1 nsLTP		3 (8%)	3 (8%)	6 (25%)	12 (12%)	0.64	4 (14%)	0.27	0.12	0.86	0.51	.84
≥1 CCD		12 (30%)	4 (11%)	10 (42%)	26 (26%)	0.46	20 (69%)	0.86	0.26	0.31	**0.22**	**<.0001**
Profilin or MUXF		10 (25%)	4 (11%)	9 (38%)	23 (23%)	0.55	25 (86%)	1.72	0.23	0.14	**0.16**	**<.0001**
≥1 of the 5 allergens: Tri a 27, Tri a 28, Tri a 29.02, Tri a 32, Tri a 39		20 (50%)	24 (65%)	12 (50%)	56 (55%)	1.70	0 (0%)	0.03	0.55	1	**0.84**	**<.0001**
≥1 of the 6 allergens: Tri a 21, Tri a 27, Tri a 28, Tri a 29.02, Tri a 32, Tri a 39		22 (55%)	24 (65%)	13 (54%)	59 (58%)	1.45	0 (0%)	0.04	0.58	1	**0.84**	**<.0001**
≥1 of the 7 allergens: Tri a 15, Tri a 21, Tri a 27, Tri a 28, Tri a 29.02, Tri a 32, Tri a 39		23 (58%)	24 (65%)	13 (54%)	60 (59%)	1.28	0 (0%)	0.04	0.59	1	**0.84**	**<.0001**

Values in boldface indicate statistical significance. *P* values indicate the significance of AUCs differing from .5.
*Only 38 or 99 sera were tested.
†Only 37 or 98 sera were tested.
‡Only 28 sera were tested.

Presence of single allergen components in wheat and rye flour extracts

Inhibition experiments were performed to prove whether the identified single allergens are present in wheat flour. Competitive inhibition of IgE binding depends on sufficient allergen amounts in the extracts used for inhibition. In our experiments concentrations were used that produced complete autoinhibition in dose-response curves with wheat flour,[19] and the cereal extracts used for inhibition produced similar IgE results as wheat and rye flour on ImmunoCAPs when bound to streptavidin ImmunoCAPs[24] and higher test efficiencies than commercial wheat and rye flour solutions when used in skin prick tests.[2] However, the amount of individual allergens in the wheat flour extract could still be limited. In these cases no or only partial inhibition would result. The allergens with the highest mean inhibition by wheat flour extracts were Tri a 27 (89%) and the α-amylase inhibitors (75% to 89%). The lowest inhibition by wheat flour was obtained with profilin (25%), indicating a low concentration of wheat profilin (Tri a 12.0102) in wheat flour. Similar inhibition rates as with wheat flour were obtained with rye flour for most allergens. Because of the close relation of Triticeae,[32,33] homologous groups of proteins exist in wheat, rye, and barley, such as

TABLE II. Optimal cutoff points of sIgE for discrimination between bakers and control subjects and performance measures

	Optimal cutoff point (kU_A/L)	Sensitivity	Specificity	PPV	NPV	Youden index	Accuracy	Odds ratio	CI
≥1 of the 5 allergens: Tri a 27, Tri a 28, Tri a 29.02, Tri a 32, Tri a 39	≥0.12	0.70	0.97	0.99	0.48	0.67	0.76	66	15-279
≥1 of the 6 allergens: Tri a 21, Tri a 27, Tri a 28, Tri a 29.02, Tri a 32, Tri a 39	≥0.18	0.65	0.97	0.99	0.44	0.62	0.72	53	7-405
≥1 of the 7 allergens: Tri a 15, Tri a 21, Tri a 27, Tri a 28, Tri a 29.02, Tri a 32, Tri a 39	≥0.22	0.62	0.97	0.98	0.42	0.59	0.70	46	6-355
Profilin and MUXF	≤0.10	0.64	0.97	0.98	0.44	0.61	0.72	51	7-387
Wheat flour (f4)	≥5.33	0.59	0.97	0.98	0.41	0.56	0.68	20	4-88
Rye flour (f5)	≥4.52	0.64	0.96	0.98	0.41	0.61	0.71	23	5-101

The Youden index is defined as follows: Sensitivity + Specificity − 1. Accuracy is defined as follows: (Positive bakers + Negative control subjects)/(All bakers + All control subjects). Odds ratio is defined as follows: (Positive bakers × Negative control subjects)/(Negative bakers × Positive control subjects).
NPV, Negative predictive value; *PPV*, positive predictive value.

α-amylase inhibitors[34,35] and 1-cys-peroxiredoxin (Tri a 32).[7] However, there is little knowledge on other single allergens in rye flour. Thus the similar inhibition rates of IgE binding to single wheat flour allergens observed with rye and wheat flour indicate further homologous single allergens in *Secale cereale*. With the exception of αβ-gliadin and nsLTP 9.1, all other tested wheat flour allergens seem to have cross-reacting homologues in rye flour. However, the absence of inhibition with only 2 bakers' sera does not rule out common structures and epitopes, whereas competitive inhibition proves shared epitopes between wheat and rye flour proteins. A high number of shared epitopes in both Triticeae also explains the strong correlation between sIgE concentrations to wheat and rye flour in bakers' sera ($r_{log} = 0.90$).

IgE cross-reactivity to single allergen components and grass pollen extract

Grass pollen and wheat flour allergens also share IgE epitopes, according to the results of the respective inhibition experiments. In addition to the CCDs, 7 of the 18 recombinant wheat allergens showed cross-reactivity with grass pollen: profilin, nsLTP 9.1, nsLTP 9.2, ω5-gliadin, thioredoxin, triose-phosphate isomerase, and GAPDH. Consequently, with the exception of GAPDH, these allergens bind IgE in the grass pollen–sensitized control subjects to a level comparable with or even stronger than in the bakers, whereas allergens without grass pollen inhibition (with the exception of 2 positive results for Tri a 29.01) bind low sIgE concentrations in control subjects (Fig 2). The observed difference in inhibition results between sera of wheat-sensitized patients with hay fever and patients with baker's asthma[19] can thus be explained by different IgE reactivity profiles. IgE of patients with grass pollen allergy is bound to wheat flour allergens with shared epitopes, and therefore binding to wheat flour can be inhibited by grass pollen similarly to that seen with autoinhibition, whereas the bakers' IgE is bound predominantly to specific cereal flour allergens and only partially inhibited by grass pollen components.

Because of limited serum volumes, inhibition experiments were performed with only 2 sera in most cases, and therefore our results should be interpreted as clues but not final evidence, especially in the case of absent inhibition. The knowledge of cross-reactive allergens might be helpful in understanding the interdependency between hay fever and baker's asthma. The prevalence of sIgE to wheat flour using ImmunoCAP in a nationwide representative sample of children and adolescents in Germany was 9.9%, with higher frequencies in boys than in girls and increased prevalence with increasing age.[36] About 42% of these wheat-sensitized adolescents had current hay fever.[37] Thus even before wheat flour exposure at the workplace, apprentices frequently have positive test results. Especially in studies for dose-response relationships or evaluation of preventive measures in bakeries, differentiation of occupational sensitization and sensitization caused by cross-reactivity would be helpful.

Completeness of the allergen panel to identify wheat flour sensitization

Even though we included 21 single-allergen CAPs in our study, this panel did not detect all bakers selected based on a positive wheat flour CAP value. Twenty (20%) bakers did not react to any single allergen with sIgE levels of 0.35 kU_A/L or greater, and 9 (9%) were less than the detection limit of 0.1 kU_A/L. This can be explained by several hypotheses. First, the recombinant allergens could be differently folded than natural ones; for example, the fusion with MBP could have modified epitope accessibility. Second, the recombinant allergens lack posttranslational modifications, such as glycosylation, which differ from the carbohydrates in HRP and MUXF. Third, in natural extracts most allergens occur as several variants and isoforms that comprise more epitopes than any single allergen variant alone. Fourth, some allergens are still missing. Most probably, all these points contribute to the difference.

With these limitations in mind, the highly significant correlation between the sum of single-allergen IgE concentrations and the wheat flour CAP value, both in bakers and in control subjects, is promising. Not only missing epitopes but also identical epitopes on several single allergens, such as on the partially homologous α-amylase inhibitor components or the 2 CCDs, can disturb the correlation between the wheat flour CAP value and the sum of IgE concentrations because IgE concentrations to those epitopes are added together in the latter

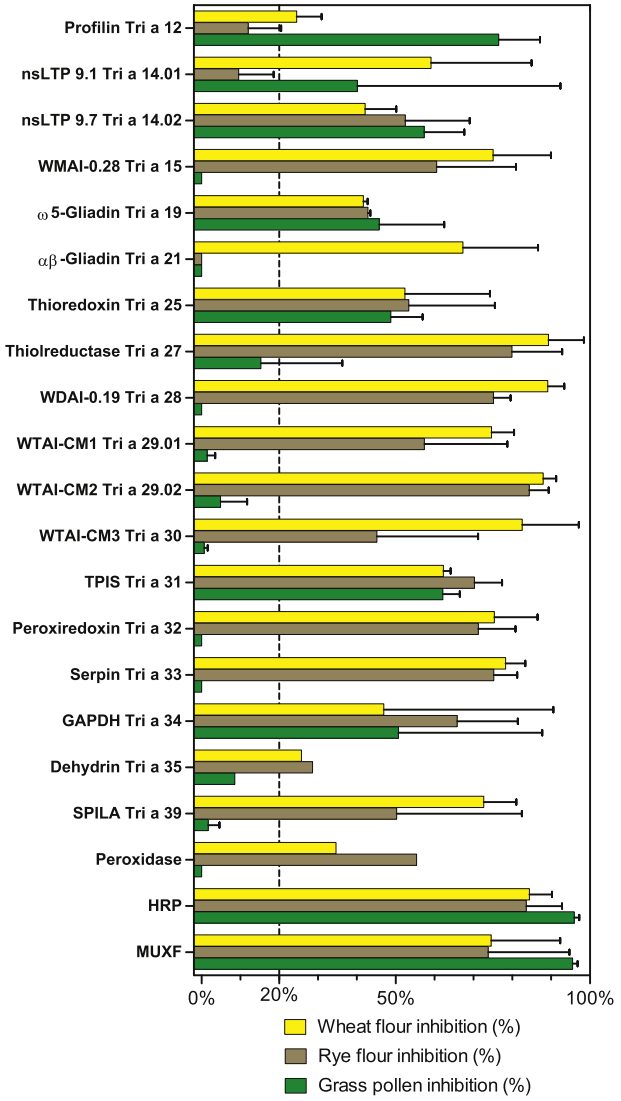

FIG 3. Inhibition of sIgE binding to single allergens by wheat flour, rye flour, or grass pollen proteins. Each *bar* indicates the mean and SD of the inhibition percentage obtained with 2 bakers' sera from different countries. Three sera for Tri a 39 and only 1 serum for Tri a 35 and the peroxidase were used, and in the case of Tri a 19, the second serum was a control serum. Inhibition greater than 20% is evaluated as significant.

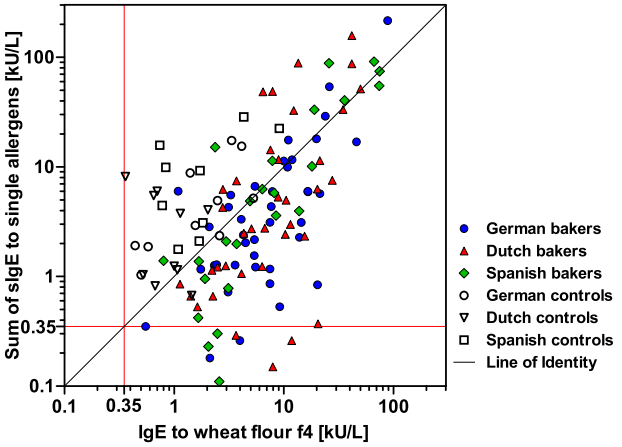

FIG 4. sIgE concentrations to wheat flour in relation to the sum of sIgE concentrations to single allergens. The *solid lines* at 0.35 kU_A/L mark the usual cutoff for positive values.

case. Therefore the summed single-allergen IgE concentrations usually produce higher values than results with a mixture of single allergens or natural extracts.[38] This is not the case for most of the bakers, indicating that missing epitopes play a greater role than redundant ones for this group in our study. In contrast, for most control subjects, higher sum values than wheat flour IgE values were obtained. In these cases limited amounts of the cross-reacting allergens profilin, thioredoxin, or nsLTP in wheat flour extracts and thus on wheat flour CAPs might be the explanation because IgE values higher than those to wheat flour were obtained for profilin in 11 control subjects, for thioredoxin in 3 control subjects, for nsLTP 9.7 in 2 control subjects, and for nsLTP 9.1 in 1 control subject (data not shown). These results are in line with the only partial inhibition of IgE binding to these allergens obtained with the wheat flour extract.

Wheat allergen components for diagnosis of baker's allergy

For component-resolved analysis of occupational wheat flour allergy, the allergens exclusively recognized by IgE from wheat flour–exposed patients should play a key role. Secondary wheat flour sensitization of primary grass pollen–sensitized subjects is a common phenomenon,[39] and therefore such subjects from the same countries as the bakers were selected as the control group. All allergen components and combinations were evaluated concerning their ability to discriminate between both selected groups: the bakers given a diagnosis of wheat flour allergy and the control subjects without wheat flour exposure but with wheat flour–specific IgE. To diagnose occupational wheat flour allergy, a combination of IgE tests with the following 5 allergens produced the best results in ROC analyses (AUC = 0.84) and, with a cutoff value of 0.12 kU_A/L or greater, produced the best performance measures (97% specificity and 70% sensitivity): thiol reductase (Tri a 27), WDAI-0.19 (Tri a 28), WTAI-CM2 (Tri a 29.02), SPILA (Tri a 39), and 1-cys-peroxiredoxin (Tri a 32). The most frequently detected allergen, thiol reductase (27% of bakers, ≥0.35 kU/L) was identified previously by using 2-dimensional immunoblots[40-42] as 27-kDa protein spots with different isoelectric points but had not been used as a single component other than in our previous study in German bakers. The important role of α-amylase inhibitors for baker's asthma is well accepted[1,33,43]; however, it was not clear which and how many of the subunits should be included for component-resolved analysis. According to high correlations between the partially homologous subunits, it might be sufficient to include 2 components. SPILA was the allergen to which the highest sIgE values were observed in the baker group, which is in line with its previously shown relevance for baker's asthma.[8,14,21] The importance of 1-cys-peroxiredoxin is supported by a study with bakers from Italy and Austria.[7] Recombinant 1-cys-peroxiredoxin (with 97% sequence identity to wheat 1-cys-peroxiredoxin [Tri a 32.0101]) was the most frequently recognized allergen in dot-blot analyses, with 10 positive results of 28 tested sera (36%), and it induced specific and dose-dependent basophil degranulation. However, in comparison with the purified native 1-cys-peroxiredoxin, IgE binding of this recombinant allergen in ELISAs was clearly

increased. This recombinant Tri a 32 allergen variant was also the most reactive wheat allergen in 81 Italian bakers, with 13 positive results (16%).[8] Specific IgE to this allergen was associated with work-related asthma and dermatitis.

In addition to the 5 allergens of the best combination, 2 α-amylase inhibitor components (Tri a 15 and Tri a 30), αβ-gliadin (Tri a 21) and serpin (Tri a 33), were specific for baker's allergy and showed no cross-reaction to grass pollen in the inhibition experiments. However, because mostly the same bakers had sIgE as already detected with the 5 most important allergens, addition of these allergens in combination did not improve the results for our study group.

Allergens with significantly higher sIgE levels in control subjects than in bakers were MUXF, HRP, and profilin. This fact and higher inhibition rates by grass pollen than by flour extracts indicate that they are often not primary allergens for baker's asthma. A negative profilin and MUXF result of 0.1 kU_A/L or less in this wheat-sensitized study group provided 98% precision to identify a baker.

Allergens with similar frequencies in IgE binding in bakers and control subjects and, consequently, in ROC analyses with AUC values near 50% are thioredoxin (18% of bakers ≥0.35 kU_A/L) and both nsLTPs (5% or 11% of bakers ≥0.35 kU_A/L). They belong to the allergens sharing epitopes with grass pollen, and whether they are primary or secondary allergens for bakers might vary from case to case. Addition of these allergens in component-resolved analysis improved sensitivity but not specificity.

Finally, the question has to be raised whether component-resolved diagnosis of baker's allergy to wheat flour is already able to improve the classical diagnosis based on wheat flour sIgE levels. Although we think a big step forward toward this aim was made with the data from our study, for the moment, the answer has to be negative. Other allergens not yet in our test panel, such as the wheat tetrameric α-amylase inhibitor subunit CM16,[10] or probably more allergenic isoallergens, such as serpin,[44] could help to reach the sensitivity of wheat flour, and then the increased specificity of a combination of selected components might improve diagnosis.

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Clinical implications: Knowledge of the most important IgE-binding wheat flour components might contribute to standardize diagnostics for baker's asthma and might help to discriminate between grass pollen allergy, wheat-induced food allergy, and baker's asthma.

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