Identification of wheat gliadins as an allergen family related to baker's asthma

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Background: Flour is still one of the most common causes of occupational asthma worldwide. Thus far, little is known about the relevant allergens causing baker's asthma. Therefore the reliability of current diagnostic procedures is insufficient. Only few of the suspected causative wheat allergens have been hitherto characterized on the molecular level.
Objective: The aim was to identify and characterize unknown wheat allergens related to baker's asthma to improve the reliability of diagnostic procedures.
Methods: A wheat pJuFo cDNA phage display library was created and screened for IgE binding to wheat proteins with pooled sera from patients with baker's asthma. After identifying an αβ-gliadin, the frequency of sensitization was investigated by means of ELISA screening of 153 bakers' sera with the recombinant αβ-gliadin. Furthermore, the allergenicity of native total gliadin (αβ, γ, ω) was analyzed by means of ImmunoCAP.
Results: One cDNA clone was identified as an αβ-gliadin. Serum IgE antibodies to the recombinant allergen were found in 12% of bakers with occupational asthma. Of the asthmatic bakers, 33% showed sensitization to native total gliadin; 4% of them had negative results on routine IgE testing with wheat extract.
Conclusions: Gliadins represent a newly discovered family of inhalable allergens in baker's asthma. This finding demonstrates that water-insoluble proteins might also represent causative allergens. (J Allergy Clin Immunol 2008;121:744-9.)

Key words: *Baker's asthma, wheat, gliadins, recombinant αβ-gliadin, recombinant allergens, cDNA phage display*

Baker's asthma is still a serious kind of occupationally related obstructive airway disease worldwide, affecting 4% to 25% of bakery workers.[1-4] In the West wheat is the primary cereal used in bread baking. Sixty percent to 70% of bakers with rhinitis or asthma have increased specific IgE levels to wheat, rye, or both flour extracts.[5,6] Further causative allergens are derived from taxonomically unrelated soybean flour, buckwheat, and other noncereal components, such as fungal α-amylase, spices, baker's yeast, egg powder, and other additives.[7,8]

Reliable diagnostic tools for baker's asthma and especially wheat sensitization are still not available. Commercially available native wheat extracts commonly used for skin prick tests and serologic analyses differ in antigen concentration and composition because of the nonstandadized allergen sources and preparation procedures. This might lead to false-negative results. Therefore there is a need for the improvement and standardization of diagnostic tools for baker's asthma (ie, by means of highly specific and sensitive recombinant diagnostic tests).

Wheat proteins are subdivided into the water/salt-soluble fraction (including albumins and globulins) and the water/salt-insoluble gluten (containing gliadins and glutenins). The latter represent about 80% of all wheat proteins. The mainly monomeric gliadins exhibit intramolecular disulfide linkages, thus being compact and of globular shape. The subunits of the gliadin family are the αβ-, γ-, and ω-gliadins based on their electrophoretic mobility. The different gliadin subunits share amino acid sequence and 3-dimensional structure homologies.[9]

By means of Western blotting and 2-dimensional immunoblot analyses, preliminary studies showed bakers' sera reacting with a wide range of wheat proteins from less than 6.5 kd up to 100 kd, with a great interindividual heterogeneity in sensitization patterns.[10,11] Thus far, only few flour allergens have been described at the molecular level: α-amylase inhibitors,[12] acyl-CoA oxidase and fructose-bisphosphate aldolase,[13] a wheat glycoprotein with peroxidase activity,[14] triose-phosphate isomerase (TPIS),[15] and recently thioredoxin,[16] all of which belong to the water/salt-soluble protein fraction.

Studies by Walsh et al,[17] Weiss et al,[13,18] and Sandiford et al[19] demonstrated IgE binding in the water-insoluble glutenin fraction (including the gliadin families) of wheat flour. Nevertheless, the water-soluble albumin fractions were believed to represent the most relevant allergens.[11,20,21]

The aim of our study was to identify and characterize unknown wheat allergens related to baker's asthma to improve diagnostic tests. We used the phage display technique, which has been previously demonstrated to be a promising method in the molecular characterization of allergens from birch and grass pollens and peanut proteins.[22,23]

METHODS

Patients' sera

Sera for IgE immunodetection were obtained from 153 bakers with rhinitis and bronchial asthma caused by type I allergy to flour (kindly supplied by Professor K.-Ch. Bergmann, formerly of the Allergie- und Asthma Klinik, Bad Lippspringe, Germany). The diagnosis was made by means of clinical history and immunologic testing. In 36 cases in which no agreement between clinical history and immunologic testing was found and airway resistance was normal or only slightly increased (specific airway

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resistance [sRaw] $\leq$ 1.2 kPa x s), the diagnosis was confirmed by means of inhalation challenge tests with wheat flour. None of the patients reported symptoms after eating wheat-derived products. All of the 153 bakers had signed an informed consent form. The 153 subjects (20 female and 133 male subjects) had an average age of 40 years. For the biopanning procedure, pooled sera from 21 of the 153 bakers with occupational asthma (2 female and 19 male subjects) were used; all of them were classified as ImmunoCAP class 3 (relevant sensitization) for wheat flour. The control sera were from 100 subjects (40 female and 60 male subjects with an average age of 42 years) not occupationally exposed to wheat flour (80 subjects worked in a psyllium-processing pharmaceutical industry and 20 worked in a coffee roaster). In none of the control sera had an obstructive airways disease ever been diagnosed; furthermore, none were sensitized to occupational allergens. All sera had been stored at −20°C until analysis was performed.

Construction of the wheat phage display library

A phage display library of developed wheat caryopses (*Triticum aestivum* L.) was produced with the cDNA synthesis kit from Stratagene (Amsterdam, The Netherlands). A modification of the producer's protocol was achieved by using 5 μg of total RNA to perform a first-strand synthesis. After ligation of the *Eco*RI adaptors to the double-stranded cDNA, a PCR reaction to amplify the obtained cDNA was carried out by using the following primers (obtained from Invitrogen, Karlsruhe, Germany): *ZAP5′ long*, GGTAGTTATGAGAATTCGGCACGAGG; *ZAP3′*, GAGAGAGAACTAGTCTCGAG.

PCR probes contained 100 mmol/L Tris HCl, 100 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 300 mmol/L betaine, 10 pmol of each primer, and 10 pmol of each deoxyribonucleoside triphosphate. The PCR program was a touchdown PCR (3 minutes at 94°C for 19 cycles [94°C for 45 seconds, 66°C for 45 seconds, and 72°C for 120 seconds] with a temperature decrease of 0.3°C, a subsequent 9 cycles [94°C for 45 seconds, 57°C for 45 seconds, and 72°C for 120 seconds], and a final step of 72°C for 5 minutes).

The resulting PCR fragments were ligated into the pCR2.1 Topo vector (Invitrogen) and transformed into 100 μL of *Escherichia coli* OneShot (Invitrogen) by means of electroporation. The transformed cells were incubated in 250 μL of SOC medium and subsequently used to inoculate 350 mL of LB medium containing 50 μg/mL ampicillin. Plasmid preparations were performed to gain the plasmids. Five hundred micrograms of plasmid was cut with *Eco*RI/*Xho*I, and the resulting inserts were ligated into pJuFo-2 (kindly supplied by Professor R. Crameri, Schweizerisches Institut für Allergïe- und Asthmaforschung, Davos, Switzerland) according to the protocol of Crameri et al.[24]

Biopanning

This strategy covalently links gene products displayed on the phage surface as fusion proteins to the respective cDNAs integrated into the phage genome. For the selection of IgE-binding proteins from the phage display cDNA library, 3 to 5 rounds of affinity enrichment (biopanning) were carried out. Biopanning was performed with a modification of the protocol of Crameri et al.[24] Briefly, overnight coating at 4°C with the antibody solution (mouse mAb, anti-human clone MH25-1; Progen, Heidelberg, Germany) was used to capture IgE from the pooled sera. The cDNA library was added after the blocking, incubation with diluted sera, and washing steps. After phage removal and extensive washing, binding phages were eluted with elution buffer (0.1 mol/L glycine, pH 2.2). The eluate was immediately neutralized with 14 μL of Tris base (0.5 mol/L), and the eluted phagemide was used to infect 5 mL of freshly grown *E coli* XL1-Blue (Stratagene) for 30 minutes at 37°C. One microliter of ampicillin (100 mg/mL) was added to the first infection culture. After the next incubation time (32°C at 250 rpm for 30 minutes), another 4 μL of ampicillin was added (incubation overnight at 32°C and 300 rpm). The amplification of the infected *E coli* cells was completed by infection with the VCSM13 helper phage (Stratagene). After incubation (8 hours at 30°C and 300 rpm), the culture was spun down (5000 rpm at 4°C for 20 minute), and the phage was precipitated by adding one-quarter volume of 20% (wt/vol) polyethylene glycol-8000 and 15% (wt/vol) NaCl overnight at 4°C to the supernatant. In a further centrifugation step (5000 rpm at 4°C for 20 minutes), the precipitated phages were spun down and dissolved in 2 mL of cold Tris-buffered saline followed by a cleaning step (14,000 rpm at 4°C for 10 minutes). The phages were precipitated for a second time with one-quarter volume of the cold PEG-NaCl solution (14,000 rpm at 4°C for 10 minutes), and resuspended in an adequate volume of Tris-buffered saline. The phages were stored at 4°C. For long-term storage, PEG-NaCl solution was added.

Cloning of an αβ-gliadin

After affinity selection, a total of 89 clones from the enriched repertoires were randomly hand-picked, and plasmid DNA was prepared. The cDNA inserts were analyzed by means of restriction analysis with *Eco*RI and *Xho*I. Repeatedly appearing clones with cDNA inserts of the same size were sequenced. Sequencing reactions were performed on an ABI Prism sequencer by using the ABI Prism Big Dye reaction kit (Applied Biosystems, Darmstadt, Germany). The sequences were analyzed with the software Chromas Lite and BLAST searches run on the National Center for Biotechnology Information Web page (http://www.ncbi.nlm.nih.gov/BLAST). The inserts from the pJuFo clones were subcloned into the high-level expression vector pQE-30 UA (Qiagen, Hilden, Germany) after amplifying with the following oligonucleotides: forward, 5′-GAA TTC GGC ACG AGA GCA-3′; backward, 5′-CAT TAA GCG CGG CGG GTG-3′.

All primers used in the PCR amplification were synthesized by Invitrogen. The ligation was carried out with the QIAexpress UA cloning kit (Qiagen) and transformed into M15 electrocompetent *E coli* cells by means of electroporation. Recombinant clones in the correct direction containing an insert of the correct size were selected, and the sequence was rechecked.

Expression and purification of recombinant proteins and SDS-PAGE

For the expression of recombinant proteins, the high-level expression vector pQE-30 UA and the *E coli* strain M15 (both from Qiagen) were used. The vector encodes a fusion protein with an aminoterminal hexahistidine-tag (6× His-tag), the prerequisite of which is purification with Ni-NTA. For the production of the recombinant proteins, 100 mL of media containing antibiotics was inoculated with an overnight culture and grown at 37°C until an OD_{600} of 0.6 was achieved. Gene expression was induced by adding isopropyl-β-D-thiogalactopyranoside in a final concentration of 1 mmol/L for 4 hours. The cell culture was harvested, and the pellet was stored at −20°C. The recombinant protein was purified over Ni-NTA under denaturing conditions in the presence of 8 mol/L urea buffer and eluted finally through pH-shift in a volume of 4 fractions of 500 μL, according to the instructions of the manufacturer. The protein content was measured by using the Bradford assay.[25] The purity of the eluates was determined by means of SDS-PAGE (NuPAGE, 10% bis-tris, Invitrogen). Before electrophoresis, the samples were prepared with LDS sample buffer (Invitrogen) and heated for 10 minutes to 70°C. Electrophoresis was run at a 200-V constant current until the tracking dye bromphenol blue reached the bottom of the gel. Afterward, the gel was stained with Coomassie. The molecular weights of standard proteins (Invitrogen) were myosin at 188 kd, phosphorylase at 98 kd, BSA at 62 kd, glutamic dehydrogenase at 49 kd, alcohol dehydrogenase at 38 kd, carbonic anhydrase at 28 kd, myoglobin red at 17 kd, lysozyme at 14 kd, aprotinin at 6 kd, and insulin b chain at 3 kd.

His-tag detection

The detection of the His-tagged fusion protein was done with the InVision His-tag In-gel Stain (Invitrogen). This method is sensitive and highly specific and enables the visualization of His-tagged fusion protein bands directly in a polyacrylamide gel after electrophoresis without Western blotting detection. It uses a fluorescent dye conjugated to Ni^{2+}, which binds with a high affinity to the oligohistidine sequence, and thereby a clear and specific visualization of the His-tagged protein is obtained. After electrophoresis, the proteins were