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Western Blot Analysis of Water-Soluble Wheat Flour (*Triticum vulgaris*) Allergens

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Abstract. Water-soluble wheat flour allergens were analyzed using the western blot technique. The sera of 130 bakers (42 with wheat flour allergy, 88 without allergy) were analyzed for IgE, IgG, and IgG4 binding towards wheat flour allergens. Three major allergens with molecular weights of 47, 17, and 15 kD were identified (Tri v Bd 47, Tri v Bd 17, and Tri v Bd 15; IUIS allergen nomenclature) by their ability to bind IgE from approximately 50% of the allergic sera. The specificity of the IgG was mostly directed against three high molecular weight components (134, 122, and 98 kD). In contrast, IgE and IgG4 was found frequently against the major allergens. 40% of healthy donors versus 12% of the asthmatic bakers had no detectable IgG4 against wheat flour extract. The biological activity of the water-soluble wheat flour components has been shown by their ability to induce histamine release from basophil leukocytes obtained from patients with bakers' asthma. The release of histamine from basophil leukocytes significantly correlated with the presence of IgE antibodies against wheat flour components of 47, 17 and 15 kD. A regulatory role of wheat flour specific IgG4 in allergic diseases could not be evaluated by western blot analysis.

Introduction

Hypersensitivity to inhaled flour allergens provides an excellent model of occupationally induced allergic asthma. It represents a significant proportion of allergic diseases. Allergic diseases of the lung increased during the last decades from 12% in 1961 to 25% in 1984 [1]. Suitable techniques for prevention and early diagnosis of wheat flour sensitive airway diseases are presently not available. The exact identification and characterization of the wheat flour allergens are one requirement for the development of new diagnosis and therapeutic concepts. Baking additives such as α -amylase, cellulase, or soybean flour have been described as allergens [2, 3]. But wheat flour and rye flour on their own have been identified as major allergens, although the individual allergenic components have not been satisfactorily characterized. In the past we generated monoclonal antibodies for the standardization of antigenic and allergenic components [4]. It was the purpose of this study to analyze wheat flour allergens by using the western blot technique. Different wheat flour extracts were analyzed with regard to their reactivity towards monoclonal antibodies.

Materials and Methods

The sera of 130 healthy as well as allergic bakers and pastry cooks who were daily exposed to wheat flour at their workplace were investigated with regard to their IgE- and IgG-binding pattern towards wheat flour allergens. With regard to their working anamnesis the donors were divided into three groups: (1) bakers without allergy ($n = 88$); (2) bakers with rhinitis at the workplace ($n = 26$), and (3) bakers with asthma at their workplace ($n = 16$).

Monoclonal Antibodies

The production of monoclonal antibodies has been described by Theobald et al. [4]: the panel of four monoclonal antibodies recognized components at molecular weights of 17 and 15 kD (W201), 32, 17, and 15 kD (W102), 67 and 32 kD (W106), and 67, 61, 47, and 39 kD (aB2).

Wheat Flour Extracts

Commercially available wheat flour (type 405; Aurora, Köln, FRG) was extracted by phosphate-buffered saline (PBS) containing NaCl (154 mmol/l), Na_2HSO_4 (8.05 mmol/l), and KH_2PO_4 (1.96 mmol/l). 1 g of wheat flour was mixed with 4 ml of PBS. The mixture was stirred for 1 h at room temperature. The extract was separated from the flour by centrifugation (10,000 g) for 20 min and stored at -20°C . The protein content of this extract was 4 mg/ml, as determined by the method described by Lowry et al. [5]. Furthermore, wheat flour was extracted by using sodium dodecyl sulfate (SDS), urea, ethanol, distilled water, acetic acid, isopropanol, and methylenamine. The extracts have been dialyzed against PBS,

lyophilized, and dissolved in deionized water at a final concentration of 4 mg/ml.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method described by Lughtenberg et al. [6]. The wheat flour extract was separated on a 12.5% polyacrylamide gel. The following marker proteins were used: Beriglobin® (150 kD; Behringwerke, Marburg, FRG), bovine serum albumin (69 kD; Boehringer, Mannheim, FRG), ovalbumin (45 kD; Sigma, Munich, FRG), α -chymotrypsinogen (24 kD; Serva, Heidelberg, FRG), and cytochrome C (12.5 kD; Sigma). After electrophoresis the gels were stained with Coomassie brilliant blue R250 (Serva, Heidelberg, FRG). The molecular weights of the wheat flour components were determined by linear regression in comparison with the marker proteins.

Immunoblot, Autoradiography

The extract was separated by SDS gel electrophoresis on a 12.5% acrylamide gel. After electrophoresis the gels were transferred to nitrocellulose by the western blot technique, as described by Towbin et al. [7]. After blotting, the filters were cut into strips of 0.5×10 cm. Nonspecific binding was eliminated by incubating the filter strips for 1 h in PBS containing 0.05% Tween 20 and 10% fetal calf serum. Subsequently, the blots were washed three times with a buffer containing PBS, 0.05% Tween 20, and 0.1% bovine serum albumin. Diluted sera (1:10 for IgE detection and 1:20 for IgG detection) were added to the blots and incubated overnight at room temperature in a rotation rack. The strips were rinsed, and the bound immunoglobulin was detected with a secondary ^{125}I -labelled antibody (10^6 cpm/4 ml; Tago, Hamburg, FRG) by autoradiography on Kodak X-Omat AR 5 film (-80°C , 1–4 days). Radiolabelling of goat antihuman IgE and IgG antibodies was done by the chloramine T method as described by Klinman and Taylor [8].

Histamine Release

Histamine release was performed as described by Siraganian [9]. Briefly, human basophil leukocytes were obtained from peripheral blood by dextran sedimentation. The crude allergenic extract was diluted 1:25, 1:250, and 1:2,500 in PBS. The histamine content was measured after incubation with allergen for 60 min by using a Technicon autoanalyzer. The allergen-induced histamine release (HR) was determined by the following equation:

$$\text{HR (\%)} = \frac{(\text{allergen-induced HR}) - (\text{spontaneous HR})}{(\text{perchloric acid induced HR})} \times 100.$$

Enzyme-Linked Immunosorbent Assay

This assay for extract characterization was performed as described elsewhere [10]. Briefly, flexible microtiter plates were coated with the extracts in the presence of 1.25% glutaraldehyde. Unbound protein was washed after incubation for 2 h at 37°C with PBS containing 0.05% Tween 20. Subsequently, incubation with the monoclonal antibodies for 2 h at 37°C was carried out. After washing, the bound monoclonal antibody was detected with a secondary peroxidase-labelled antibody (goat antimouse Ig: Nordic, Tilburg, The Netherlands). Incubation proceeded for 2 h at 37°C with the antibody diluted 1:200. The bound peroxidase activity was determined with 0.03% H_2O_2 in citric acid buffer and 0.075% (w/v) 2,2'-azino-bis(3-ethyl benzthiazoline sulfonic acid) (Sigma).

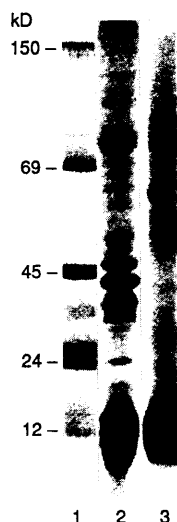


Fig. 1. Separation of wheat flour extract by SDS-PAGE and subsequent staining with Coomassie brilliant blue. 1 = Molecular weight markers; 2 = PBS-405 extract; 3 = binding pattern of the monoclonal antibody W201.

Statistical Analysis

Significant differences between the different groups were evaluated by the chi-square test. The levels of significance are given in the text.

Results

SDS-PAGE

The antigenic and allergenic components of the water-soluble wheat flour extracts were studied by SDS-PAGE and subsequent western blot analysis.

The pattern of the PBS-wheat flour extract after separation on a 12.5% SDS-polyacrylamide gel and subsequent staining with Coomassie brilliant blue is shown in figure 1. Several bands with molecular weights between 134 and 9 kD were detected. Eight major bands were obtained with molecular weights of 47, 39, 33, 29, 17, 15, 13, and 10 kD. Furthermore, 22 smaller bands could be detected by the Coomassie staining procedure.

Immunoblotting

We then analyzed the IgE- and IgG-binding patterns towards wheat flour allergens in individuals exposed to wheat flour dust. Therefore, water-soluble wheat flour allergens were separated on SDS-PAGE and electroblotted onto nitrocellulose sheets. The im-

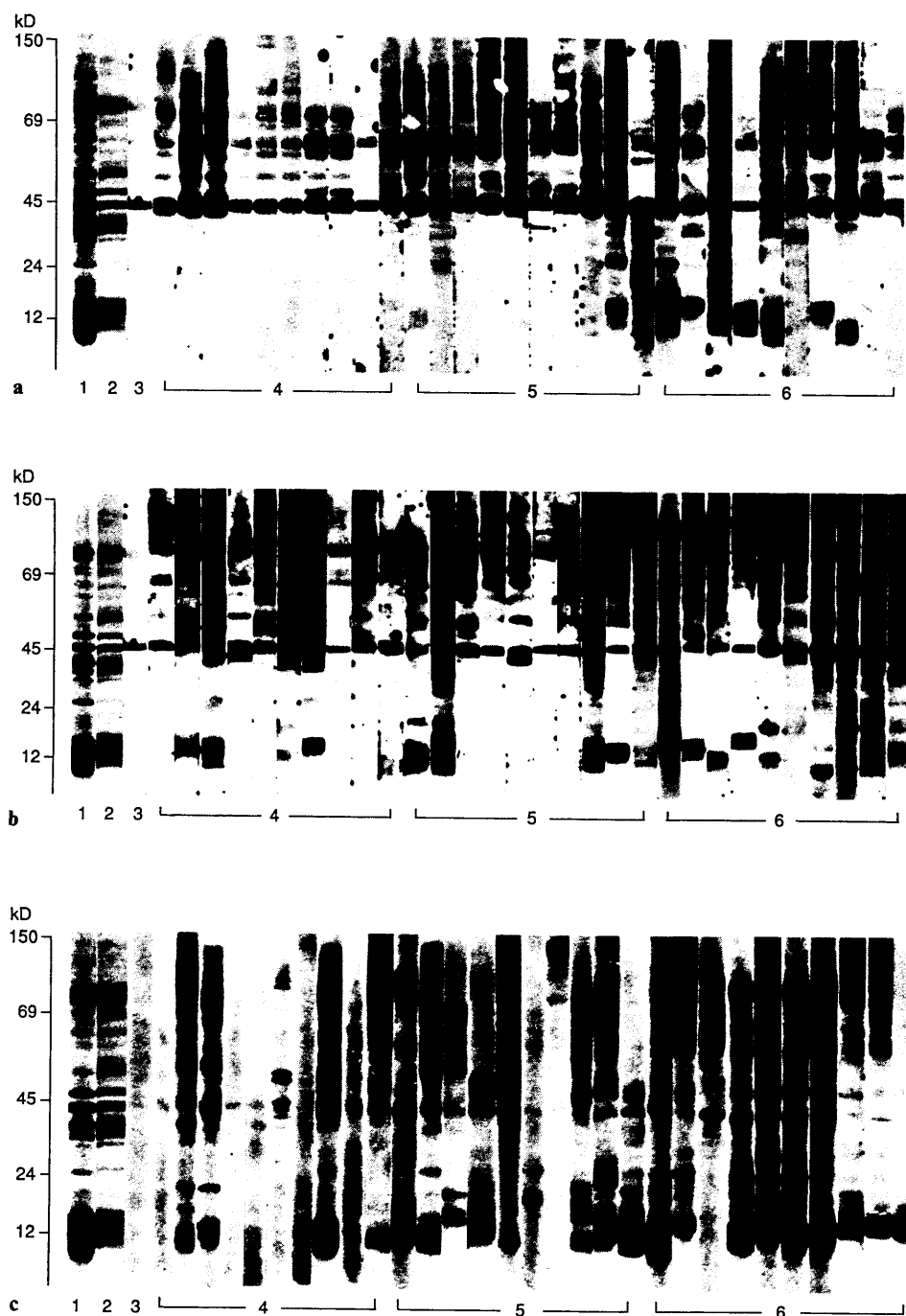


Fig. 2. Identification of Ig-binding components by autoradiography (western blot). a IgE. b IgG. c IgG4. 1 = Coomassie brilliant blue staining of separated wheat flour extract; 2 = amido black staining of the transferred proteins on nitrocellulose; 3 = nonspecific binding; 4 = strips incubated with human sera from nonallergic bakers; 5 = bakers with rhinitis; 6 = bakers with asthma.

munoglobulin-binding pattern of bakers with allergic airway diseases (asthma, rhinitis) as well as healthy volunteers was compared.

As is apparent, the control (buffer instead of serum = nonspecific binding; fig. 2a-c, lane 3) exhibits a regular binding of the radiolabelled antibody to five wheat flour proteins (83, 71, 63, 52 and 39 kD). Satu-

ration of the nitrocellulose with Tween 20, bovine serum albumin, fetal calf serum, horse serum, or non-fat dry milk had no effect on the nonspecific binding (data not shown). Antibodies directed towards human IgG and IgG4 or murine IgG also revealed the same nonspecific binding pattern to five wheat flour components.

Analysis of IgE Binding

Figure 2a shows a typical IgE-binding pattern of healthy bakers (group 4) or bakers with allergic rhinitis (group 5) or with allergic asthma (group 6). The pattern appears to be very heterogenous, but the frequency of IgE binding, as presented in figure 3a, demonstrates that the 74- and 69-kD components were recognized by approximately 60–70% of all sera (healthy as well as allergic). Three further components with molecular weights of 47, 17 and 15 kD were recognized by IgE in the sera of approximately 50% of the asthmatic bakers. By comparing the frequency of IgE binding towards wheat flour components in allergic and nonallergic bakers, statistically significant differences were observed for these three bands. IgE with specificity towards the 47-kD proteins were detectable in 56% of the asthmatic bakers and in 42% of the bakers with rhinitis. In nonallergic bakers, IgE with the same specificity was detectable only in 15.8%. These differences appear to be significant by the chi-square test ($p < 0.01$). Bindings towards the 17-kD (50%) and the 15-kD (44%) bands were more frequently in asthmatic than in nonallergic bakers (9 and 11%, respectively). Approximately 90% of the sera which recognized the 17-kD protein exhibit also binding towards the 15-kD band. No statistically significant differences of IgE binding towards the 17- and 15-kD components in bakers with rhinitis as compared to healthy bakers could be evaluated.

Analysis of IgG Binding

Binding of IgG antibodies towards wheat flour was not detectable in 22.5% of the nonallergic bakers. Figure 2b demonstrates the IgG-binding pattern of 10 nonallergic bakers (group 4). The distribution of the IgG binding (fig. 3b) in nonallergic bakers shows that mainly IgG with specificity towards high molecular weight components (134, 122 and 98 kD) was detectable. But IgG binding towards smaller molecules was also present. In allergic bakers (fig. 2b; groups 5 and 6, rhinitis and asthma, respectively), IgG binding towards the smaller proteins was more frequently detected as compared to nonallergics. Three bands with molecular weights of 47, 17 and 15 kD were recognized by the sera of approximately 43% (47 kD) to 65% (17 and 15 kD) of the asthmatic bakers.

Analysis of IgG4 Binding

Figure 2c shows the pattern of wheat flour specific IgG4 in the sera of bakers with and without allergic

symptoms. In bakers without allergic symptoms (group 4) we demonstrated IgG4 towards the 17- and 15-kD components: 35% of the nonallergic bakers had specific IgG4 towards these two allergens. In allergic bakers IgG4 directed against the 17- and 15-kD proteins predominated (fig. 2c, groups 5, 6). The statistical evaluation of the frequency of IgG4 binding towards the 17- and 15-kD components demonstrated no differences in allergic bakers as compared to healthy donors (fig. 3c). However, IgG4 against the 47-kD component was only rarely obtained in healthy as well as allergic donors.

Histamine Release

Sensitization towards water-soluble wheat flour components correlated with the allergen-induced in vitro histamine release from peripheral basophil leukocytes. The histamine release was enhanced in a dose-dependent manner by the wheat flour extract at dilutions between 1:25 and 1:2,500. At a dilution of 1:250 the histamine release in the nonallergic group was $5.13 \pm 2.5\%$. With regard to this level the bakers were divided into two groups: (1) with an allergen-induced in vitro histamine release $> 10\%$ (mean $+ 2$ SD) and (2) a group with $< 10\%$ histamine release after allergen challenge. The frequency of IgE binding towards wheat flour components in these two groups is shown in figure 4. Binding towards the 47-, 17- and 15-kD bands occurred in the histamine-positive group (histamine $> 10\%$) more frequently than in bakers which released $< 10\%$ histamine. These differences were statistically significant, as evaluated by the chi-square test ($p < 0.01$).

Binding of Monoclonal Antibodies

The generation of monoclonal antibodies towards wheat flour extracts has been described by us in detail. The binding of the monoclonal antibody (W201) is shown in figure 1 (lane 3). The monoclonal antibody W201 exhibits a binding towards both the 17- and the 15-kD components. Using this monoclonal antibody, wheat flour extracts obtained by various procedures were compared with regard to their content of the 17- and 15-kD allergens (fig. 5). The highest amounts of 17- and 15-kD components were detectable in PBS and urea extract. Less quantities were extracted by ethanol and isopropanol and distilled water alone. Under acidic (acetic acid) or basic (KOH) extraction conditions or using a detergent (SDS), only small amounts of these two components were eluted.

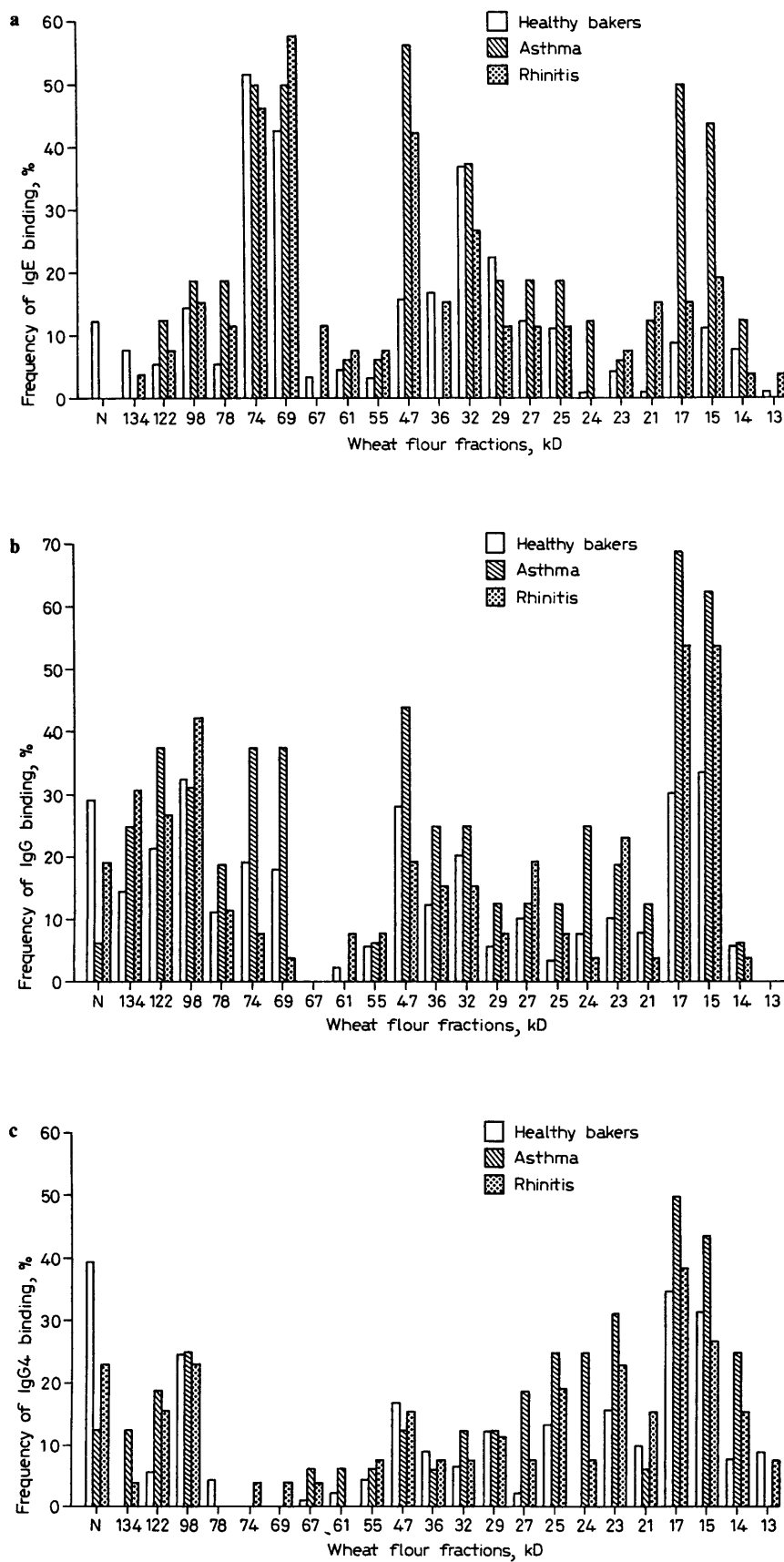
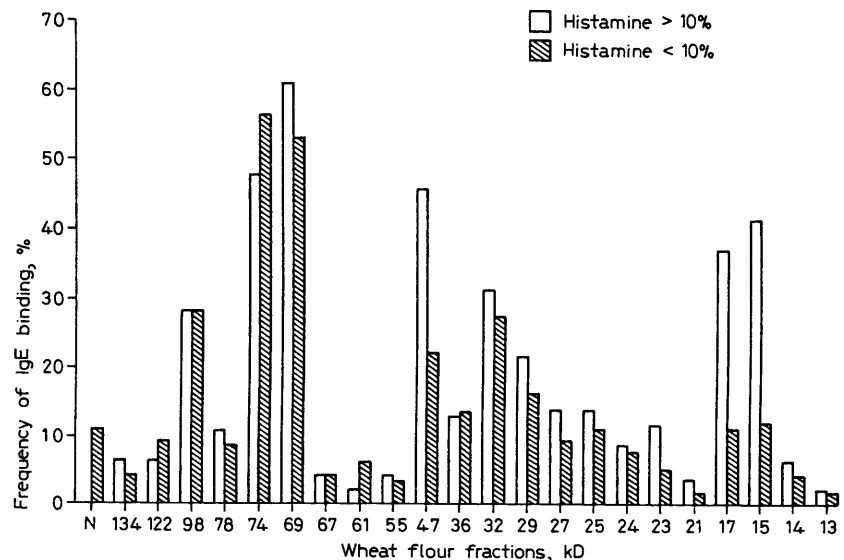


Fig. 3. Frequency histogram of Ig binding to each SDS-PAGE-separated wheat flour component from sera of healthy bakers, bakers with rhinitis, and bakers with asthma. **a** Frequency histogram of IgE binding. **b** Frequency histogram of IgG binding. **c** Frequency histogram of IgG4 binding. N = Percentage of bakers with no immunoglobulin binding.

Fig. 4. Frequency histogram of IgE binding to each SDS-PAGE-separated wheat flour component from sera of patients with wheat flour induced in vitro histamine release <10% and >10%. N = Percentage of bakers with no immunoglobulin binding.



Discussion

We investigated the binding capacity of sera from bakers exposed to wheat flour allergens. Therefore, commercially available wheat flour was extracted with PBS to obtain an extract under physiological conditions. One may argue that by these extraction procedures a proportion of wheat flour proteins is not extracted, as it has been shown by several investigators [11, 12]. But this extraction procedure appears to be equivalent to an allergen extraction occurring in the respiratory environment and leads to highly allergenic wheat flour extracts [12-14]. We demonstrated IgE and IgG antibodies with specificity towards wheat flour components in the sera of allergic as well as nonallergic bakers. All components between 134 and 12 kD exhibit IgE and IgG binding in wheat flour exposed donors, as detected by western blot analysis. Even clinically nonallergic bakers revealed wheat flour specific IgE antibodies. But IgE and IgG binding was more frequent in bakers with airway hypersensitivity as compared to donors without any allergic symptoms. These data are in line with the results reported previously by Theobald et al. [15] and Kiefer et al. [16] who demonstrated Ig binding towards wheat flour components from the sera of nonallergic donors. Bakers who reported dyspnea and cough at the work-place expressed more frequently IgE with specificity towards the 47-, 17- and 15-kD bands than nonallergic donors. In 90% of the analyzed sera which recognized the 17-kD band, IgE

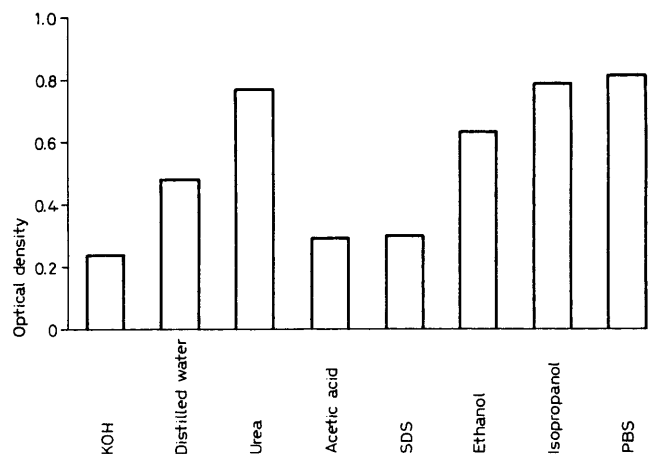


Fig. 5. Quantification of the 17- and 15-kD allergen using monoclonal antibody W201. Eight different extraction solutions were compared by a solid-phase enzyme-linked immunosorbent assay.

binding towards the 15-kD component occurred simultaneously. The monoclonal antibody W201 also recognized the 17- as well as the 15-kD wheat flour component. These data suggest that identical epitopes may be present on different wheat flour components. The analysis of different wheat flour extracts with a monoclonal antibody, which reacted with the 15- and 17-kD components, clearly shows that the conditions of allergen extraction has influences on the composition of the allergen extract.

Baldo et al. [17] reported, in agreement with our results, on a water-soluble wheat flour component with

a molecular weight between 15 and 30 kD, as was determined by gel filtration, which had a high affinity towards IgE from allergic individuals. Additionally, we demonstrated an IgE-binding component with a molecular weight of 47 kD. Bakers with allergic asthma revealed in approximately 50% of the cases IgE with specificity to this component. In bakers with wheat flour sensitive rhinitis, we demonstrated IgE binding towards the 47-kD component, but bakers with rhinitis showed rarely a sensitization against the 17- and 15-kD proteins. Sensitization against the 47-kD component thus appears to be an early sign of allergic airway disease. Development of IgE with specificity towards the 17- and 15-kD component is associated with the manifestation of bronchial hyperreactivity. A correlation of in vitro histamine release from peripheral basophil leukocytes with the IgE-binding pattern clearly revealed the importance of specific IgE towards the three components (47, 17 and 15 kD) for histamine release. Sensitization against these three proteins was associated with an allergen-inducible histamine release. Similar results were obtained by Blands et al. [14] who had identified three highly allergenic components in wheat flour extracts by crossed radioimmuno-electrophoresis. Sensitization towards these allergens was also positively correlated with the allergen-induced in vitro histamine release.

Our data clearly demonstrate that three wheat flour components with molecular weights of 47, 17 and 15 kD were of high allergenic potency. With regard to the IUIS allergen nomenclature [18] they should be called Tri v (*Triticum vulgaris*) Bd 47, Tri v Bd 17, and Tri v Bd 15.

By the western blotting procedure we obtained a nonspecific binding of immunoglobulins to five wheat flour components. This may be due to lectinlike binding which has been described for several allergens [19].

Binding of IgG antibodies towards wheat flour components appears to be more frequent in allergic than in nonallergic bakers. The major binding pattern was identical to that obtained for IgE. The induction of the IgG response in allergic bakers is in contrast to the low immunization of nonallergic individuals. Elevated resorption of wheat flour proteins may be caused by alteration of the epithelial barrier. The occurrence of IgG antibodies thus could be regarded as a secondary effect in the pathogenesis of allergic asthma. A protective effect of the IgG, especially the IgG4 response as described by other investigators for

different allergens [20] was not demonstrated. Allergic bakers express more frequently IgG4 binding towards wheat flour components than healthy donors. This could be due to the inflammation-induced alteration of the bronchoalveolar wall which subsequently induces a higher resorption of antigenic components. It is speculative to what extent IgG4 antibodies are involved in the human allergic response [21].

Our data clearly demonstrate that the water-soluble wheat flour fraction contains three components with high allergenic potency. Purification and characterization by molecular biological techniques will lead to a better understanding of the mechanism involved in the induction of allergic diseases. The characterization of the major allergens is a prerequisite for the standardization of allergen extracts for the in vivo and in vitro diagnosis as well as novel therapeutic strategies.

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