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Identification and characterization of wheat grain albumin/globulin allergens

Bakers' asthma, an immediate-type allergic response to the inhalation of cereal flours, is an important occupational disease among workers of the baking and milling industries, and the salt-soluble proteins of wheat and rye flour dust are considered the most relevant allergens. In order to identify and characterize the major IgE-binding proteins, the polypeptide composition of the albumin/globulin protein fraction obtained from different cultivars was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and high-resolution two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients in the first dimension (IPG-Dalt), followed by immunoblotting with sera from asthmatic bakers. Relevant allergens were isolated by micropreparative IPG-Dalt and blotting onto polyvinylidenedifluoride membranes and identified by amino acid composition analysis or N-terminal amino acid sequence analysis. SDS-PAGE, IPG-Dalt, and immunoblotting demonstrated that the sera of the bakers allergic to flour contained IgE antibodies which bound to numerous albumin/globulin polypeptides in the 70, 55, 35, 26–28, and 14–18 kDa areas. More detailed investigations using IPG-Dalt revealed cultivar-specific differences in IgE-binding. It was also demonstrated that the majority of the allergens were not single polypeptide spots, but consisted of up to ten isoforms of similar molecular mass but different isoelectric points. Amino acid composition analysis and N-terminal amino acid sequence analysis, which were performed for nine allergens located in the 14–18, 26–28, and 35 kDa areas, revealed homologies to amylase/protease inhibitors, acyl-CoA oxidase and fructose-bisphosphate-aldolase from wheat, barley, maize, and rice, respectively*.

1 Introduction

Cereal-induced occupational asthma (bakers' asthma) primarily affects persons working in the baking and milling industries. The frequency of this disease varies considerably in different parts of the world [1]. In Germany, bakers' asthma is the most common cause of occupational asthma requiring compensation, and during the last fifteen years, the number of notified cases has steadily increased from 569 cases (in 1980) to 1764 cases (in the year 1995). Research has been directed primarily towards the identification of the allergens responsible for the induction of this inhalation-induced hypersensitivity. Bakers' asthma is an IgE-mediated type I allergic reaction to flour dust, and (though to a lesser extent) to storage mite and baking additives [2, 3]. IgE antibodies to a number of wheat flour components have been demonstrated in allergic bakers' sera [4–8], and the strongest reactivity was observed with albumins/globulins. Although the albumins and globulins are considered to be the most important inhalant allergens, physicochemical data on these proteins are still lacking. More detailed information on these allergens is of

importance, not only for the development of better diagnostic tools, but also for the prevention of this disease by processing less allergenic flours. In the present paper we summarize the results of our studies on the identification and characterization of relevant albumin/globulin allergens in different wheat cultivars by two-dimensional gel electrophoresis with immobilized pH gradients (IPG-Dalt), immunoblotting, amino acid composition analysis, amino acid sequence analysis, and protein database searching.

2 Materials and methods

2.1 Apparatus and chemicals

All apparatus for IEF and horizontal electrophoresis (Multiphor II electrophoresis chamber, Macrodrive V power supply, gradient mixer) and blotting (Novablot, Macrodrive I power supply, GelRemover), Immobilines, Pharmalyte pH 3–10, GelBond PAGfilm, and ¹²⁵I anti-human IgE (Phadebas; RAST) were from Pharmacia-Biotech (Uppsala, Sweden). The Dalt-apparatus for multiple runs was from Pharmacia-Hoefer (San Francisco, CA, USA). Acrylamide (2 × cryst.), N,N'-methylenebisacrylamide, SDS, and Amido Black 10B dye was from Serva (Heidelberg, Germany). Polyvinylidene difluoride (PVDF) membranes were from Millipore (Bedford, MA, USA; Immobilon P) and Pall (Dreieich, Germany; Fluoro Trans[®]). Polyvinylpyrrolidone (PVP, M_w 40 000), iodoacetamide, dithiothreitol (DTT), CHAPS, Tris(hy-

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Nonstandard abbreviations: IPG-DALT, two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradient

Keywords: Allergy / Bakers' asthma / Immobilized pH gradient / Amino acid sequence analysis / Two-dimensional polyacrylamide gel electrophoresis / Wheat

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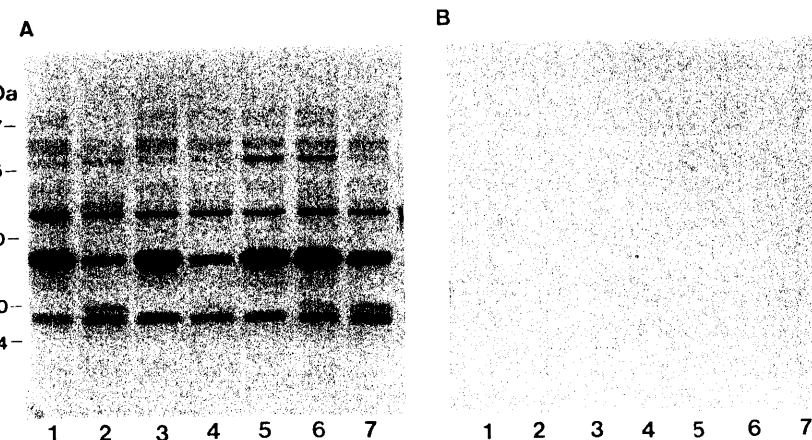


Figure 1. Wheat grain albumin/globulins separated by SDS-PAGE (10–15%T) and electroblotted onto a PVDF membrane. Immunoblotting with (A) patients' sera (asthmatic bakers) and (B) control sera (serum pool of healthy donors and individuals allergic to house dust mite), followed by secondary ¹²⁵I-labeled anti-human IgE and autoradiography. Samples: wheat cultivars (1) Apollo, (2) Aron, (3) Avis, (4) Boheme, (5) Bussard, (6) Fregatt, (7) Futur.

droxymethyl)aminomethane (Tris), and alkaline phosphatase-labeled anti-human IgE were from Sigma (St. Louis, MO, USA). Serum albumin (BSA, fraction V), urea, glycerol, and all other chemicals (analytical grade) for electrophoresis and staining procedures were from Merck (Darmstadt, Germany). Fount India ink was obtained from Pelikan (Hannover, Germany), and Kodak X-OMAT AR X-ray films were from Kodak (Rochester, NY, USA).

2.2 Sera

Sera were collected from 20 bakers who reported asthmatic symptoms or rhinitis after exposure to flour. A diagnostic procedure, including intra-cutaneous testing with a wheat-flour extract and, in several cases, challenge tests (nasal and bronchial), confirmed that these persons were allergic to flour. Specific IgE antibodies to wheat flour proteins were evaluated by IgE RAST (Pharmacia) according to the manufacturer's instructions using commercially available allergen discs. The levels of specific IgE of the subjects allergic to flour, as determined by the RAST method, were ≥ 3.5 units per mL serum (equal to RAST score ≥ 3). A control serum was pooled from six exposed, but asymptomatic bakers, as well as from persons allergic to house dust mite, and no specific IgE antibodies to wheat flour proteins were detected in these control sera by the RAST method. All sera were stored at -20°C until use.

2.3 Protein extraction procedure and sample preparation for electrophoresis

Samples of twenty soft winter wheat (*Triticum aestivum* L.) cultivars were provided by Bertha Killermann (Bayrische Landesanstalt für Bodenkultur und Pflanzenbau, Freising-Weihenstephan). The majority of these varieties

is grown for breadmaking purposes in Germany. Grain samples were ground in a mortar with a pestle until a fine powder was obtained that passed through a 0.5 mm wide sieve. Salt-soluble flour proteins (albumins and globulins) from whole flour were then extracted according to the following protocols.

2.3.1 Protein extraction and sample preparation for SDS-PAGE

Tris buffer-extractable wheat flour proteins were prepared as described previously [8]. Briefly, samples of the ground grain (1.0 g) were extracted with 4 mL of Tris-HCl buffer (50 mM, pH 8.8), and aliquots (200 μL) of the supernatant were mixed with 500 μL of SDS sample buffer (50 mM Tris-HCl, pH 8.8, 1% SDS, 0.5% DTT and 20% glycerol) and heated to 95°C (5 min).

2.3.2 Protein extraction and sample preparation for IPG-Dalt

Salt-soluble wheat proteins were extracted from flour (150 mg) with 1.0 mL of buffer (0.8% w/v Pharmalyte pH 3–10) for 30 min (4°C) with occasional vortexing. Then the sample was centrifuged for 1 h at 40 000 g. The clear supernatant was removed, diluted with an identical volume of solubilization buffer (9 M urea, 1% dithiothreitol, 2% CHAPS and 0.8% w/v carrier ampholytes, pH 3–10) and stored at -78°C until used.

2.4 Electrophoresis

2.4.1 Horizontal SDS-PAGE of wheat grain proteins

Horizontal SDS-PAGE was carried out as described previously [8]. The SDS-gels contained a 4% polyacrylamide

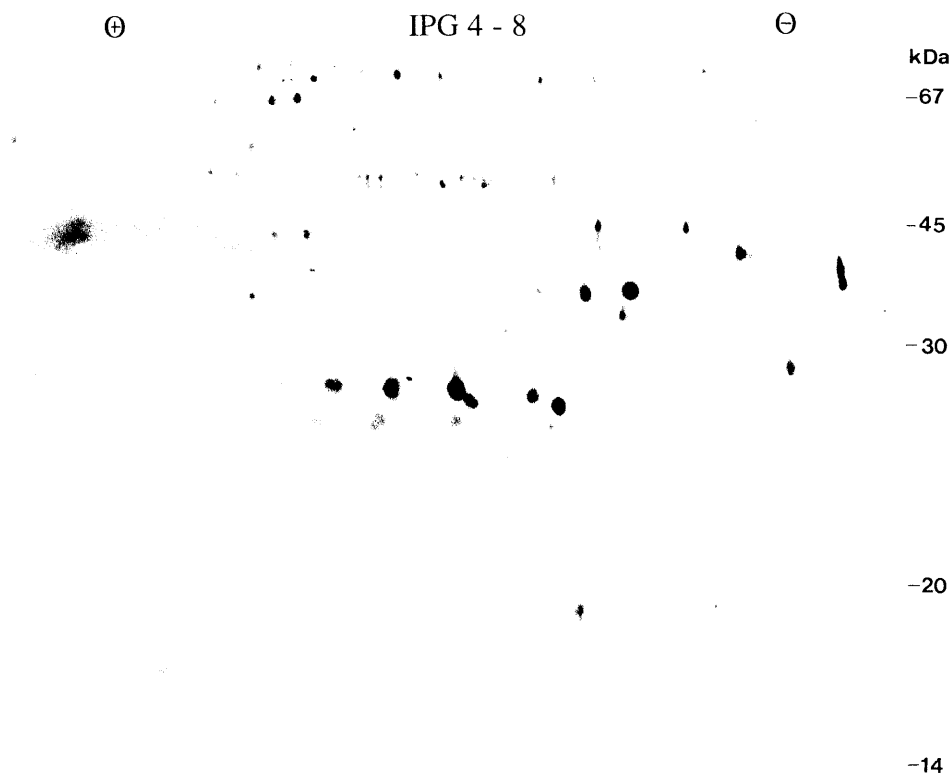


Figure 2. Detection of salt-soluble wheat flour allergens after IPG-Dalt 4–8 and blotting onto a PVDF membrane. Immunostaining with patients' sera and secondary alkaline phosphatase-labeled anti-human IgE.

stacking gel and a resolving gel of a linear gradient from 10–15% polyacrylamide (4% C constant). The buffer system of Laemmli [9] was employed. Samples (3 μ L when the proteins were silver-stained, and 6 μ L when the proteins were blotted onto an immobilizing membrane) were applied into precast application slots. Upon completion of electrophoresis, the proteins were silver-stained [10, 11] or blotted onto an immobilizing PVDF membrane (see Section 2.5).

2.4.2 IPG-Dalt of wheat grain proteins

IPG-Dalt was carried out according to Görg *et al.* [12–14]. IPG gels with linear gradients, pH 4–8 (4% T, 4% C, size 250 $\times$ 180 $\times$ 0.5 mm³) were cast as described [13]. The washed and dried IPG gels were then cut into 5 mm wide IPG strips which were rehydrated overnight to their original thickness of 0.5 mm with a solution consisting of 8 M urea, 0.5% CHAPS, 0.2% DTT, and 0.2% Pharmalytes pH 3–10. The rehydrated IPG gel strips were placed onto the cooling plate of a flatbed focusing

chamber. For analytical IPG-Dalt, a sample volume of 20 μ L was applied into silicon frames near the anode, whereas for micropreparative IPG-Dalt a sample volume of 100 μ L was applied with the help of sample application cups (Pharmacia DryStrip kit) placed onto the surface of the IPG gel strips. When IPG-Dalt was followed by electroblotting, about 500 μ g of protein was loaded onto each IPG gel strip; for analytical runs followed by in-gel silver staining, 60–80 μ g were applied. IEF was performed at 20°C [15] for 40 000 and 60 000 Vh for analytical and micropreparative runs, respectively. Following IEF, the IPG gel strips were stored in a plastic bag at –78°C. Prior to the second dimension of IPG-Dalt, the IPG gel strips were equilibrated for 2 $\times$ 10 min in 2 $\times$ 10 mL equilibration solution (Tris-HCl buffer 50 mM, pH 8.8, containing 6 M urea, 30% w/v glycerol, 2% w/v SDS). DTT (1% w/v) was added to the first equilibration solution, and iodoacetamide (4% w/v) to the second. Second-dimensional SDS-PAGE (13% T, 2.6% C) was carried out simultaneously on ten slab gels (210 $\times$ 185 $\times$ 1.0 mm³) in a Dalt tank [13, 14, 16].

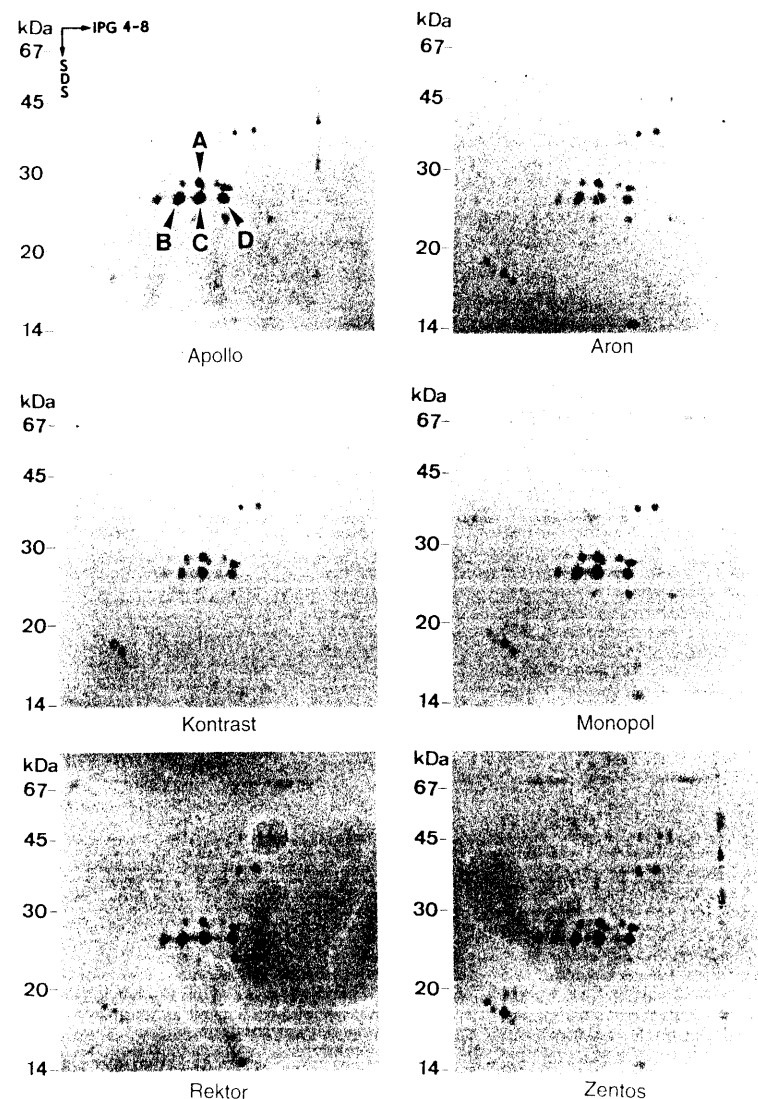


Figure 3. Wheat grain albumin/globulins of six different cultivars separated by IPG-Dalt 4–8 and electroblotted onto a PVDF membrane. Immunostaining with patients' sera and secondary alkaline phosphatase-labeled anti-human IgE. Samples: wheat cultivars "Apollo", "Aron", "Kontrast", "Monopol", "Rektor", and "Zentos".

2.5 Electrophoretic transfer (blotting) and immunostaining

Following SDS-electrophoresis, the polyacrylamide gel was removed from the plastic support with the help of a

film remover (Pharmacia). The electrophoretically separated proteins were then electroblotted onto immobilizing PVDF membranes using the semidry blotting apparatus described by Kyhse-Andersen [17]. The blotting buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS

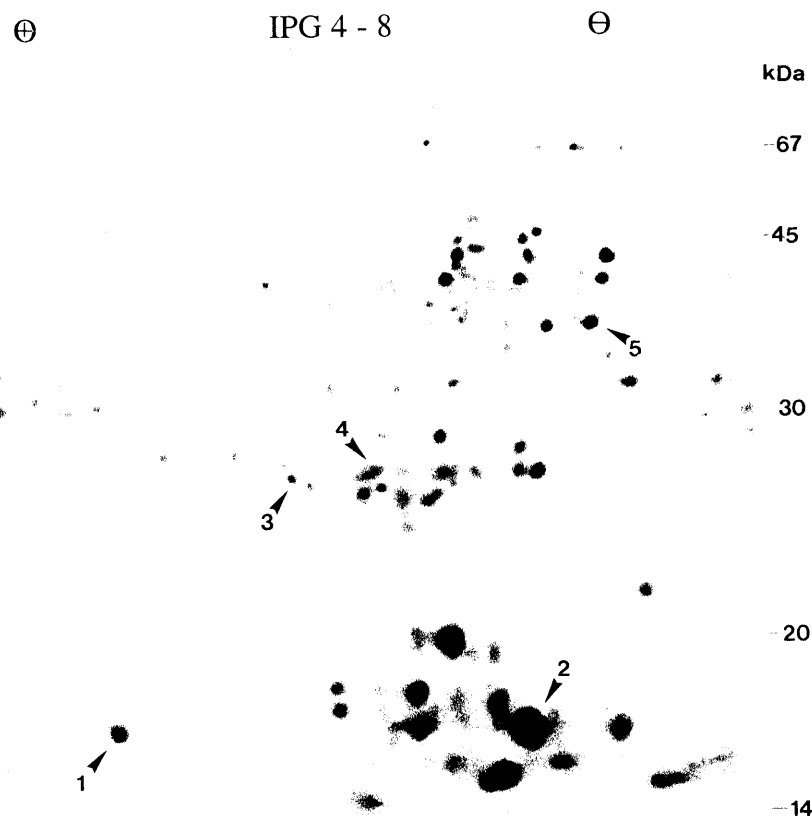


Figure 4. Salt-soluble wheat flour proteins separated by micropreparative IPG-Dalt 4–8 and electroblotted onto a PVDF (FluoroTrans®) membrane. Coomassie stain. Major IgE-binding polypeptide spots which revealed homologies to already known proteins according to their amino acid composition and N-terminal amino acid sequences, respectively, are labeled with arrows.

and 20% methanol. The transfer was performed at 0.8 mA/cm² for 1 h at room temperature. If blotting was followed by amino acid sequence/composition analysis, the 2-D gels were equilibrated in 50 mM Tris base/50 mM boric acid, pH 8.5 (blotting buffer), for 10 min and electroblotted onto FluoroTrans[®] membranes at 1 mA/cm² for 90 min. After the electrophoretic transfer was terminated, the membranes were washed three times (5 min each) with saline (150 mM). Then the membranes were air-dried and stored at 4°C in a sealed plastic bag until use. In order to evaluate whether the electrophoretic transfer had been performed correctly and the transferred proteins had bound to the immobilizing membrane, several PVDF membranes were stained with India ink [18]. For immunostaining, nonoccupied binding sites on the PVDF membranes were blocked with 1% PVP in Tris-buffered saline (TBS). The membranes were then

incubated with the sera from asthmatic bakers or control persons, and bound IgE was demonstrated by ¹²⁵I-labeled or alkaline phosphatase-conjugated anti-human IgE and autoradiography, or BCIP/NBT-staining as described previously [8, 19]. To detect glucose and/or mannose containing glycoproteins, several PVDF-membranes were incubated with the lectin concanavalin A, followed by horseradish peroxidase staining (substrate/chromogen solution: hydrogen peroxide and 4-chloro-1-naphthol) as described previously [8].

2.6 Amino acid composition analysis and N-terminal amino acid sequence analysis

Nine of the major IgE binding polypeptides were subjected to amino acid composition analysis and

Table 1. N-terminal amino acid sequences of IgE-binding polypeptides (indicated by arrows in Fig. 4) that exhibit homologies to already described proteins^a

Subject	N-terminal amino acid sequence															Molecular mass
Polypeptide No. 1	S	G	P	W	M	E	Y	P	T	Q	A	A	I	V		≈ 16 kDa
Polypeptide No. 2	S	G	P	W	M	X	Y	P	G	Q	A	F	Q	V		≈ 17 kDa
Wheat α-amylase inhibitor (IAAI)	S	G	P	W	M	C	Y	P	G	Q	A	F	Q	V		13.3 kDa
Polypeptide No. 3	P	G	L	A	I	G	D	T	V	A	N	L	E	V		≈ 27 kDa
Polypeptide No. 4	P	G	L	T	I	G	E	A	V	P	N	L				≈ 27 kDa
Acyl-CoA oxidase (rice)	P	G	L	T	I	G	D	I	V	P	N	L				n.d.
28 k Protein 4209 (rice)	P	G	L	V	I	G	D	I	V	P	N	P	E			≈ 28 kDa
HvB15C gene product (barley)	P	G	L	T	I	G	D	T	V	P	N	L	E	L		n.d.

a) Gaps in the sequence are denoted by X; n.d., no data available

N-terminal amino acid sequence analysis: The albumin/globulin proteins were separated by micropreparative IPG-Dalt, blotted onto an immobilizing PVDF-membrane and visualized with Coomassie Brilliant Blue R-250 staining (0.1% in destaining solution; 2 min). After destaining in water/methanol/acetic acid (45:45:10), the blots were rinsed for 15 min in deionized water and air-dried. Then the protein spots of interest were excised from the blotting matrix and placed into an ABI Model 477A pulsed-liquid protein sequencer equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer. Routinely, ten to fifteen Edman degradation cycles were performed for each spot [19]. The partial protein sequences were compared for homologies to already known proteins using the BLAST algorithm [20]. Alternatively, PVDF-bound polypeptides were hydrolyzed with 6 N HCl and subjected to amino acid composition analysis [21]. Searches for homologies were performed using ExPASy's amino acid composition similarity search tool [http://expasy.hcuge.ch/ch2d/aacompi.html].

3 Results and discussion

SDS-PAGE, IPG-Dalt and immunoblotting with sera from patients suffering from bakers' asthma, followed by amino acid composition analysis, amino acid sequence analysis and database searching were performed to identify and characterize wheat albumin/globulin allergens of different cultivars involved in bakers' asthma.

3.1 SDS-PAGE, IPG-Dalt and immunoblotting

SDS-PAGE and immunoblotting using sera from wheat-flour-sensitive individuals detected more than twenty allergens in the twenty wheat cultivars investigated. The highest percentage of IgE binding was observed with certain albumin/globulin polypeptides of 70, 55–65, 35, 26–28, and 14–18 kDa (Fig. 1A), whereas the serum pool obtained from exposed, but asymptomatic donors and subjects allergic to house dust mite did not contain IgE antibodies that bound to any of the wheat seed proteins (Fig. 1B). These findings are similar to those of Sandiford *et al.* using the sera from occupationally exposed individuals [22]. More detailed investigations using IPG-Dalt showed that many albumin and globulin allergens were not single polypeptides, but consisted of

up to a dozen isoforms of similar molecular mass but different pIs (Fig. 2). This charge heterogeneity may be due to slight differences in their primary structures, or to post-translational modifications such as different levels of glycosylation, as was demonstrated for the 55–65 kDa allergens by concanavalin A / peroxidase-staining in a previous study [8].

Densitometric evaluation of the autoradiogram presented in Fig. 1A revealed that the amount of IgE bound by particular polypeptides (such as the 26–28 kDa allergens) differed considerably between individual wheat cultivars. One possible explanation for this finding is that these allergens differ slightly in amino acid composition and/or post-translational modifications and might therefore possess nonidentical epitopes. A second explanation is that in certain cultivars particular allergens are present in lower amounts than in others. To verify this hypothesis, albumin/globulin proteins from six cultivars (Apollo, Aron, Kontrast, Monopol, Rektor, and Zentos) were separated by IPG-Dalt and visualized by immunoblotting using patients' sera (Fig. 3). Interestingly, all cultivars exhibited almost identical allergen patterns. The intensities, however, of particular allergen spots differed considerably between individual cultivars. For example, the 28 kDa spot labeled with the letter A was stained with similar intensity in all cultivars, whereas spots B, C, and D were much less intensely stained in cultivars "Aron" and "Kontrast" than in "Apollo", "Monopol", or "Zentos" (Fig. 3). These findings support the view that primarily quantitative effects are responsible for different IgE binding of individual cultivars. On the other hand, it cannot be excluded that factors such as slightly different antibody binding sites on individual allergens (due to different levels of glycosylation, etc.) also play a role, as has been previously shown for inhalant allergens from fungi and wheat [23, 24]. Additional analyses are necessary to fully answer this question.

3.2 Amino acid composition and N-terminal amino acid sequence analysis

Micropreparative IPG-DALT was used to purify salt-soluble wheat grain allergens for microsequencing and amino acid composition analysis: albumin/globulin polypeptides were electrophoretically separated, electroblotted onto a polyvinylidene difluoride (Fluorotrans[®]) membrane and stained with Coomassie Brilliant Blue

Table 2. Amino acid composition of the 35 kDa IgE-binding polypeptide (indicated by arrow No. 5 in Fig. 4)

Asx:	8.50	Glx:	12.30	Ser:	5.10	His:	2.10
Gly:	10.20	Thr:	4.30	Ala:	11.10	Pro:	7.40
Tyr:	3.30	Arg:	6.50	Val:	8.30	Met:	0.60
Ile:	3.90	Leu:	8.50	Phe:	2.50	Lys:	5.20

(Fig. 4). Nine polypeptide spots which had been identified as major allergens in our previous experiments (six major allergens located in the 26–28 kDa area, one 35 kDa allergen, and two 16–17 kDa allergens) were excised from the membrane and subjected to *N*-terminal amino acid sequence analysis [19]. Our results revealed that two of the 26–28 kDa polypeptides are clearly related to several acyl-CoA oxidase variants of rice and barley (see Table 1), whereas no significant homologies were found for the remaining four 26 kDa allergens analyzed. The *N*-terminal amino acid sequences of the two low molecular mass (16 and 17 kDa) allergens revealed a very high degree of homology with wheat monomeric alpha-amylase inhibitor 1 (IAA1). The latter finding is in accordance with previously published reports from other authors [25–28]. No sequence information could be gained on the 35 kDa allergen (*pI* ~ 7.2), probably because of a blocked amino terminus. In order to better characterize or identify this allergen, amino acid composition analysis was performed (Table 2). Amino acid similarity search revealed homologies to fructose-bisphosphate-aldolase in maize (*M*, 38 600; SWISS-PROT accession number P08440) and rice (*M*, 38 800; SWISS-PROT accession number P17784). In control experiments, one of the spots whose identity was known (wheat alpha-amylase inhibitor), was also hydrolyzed and high scores were obtained when using ExPASy's amino acid composition similarity search tool.

4 Concluding remarks

Our studies on the amino acid composition and amino acid sequences of wheat grain allergens revealed homologies to amylase/protease inhibitors and enzymes such as acyl-CoA oxidase. Several authors have also shown that allergens which induce bakers' asthma have enzymic and enzyme inhibitory activity: Walsh *et al.* [28], Fränken *et al.* [27], and Salcedo and co-workers [24–26] identified amylase-inhibitors as major allergens of wheat, whereas Baur *et al.* [2–3] and Sandiford *et al.* [29] suggested that baking additives such as fungal amylases and other hydrolytic enzymes are important allergens, too. Whether a relationship exists between enzymic and/or enzyme inhibitory activity and the allergenicity of proteins is not clear. Several authors suppose that allergens which exhibit enzymatic activity may more easily penetrate mucous membrane barriers and trigger allergic reactions [27, 30]. Yet, things are more complicated since most research [4–6, 31, 32] had been focused on the albumin/globulin protein fraction, which is primarily composed of enzymes or enzyme inhibitors. On the other hand, it has been demonstrated that gliadins and glutenins, which are storage proteins without any known enzymic activity, also play a role in provoking bakers' asthma [7, 8]. Therefore, research should also be intensified in this direction. With techniques similar to those

described in the present paper [21, 33–37], much faster characterization of allergens can be achieved than was possible in the past. In the long run, we are planning to identify and characterize IgE-binding wheat grain proteins of all solubility fractions (*i.e.*, albumins, globulins, gliadins, and glutenins) by micropreparative IPG-Dalt followed by amino acid composition analysis, peptide mass fingerprinting, peptide mapping, and amino acid sequence analysis by mass spectroscopy or Edman degradation, in order to establish a wheat grain allergen database which might enable us to (i) identify and compare epitopes on different wheat grain allergens, (ii) study cross-reactivities between different allergens, and (iii) breed new, less allergenic wheat cultivars, either by conventional breeding techniques or genetic engineering.

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