

of the β -galactosidase digestion in contrast with the desialylated AFP which was hydrolyzed rapidly with the same β -galactosidase preparation. We conclude from these results, together with the increased intensities of the AFP-B-P4 spot in patients with hepatocellular carcinoma in the present study, that the sugar chain structures which are responsible for the AFP-P4 present in cord serum and hepatocellular carcinomas are the nonbisected biantennary oligosaccharides, which have an exposed galactose of the Man α 1–6 antenna and an α 2–6 monosialylated galactose of the Man α 1–3 antenna with or without the fucosylated core N-acetylglucosamine, corresponding to the A-1 structure of Yoshima *et al.* [10] (B-P4-A3 in Table 1). The other monosialo-AFP, AFP-P3, which was found in a case of hepatocellular carcinoma, could have monosialylated structures with N-acetylneuraminic acid attached by the α 2–6 linkage to the galactose of the Man α 1–6 arm (B-P3-A2s in Table 1). Fucosylation of the core N-acetylglucosamine had negligible effects on the mobility of AFP bands in E-PHA affinity electrophoresis as studied by 2-D E-PHA-lentil lectin affinity electrophoresis (unpublished observation).

AFP-P5 had the highest affinity for E-PHA, suggesting the presence of bisecting N-acetylglucosamine [14]. However, the AFP-P5 in cord serum and in patients with hepatocellular carcinoma is not likely to have bisected biantennary structures because the AFP-P5 from cord serum and hepatocellular carcinoma fully reacted with Con A [5, 9]. Bisected biantennary oligosaccharides have also not been reported for human hepatoma AFP [10]. On the other hand, AFP-P5 from cord serum and sera from hepatocellular carcinomas were shown in the present study to have an electrophoretic mobility of disialo-AFP and to form monosialo-AFP-P5-A2s transiently during neuraminidase treatment. The reduced affinity for allo A following the neuraminidase digestion indicates the cleavage of the Neu5Ac α 2–6 Gal linkage of the Man α 1–3 arm leaving N-acetylneuraminic acid attached to the other arm. Since the biantennary oligosaccharides sialylated at the C-3 position of terminal galactose residues have a high affinity for E-PHA [16], the most likely candidate structure for AFP-P5 in sera from cord blood and hepatocellular carcinoma is the nonbisected biantennary complex-type oligosaccharide having a Neu5Ac α 2–3-substituted galactose of the Man α 1–6 arm and a Neu5Ac α 2–6-substituted galactose of the other arm. Thus, asialo-AFP-P4 would be formed as the final product to AFP-P5 in the neuraminidase digestion through formation of monosialo-AFP-P5-A2s and monosialo-AFP-P4-A3 as observed in this study.

Note that the AFP from hepatocellular carcinoma patients contains increased proportions of sugar chains that

have a monosialylated galactose of the Man α 1–3 antenna (AFP-B-P4) rather than a monosialylated galactose of the Man α 1–6 antenna (AFP-B-P3). The sugar chain structure identical with that of monosialo-AFP-P4 has been reported for the oligosaccharide of amylase produced by tumors [17], suggesting the importance of the biantennary oligosaccharide monosialylated on the galactose of the Man α 1–3 arm and with the exposed terminal galactose of the other arm as a new tumor marker. The results of the present study also substantiate the usefulness of 2-D-EAGE-lectin affinity electrophoresis or the 2-D affinity electrophoresis with two lectins having different affinities in characterizing the sugar chain heterogeneity of glycoproteins and also in consolidating the specificities of lectins to glycoproteins.

This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, 1992.

Received February 3, 1993

5 Reference

- [1] Taketa, K., Sekiya, C., Namiki, M., Akamatsu, K., Ohta, Y., Endo, Y. and Kosaka, K., *Gastroenterology* 1990, 99, 508–518.
- [2] Aoyagi, Y., Isemura, M., Yoshizawa, Z., Suzuki, Y., Sekine, C., Ono, T. and Ichida, F., *Biochim. Biophys. Acta* 1985, 830, 217–223.
- [3] Mackiewicz, A. and Brochowski, J., In: Bog-Hansen, T. C. (Ed.), *Lectins: Biology-Biochemistry-Clinical Biochemistry*, Vol. 1, Walter de Gruyter, Berlin 1981, pp. 315–326.
- [4] Tsuchida, Y., Fukui, M., Sakaguchi, H. and Ishiguro, T., *Tumor Biol.* 1989, 10, 289–296.
- [5] Taketa, K., Ichikawa, E., Nakabayashi, H., Sato, J., Kato, K., Akai, S., Ohkawa, R., Ohkawa, K., Taga, H. and Hirai, H., *Tumor Biol.* 1985, 6, 519–531.
- [6] Taketa, K., Ichikawa, E., Akamatsu, K., Ohta, Y., Sekiya, C., Namiki, M., Takami, H., Kaneda, H., Higashino, K., Taga, H. and Hirai, H., *Tumor Biol.* 1985, 6, 533–544.
- [7] Taketa, K., Ichikawa, E., Umetsu, K., Suzuki, T., Taga, H. and Hirai, H., *J. Tumor Marker Oncol.* 1987, 2, 133–139.
- [8] Taketa, K., *Electrophoresis* 1987, 8, 409–414.
- [9] Taketa, K., Ichikawa, E., Sato, J., Taga, H. and Hirai, H., *Electrophoresis* 1989, 10, 825–829.
- [10] Yoshima, H., Mizuuchi, T., Ishii, M. and Kobata, A., *Cancer Res.* 1980, 40, 4276–4281.
- [11] Yamashita, K., Hitoi, A., Tsuchida, Y., Nishi, S. and Kobata, A., *Cancer Res.* 1983, 43, 4691–4695.
- [12] Ogata, S., Muramatsu, T. and Kobata, A., *J. Biochem.* 1975, 78, 687–696.
- [13] Narashimhan, S., Freed, J. C. and Schachter, H., *Carbohydr. Res.* 1989, 149, 65–83.
- [14] Yamashita, K., Hitoi, A. and Kobata, A., *J. Biol. Chem.* 1983, 258, 14753–14755.
- [15] Yamashita, K., Umetsu, K., Suzuki, T., Iwaki, Y., Endo, T. and Kobata, A., *J. Biol. Chem.* 1988, 263, 17482–17489.
- [16] Kobata, A. and Yamashita, K., *Methods Enzymol.* 1989, 179, 46–54.
- [17] Yamashita, K., Tachibana, Y., Takeuchi, T. and Kobata, A., *J. Biochem.* 1981, 90, 1281–1289.

Walter Weiss¹
Claus Vogelmeier²
Angelika Görg¹

¹Lehrstuhl für Allgemeine Lebensmitteltechnologie, Technische Universität München, Freising-Weihenstephan
²Pneumologische Abteilung, Medizinische Klinik I, Klinikum Großhadern, Universität München

Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma

In order to identify and characterize the wheat grain allergens involved in bakers' asthma, proteins were sequentially extracted from whole-meal flour. The polypeptide composition of the individual solubility fractions (albumin/globulin, gliadin and glutenin) was analyzed by sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE), and high-resolution two-dimensional gel electrophoresis with immobilized pH gradient 4–9 in the first dimension (IPG-Dalt). The resolved polypeptides were transferred onto an immobilizing polyvinylidene difluoride membrane and incubated with a pooled serum from four asthmatic bakers. Bound IgE was demonstrated by autoradiography using ¹²⁵I-labeled anti-human IgE. Our study demonstrated that the serum of the bakers allergic to flour contained IgE antibodies which bound to numerous polypeptides of all three solubility fractions. The highest percentage of IgE binding was observed with certain albumin and/or globulin polypeptides, whereas the gliadins and glutenins exhibited considerably less allergenicity. SDS-PAGE revealed that the protein which bound the highest percentage of IgE from the sera of the allergic bakers is a 27 kDa albumin. More detailed investigations using IPG-Dalt demonstrated that this allergen is not a single polypeptide but consists of several polypeptide spots that differ in their isoelectric points. Quantitative studies using computer-assisted laser densitometry revealed that the amount of patients' IgE bound by these particular polypeptides differed considerably between the seven wheat cultivars examined, ranging from 13% to 53% of the total radioactive uptake.

1 Introduction

In several European countries, bakers' asthma, a form of allergic asthma, is one of the most common causes of occupationally related obstructive airway diseases [1]. Although bakers' asthma is one of the oldest occupational lung diseases – its first description was given by Ramazzini almost 300 years ago (*cf.* [1]) – the causative agents responsible for this illness were unknown until the last decades when it was realized that this disease is the result of an IgE-mediated type I reaction to cereal flour components. Since then, a number of attempts have been made to identify the responsible allergens. In particular, Baldo, Wrigley and co-workers have carried out a number of studies in Australia since 1978 [2–6].

Using a solid-phase radio-immunoassay (the radioallergen sorbent test, RAST) they were able to demonstrate IgE antibodies to a number of flour components in the sera of allergic bakers, but the strongest reactivities were observed with water-extractable albumins and (though less strong) globulins, compared to the less soluble gliadins and glutenins [2]. In an extended study [3] in which

a modified RAST procedure was utilized, additional IgE binding was also demonstrated in the gliadin and glutenin protein fractions. In order to better characterize the individual protein fractions, additional chromatographic and electrophoretic studies were performed [4–6]. When the water-soluble proteins were fractionated by gel filtration, the IgE antibodies from the sera of three allergic bakers reacted primarily with components of the 15–35 kDa fraction [4]. Two-dimensional (2-D) gel electrophoresis, with isoelectric focusing (IEF), pH 5–9, in the first dimension and polyacrylamide gel electrophoresis (PAGE) at pH 3 in the second dimension, followed by immunoblotting, revealed that the major allergens were low *M_r* albumins and/or globulins, with a full range of pI's, and slightly basic gliadins [5, 6]. Glutenins could not be studied because they did not migrate into these gels.

Unfortunately, it is not clear whether the results of the studies by Baldo, Wrigley and co-workers (obtained with Australian wheat cultivars) are generally valid, since the situation may be different in other countries. Wheat cultivars used elsewhere for processing may be related only distantly to those studied in the investigations of Baldo and Wrigley, and might, therefore, contain other sets of allergens.

Therefore, the aim of the present study was to achieve a more detailed characterization of the wheat grain allergens involved in bakers' asthma in Germany. Proteins were sequentially extracted from whole-meal flour according to a modified Osborne procedure [7]. The polypeptide composition of these solubility fractions (including the glutenins) was then analyzed by high-resolution one- and two-dimensional gel electrophoresis techniques, and IgE-binding proteins (= allergens) in

Correspondence: Priv. Doz. Dr. A. Görg, Technische Universität München, Lehrstuhl für Allgemeine Lebensmitteltechnologie, D-85350 Freising-Weihenstephan, Germany

Abbreviations: BSA, bovine serum albumin; Con A, concanavalin A; cpm, counts per minute; cv, cultivar; 2-D, two-dimensional; DTT, dithiothreitol; HMW, high molecular weight; HRP, horseradish peroxidase; IEF, isoelectric focusing; IgE, immunoglobulin E; IPG-Dalt, two-dimensional gel electrophoresis with immobilized pH gradient in the first dimension; LMW, low molecular weight; *M_r*, relative molecular mass; PAGE, polyacrylamide gel electrophoresis; pI, isoelectric point; PVDF, polyvinylidene difluoride; PVP, polyvinylpyrrolidone; RAST, radioallergen sorbent test; SDS, sodium dodecyl sulfate; TBST, RAST buffered saline + Tween 20

these extracts were detected by immunoblotting. Additionally, the individual solubility fractions of seven different wheat cultivars were studied by sodium dodecyl sulfate (SDS)-electrophoresis and immunoblotting to examine whether there are qualitative and/or quantitative differences between the IgE-binding patterns of these cultivars.

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) were from Pharmacia-LKB (Uppsala, Sweden). Immobilon, 2-D Pharmalyte pH 3–10, GelBond PAGfilm, and ^{125} I anti-human IgE (Phadebas; RAST) were also obtained from Pharmacia. Polyvinylidene difluoride (PVDF) membranes (Immobilon P, pore size 0.45 μ m) were from Millipore (Bedford, MA, USA). Acrylamide (2 $\times$ cryst.), *N,N*-methylenebisacrylamide, SDS, Amido Black 10B dye, horseradish peroxidase (HRP, 400 U/mg) and concanavalin A (Con A) were from Serva (Heidelberg, Germany). Polyvinylpyrrolidone (PVP, *M*, 40000), 4-chloro-1-naphthol, Tris(hydroxymethyl)aminomethane, iodoacetamide, dithiothreitol (DTT), Triton X-100 and rabbit anti-gliadin (wheat) IgG-peroxidase conjugate 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 from Kodak (Rochester, NY, USA).

2.2 Sera

Sera were obtained from four bakers who reported asthmatic symptoms and/or rhinitis after exposure to flour. A diagnostic procedure, including prick-testing with a wheat-flour extract, confirmed that these persons were allergic to flour. Specific IgE antibodies to wheat flour proteins were evaluated by the IgE RAST (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions using commercially available allergen discs. The levels of specific IgE of the four subjects allergic to flour as determined by the RAST method, were 48, 30, 27 and 17.5 units per mL serum (equal to a RAST score of 4). Control sera from healthy, non-allergic adults were obtained from members of the hospital staff; no specific IgE antibodies to wheat flour proteins were detected in the sera of these healthy controls by the RAST method. All sera were stored at -20°C until use.

2.3 Sample preparation

2.3.1 Seed material

Samples of fifteen soft winter wheat (*Triticum aestivum* L.) cultivars were provided by a local milling company.

The majority of these varieties is grown for bread-making purposes in Germany. Seven of these cultivars (Apollo, Ares, Avis, Boheme, Bussard, Fregatt, Futur), which exhibited major differences regarding their polypeptide band patterns as determined by SDS-gel electrophoresis, were selected for the following study.

2.3.2 Protein extraction procedure and sample preparation for electrophoresis

Grain samples of wheat were ground in a mortar with a pestle until a fine powder was obtained that passed through a 0.5 mm wide sieve. Flour proteins from whole meal were then extracted according to the following protocols.

2.3.2.1 Protein extraction and sample preparation for SDS-electrophoresis

Wheat flour protein fractions were prepared using a modified sequential extraction method originally described by Osborne [7]. This procedure resulted in three protein fractions: (i) salt-soluble proteins (albumins and globulins), (ii) aqueous alcohol-extractable proteins (gliadins), and (iii) SDS/DTT-extractable proteins (glutenins; see Fig. 1). Briefly, samples (1 g) of the ground grain were extracted with 4 mL of Tris-HCl buffer (50 mM, pH 8.8) for 1 h at 4°C with vortexing at 15 min intervals, and centrifuged (20000 g, 20 min). The supernatant, containing the albumin and globulin proteins, was carefully removed and stored at -80°C . The pellet was then extracted twice with Tris-HCl buffer, and the supernatants of these extraction steps were discarded.

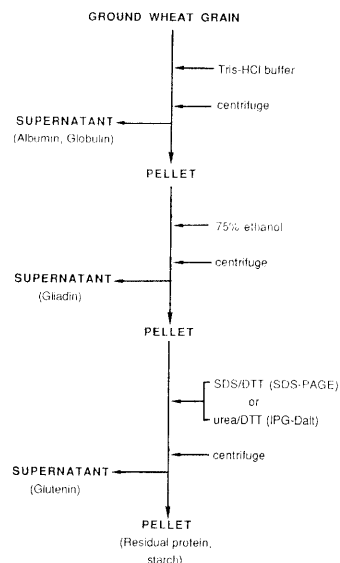


Figure 1. Schematic representation of the sequential extraction procedure for preparing albumins and globulins, gliadins and glutenins.

Following a water wash to remove buffer ions, the pellet was stirred with 4 mL of 75% v/v ethanol for 2 h at 25°C and then centrifuged as above. The supernatant, containing the gliadins, was removed and stored at -80°C . In order to minimize cross-contamination (carry-over) between the gliadin and glutenin fractions, the ethanol extraction step was also repeated twice. Residual ethanol was removed from the pellet by an inserted water wash, and then the glutenins were extracted from the pellet with 4 mL of SDS/DTT buffer (50 mM Tris-HCl, pH 8.8, 1% SDS, 0.5% DTT) for 2 h at room temperature with occasional vortexing. After centrifugation the supernatant was stored at -80°C . Prior to SDS electrophoresis, aliquots (0.5 mL) of the supernatants from the sequential extraction procedure were mixed with 1.25 mL of SDS sample buffer (50 mM Tris-HCl, pH 8.8, 1% SDS, 0.5% DTT, 20% glycerol and 0.005% Bromophenol Blue). The mixture was heated in a boiling water bath for 3 min. After cooling to about 60°C , DTT (0.2%) was added to prevent reoxidation of the sulfhydryl groups. The extracts were then centrifuged (20000 g, 5 min) and used for SDS-electrophoresis.

2.3.2.2 Protein extraction and sample preparation for 2-D electrophoresis

The sequential extraction procedure was carried out as described (see Section 2.3.2.1) except that SDS/DTT buffer was replaced by urea/DTT buffer (6 M urea, 1% Triton X-100, 0.5% DTT, and 0.5% w/v 2-D Pharmalyte, pH 3–10) for the extraction of the glutenins (see Fig. 1). Additionally, an extract of "total" wheat seed protein was prepared by extraction of the ground seeds (500 mg) with urea/DTT buffer (5 mL). All extracts were stored at -80°C until use. To allow comparison between the protein patterns of the different solubility fractions, all extracts were diluted with an equal volume of 9 M urea containing 2% Triton X-100, 1% DTT and 0.8% w/v 2-D Pharmalyte, pH 3–10, prior to IEF.

2.4 Electrophoresis

All electrophoretic separations (SDS-PAGE, IEF and IPG-Dalt) were performed on horizontal systems using thin gels cast on GelBond PAGfilm according to Görg *et al.* [8].

2.4.1 Horizontal SDS-PAGE of wheat grain proteins

Horizontal SDS-PAGE [9] was carried out as described for barley seed proteins [10]. The SDS-gels contained a 4% polyacrylamide stacking gel, and a resolving gel of a linear gradient from 10–15% polyacrylamide (4% C constant). The buffer system of Laemmli [11] 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 (size $7 \times 2 \times 0.25$ mm³). Upon completion of electrophoresis, the proteins were either fixed in methanol/acetic acid/water (40/10/50) for 30 min and then silver stained (see Section 2.4.3), or, alternatively, blotted onto an immobilizing PVDF membrane (see Section 2.5).

2.4.2 IPG-Dalt of wheat grain proteins

Horizontal IPG-Dalt [12] was performed as described previously for barley seed proteins [13]. First-dimensional IEF was run on individual IPG 4–9 gel strips (size $120 \times 4 \times 0.5$ mm³) which had been rehydrated in 8 M urea, 0.5% Triton X-100, 0.2% DTT and 0.2% w/v 2-D Pharmalyte, pH 3–10. Samples of 20 μ L (*i.e.*, about 50 μ g of protein) were pipetted into silicone rubber frames 10 mm apart from the anode. Focusing was performed for 16000 Vh at 20°C [14]. After IEF was finished, the IPG gel strips were either stored at -80°C until use or equilibrated immediately for 2 $\times$ 15 min in 10 mL of a solution consisting of Tris-HCl buffer (50 mM, pH 8.8), 6 M urea, 30% glycerol and 2% SDS. DTT (1%) was added to the first, and iodoacetamide (5%) to the second equilibration step. Second-dimensional horizontal SDS-PAGE was performed on $250 \times 200 \times 0.5$ mm³ slab gels containing a 10–15% polyacrylamide resolving gel (linear gradient) and a 6% polyacrylamide stacking gel (4% C, homogeneous; Laemmli buffer system [11]). Following electrophoresis, the resolved polypeptides were either silver stained (see Section 2.4.3) or transferred onto an immobilizing membrane (see Section 2.5).

2.4.3 Silver staining

Proteins were either stained with silver nitrate according to Blum *et al.* [15] or Heukeshoven and Dernick [16]. In several experiments, the diamine silver staining technique of Oakley *et al.* [17] was employed.

2.5 Electrophoretic transfer (blotting)

Following 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 [18]. The blotting buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol [19]. The transfer was performed at 0.8 mA/cm² for 1 h at room temperature. After the electrophoretic transfer was terminated, the membranes were washed three times (5 min each) with Tris buffered saline (50 mM Tris HCl, pH 7.4, and 150 mM NaCl) to remove residual SDS which might interfere with subsequent staining procedures. 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 membranes, several membranes were stained with the protein stains Indian Ink [20] or Amido Black 10B (0.1% w/v in 2% acetic acid).

2.6 Detection of human IgE-, rabbit IgG- and concanavalin A-binding polypeptides

The dried, unstained PVDF membranes were wetted in methanol for a few seconds and then washed twice (5 min each) in Tris-buffered saline (TBS). In order to satu-

rate nonoccupied binding sites on the membranes, they were incubated with 1% PVP in TBS (TBS-PVP) for at least 6 h (but usually overnight) with gentle rocking. The membranes were then washed (twice (5 min each) with TBS and used for the immuno- or glycoprotein staining experiments.

2.6.1 Detection of IgE binding proteins (allergens)

The blocked immobilizing membrane was transferred to a cylindrical photographic developing drum (300 mm in length and 100 mm in diameter) and incubated with serum. Usually, 2 mL of a pooled serum from all four patients (or the healthy controls, respectively), and 40 mL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20, and 1% bovine serum albumin (TBST-BSA) were added to a 300 cm² membrane. The developing tank was inserted horizontally into a color photo processor (Jobo, Gommersbach, Germany) and slowly rotated for 18 h at room temperature. The following day the membrane was thoroughly washed (4 x 10 min) with TBST to remove unbound serum proteins, and incubated with 2 mL of ¹²⁵I anti-human IgE (approximately 2 x 10⁶ cpm) and 40 mL of TBST-BSA. After 18 h, unbound anti-IgE was removed with four more washes (10 min each) with TBST. The membrane was air-dried and the ¹²⁵I-labeled polypeptide zones or spots were detected by autoradiography using Kodak X-OMAT AR X-ray films and an intensifying screen. Exposure time was between one and six weeks, depending on the amount of ¹²⁵I anti-IgE that was bound to the membrane.

2.6.2 Detection of rabbit anti-gliadin binding proteins and concanavalin A binding glycoproteins

Wheat gliadins were visualized on the PVDF membrane using a rabbit anti-gliadin-IgG antibody, conjugated to horseradish peroxidase (Sigma A 1052). Briefly, the blocked PVDF membrane was incubated with 0.25 mL of the anti-gliadin peroxidase conjugate and 40 mL of TBST-BSA for 6–18 h at room temperature with gentle rocking. After four washes with TBST (10 min each), anti-gliadin binding proteins were detected by conversion of the colorless substrate, containing 50 mg of 4-chloro-1-naphthol dissolved in 20 mL methanol, and 20 µL of hydrogen peroxide (30%) in 80 mL phosphate-buffered saline (50 mM phosphate buffer, pH 6.0, and 150 mM NaCl), into an insoluble purple-colored dye. Staining time was about 20 min at room temperature. Sensitivity was markedly enhanced when the freshly stained PVDF membrane was illuminated with ultraviolet (UV) light [21].

Glucose and/or mannose containing glycoproteins were detected by the Con A – HRP method of [22], modified as described previously [23].

2.7 Densitometric evaluation

The autoradiograms (see Section 2.6.1) were digitized at 88 µm resolution using a Molecular Dynamics 300A laser scanning densitometer (Sunnyvale, CA, USA). The resulting images were analyzed using the QUANTITY

ONE and PDQUEST™ software package (Protein and DNA Imageware, New York, USA) running on a SPARCstation IPC microcomputer (Sun Microsystems, CA, USA).

3 Results and discussion

3.1 Characterization of the individual solubility fractions

The nomenclature of wheat seed proteins goes back to the investigations of Osborne [7] who defined these proteins on the basis of their solubility properties: (i) albumins, which are soluble in water, (ii) globulins, which are soluble in dilute salt solutions, but insoluble in water, (iii) gliadins, which are soluble in aqueous alcohols, and (iv) glutenins, which are soluble in dilute acid or alkaline solutions. Deviating from the classical Osborne procedure for sequential extraction, several of the extracting solvents originally described were replaced by others which are more compatible with electrophoresis, such as 50 mM Tris-HCl instead of 10% NaCl for the extraction of the globulins, and SDS/DTT (or urea/DTT) buffer instead of 1% KOH for the extraction of the glutenins. Because of the similar functional properties of the albumin and globulin proteins, both protein classes were extracted with Tris-HCl buffer as a single solubility fraction.

3.1.1 Horizontal SDS-PAGE

The polypeptides of the three different solubility fractions (albumins/globulins, gliadins, and glutenins) were resolved by SDS-PAGE and silver-stained for total proteins (see Fig. 2). A comparison between these solubility fractions shows that the albumin and globulin proteins are distributed over the entire *M_r* range from 15000 to 100000, whereas the gliadins and glutenins are restricted to specific regions of the SDS-gel: The α-, β- and γ-gliadins exhibit *M_r* between 30000–45000, and the Ω-gliadins between 50000–70000. The Ω-gliadins are only weakly stained with the silver nitrate stain [15]; however, their visualization can be enhanced with a diamine-silver staining technique [17] (see Section 3.1.2). The *M_r* of the glutenins varies from 35000–45000 (LMW subunits), and 80000–110000 (HMW subunits). Besides the HMW and LMW glutenin subunits, some additional polypeptide bands exhibiting *M_r* below 30000 and between 45000–60000 are also visible in the glutenin fraction. These polypeptides are most likely albumin and/or globulin impurities which had not been completely removed by the preceding extraction steps with Tris-HCl buffer and aqueous alcohol, but were eventually solubilized by the SDS/DTT buffer (see below).

3.1.1.1 Glycoprotein staining with the Con A/HRP reagent

Con A/HRP staining showed that the glucose- and/or mannose-containing glycoproteins of whole-meal flour are primarily located in the albumin/globulin protein fraction (see Fig. 3). The *M_r* of the major glycoproteins are between 35000–45000, 55000–60000 and 70000–

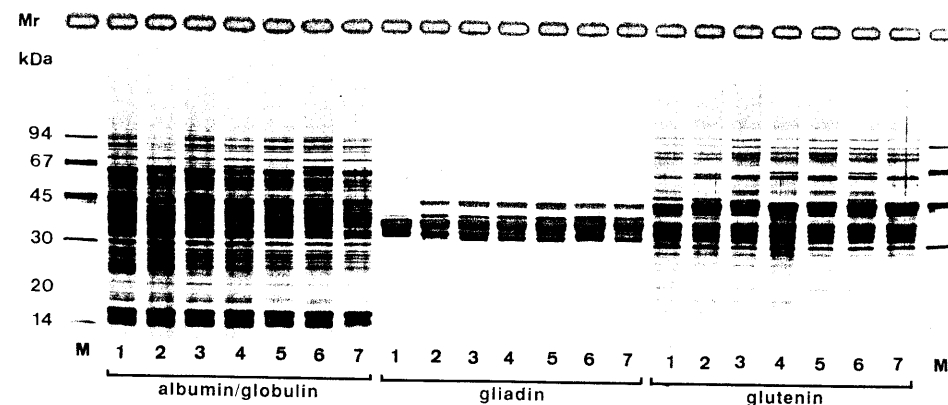


Figure 2. Horizontal SDS-PAGE (10–15% T) of albumin/globulin, gliadin and glutenin proteins from seven wheat cultivars. Silver stain [15]. Wheat cultivars: (1) Apollo, (2) Ares, (3) Avis, (4) Boheme, (5) Bussard, (6) Fregatt, (7) Futur; (M) *M_r* marker proteins.

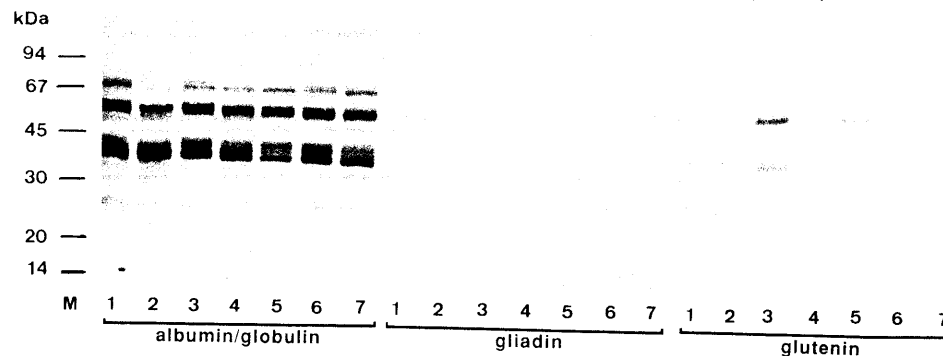


Figure 3. Detection of wheat glycoproteins by the Con A/HRP staining method after SDS-PAGE (10–15% T) and blotting onto a PVDF membrane. Samples as in Fig. 2.

75000. Some minor glycoprotein zones are visible between *M_r* 25000–30000. In contrast, no glycoproteins could be detected in the gliadin fraction, and only low amounts of glycoproteins were found in the glutenin protein fraction. However, since the patterns of the “glutenin” glycoproteins are identical to those of the albumin/globulin fraction, the glycoproteins shown in the glutenin fraction are most likely albumin/globulin impurities (Fig. 3).

3.1.1.2 Immunostaining with rabbit anti-gliadin-HRP conjugate

HRP-conjugated rabbit anti-gliadin antibodies reacted primarily with the α-, β- and γ-gliadins (*M_r* ≈ 30000–45000) of the gliadin fraction (see Fig. 4). However, some of the albumins and/or globulins (*M_r* between 20000–70000), and the LMW glutenin subunits were also recognized by these (polyclonal) antibodies. Since the patterns of the antibody-binding gliadin and glutenin polypeptides deviate from each other, we presume that

the proteins in the glutenin protein fraction which bind to the anti-gliadin antibodies are not cross-contaminating gliadins (which might have been carried over into the gliadin solubility fraction) but cross-reacting glutenins instead, which express epitopes similar or identical to those of the gliadins. This assumption is supported by the fact that gliadins and glutenins are closely related and, therefore, share common antigen binding sites.

3.1.2 IPG-Dalt

To further evaluate the polypeptide composition of the individual solubility fractions, the albumin/globulin, gliadin and glutenin protein fractions and, additionally, the total protein extract from wheat seed proteins were analyzed by high-resolution 2-D electrophoresis with immobilized pH gradients in the first dimension (IPG-DALT). IPG-DALT is the method of choice for the separation of complex protein mixtures because highly reproducible spot positions are exclusively obtained with this particular technique (A. Görg and M. J. Dunn, in

preparation). Therefore, this method enables us to compare the polypeptide patterns separated on different 2-D gels simply by superimposing the gels or autoradiograms.

3.1.2.1 Silver-stained 2-D polypeptide patterns

The silver-stained 2-D polypeptide patterns of the three solubility fractions, and of the total protein extract, are presented in Fig. 5. Depending on the sensitivity of the silver staining method applied, up to several hundred albumin and globulin polypeptide spots are visible on a single 2-D gel (Fig. 5a). The albumin/globulin polypeptides are distributed more or less uniformly over the entire pI and M_r ranges of the 2-D gel. The pI of the polypeptides is between pH 4 and 8.5, and the corresponding M_r ranges from 15000 to 100000. There is only one major gap between M_r 20000 and 25000 where only few polypeptides can be detected. In contrast to the albumins and globulins, the gliadins are restricted to well-defined regions of the 2-D gel: the α -, β - and γ -gliadins (M_r 35000–50000) form a cluster which is located at the more basic end of the gel ($pI \approx 6.5$ –9), whereas the Ω -gliadins (55000–75000) are located in the pI area from pH 4 to 7 (Fig. 5b). Similar to the gliadins, the glutenins are also predominantly found in distinct areas of the 2-D gel: the LMW glutenin subunits ($M_r \approx 35000$ –50000) exhibit pI s from pH 6–9 (and are, therefore, hardly distinguishable from the α -, β - and γ -gliadins). The HMW glutenin subunits are composed of at least two groups of different pI and M_r : a lower M_r group ($M_r \approx 85000$), which is composed of three major polypeptide spots and exhibits a neutral or slightly basic pI ($pI \approx 7$ –7.5), and a higher M_r group ($M_r \approx 110000$) which is located at the more acidic end of the 2-D gel ($pI \approx 5$ –7) (Fig. 5c). For comparative purposes, the silver-stained total protein pattern of wheat seed proteins is also presented (Fig. 5d).

The higher M_r gliadins and, especially, the glutenins are insufficiently stained by the silver nitrate staining procedures of Blum *et al.* [15] and Heukeshoven and Dernick [16]. One reason for this may be that all these proteins are soluble in acetic acid and/or in aqueous alcohol, and may therefore have been partly washed out during the fixation step prior to silver staining. Better results were obtained with the diamine-silver stain of Oakley *et al.*

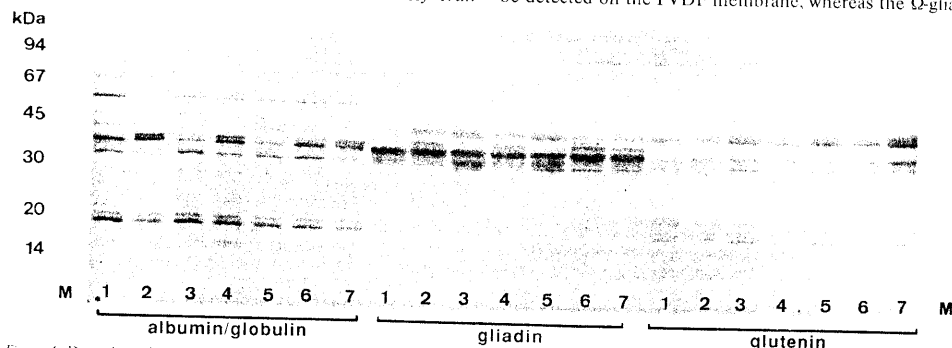


Figure 4. Detection of wheat gliadins by rabbit anti-gliadin HRP-conjugate after SDS-PAGE (10–15% T) and blotting onto a PVDF membrane. Samples as in Fig. 2.

[17] in which glutaraldehyde (10% v/v) is used as fixative and sensitizer (see Fig. 6). One drawback of this technique, however, is that residual carrier ampholyte-SDS complexes that were not completely removed during the washing steps are also heavily stained, resulting in a dark background stain in the M_r range below 25000, so that it is impossible to evaluate this part of the gel (not shown).

3.1.2.2 Total protein and glycoprotein staining of polypeptides after electrophoretic transfer

The blotted polypeptides were stained with the general protein stain India Ink to check whether all proteins which had been stained with silver nitrate in the polyacrylamide gel are also detectable on the PVDF membrane. A comparison between the silver-stained polypeptide patterns on the 2-D gel (Fig. 7a), and the India Ink-stained patterns on the PVDF membrane reveals only small qualitative differences although the α -, β - and γ -gliadins give less well-resolved spots on the India Ink-stained PVDF-membrane (Fig. 7b) than the silver-stained 2-D gel. When proteins containing glucose and/or mannose moieties were specifically stained with the Con A/HRP reagent, a surprisingly high number of glycoproteins was visualized. More than one hundred individual spots, arranged in five groups, are visible on the original PVDF membrane (Fig. 7c). These glycoprotein clusters exhibit an M_r between 15000 and 80000, and a pI between pH 4 and 8.5 (see Table 1).

3.1.2.3 Immunostaining with rabbit anti-gliadin HRP conjugate

Since certain membrane-bound polypeptides, as well as specific antibodies bound to these polypeptides, may be washed off the PVDF membrane during the various incubation and washing steps, information about the allergenic nature of individual polypeptide spots may be lost. The gliadins and glutenins are of special interest in this context. Therefore, rabbit anti-gliadin antibodies were used as a model to study this problem in more detail. As is seen from Fig. 7d, the α -, β - and γ -gliadins can easily be detected on the PVDF membrane, whereas the Ω -glia-

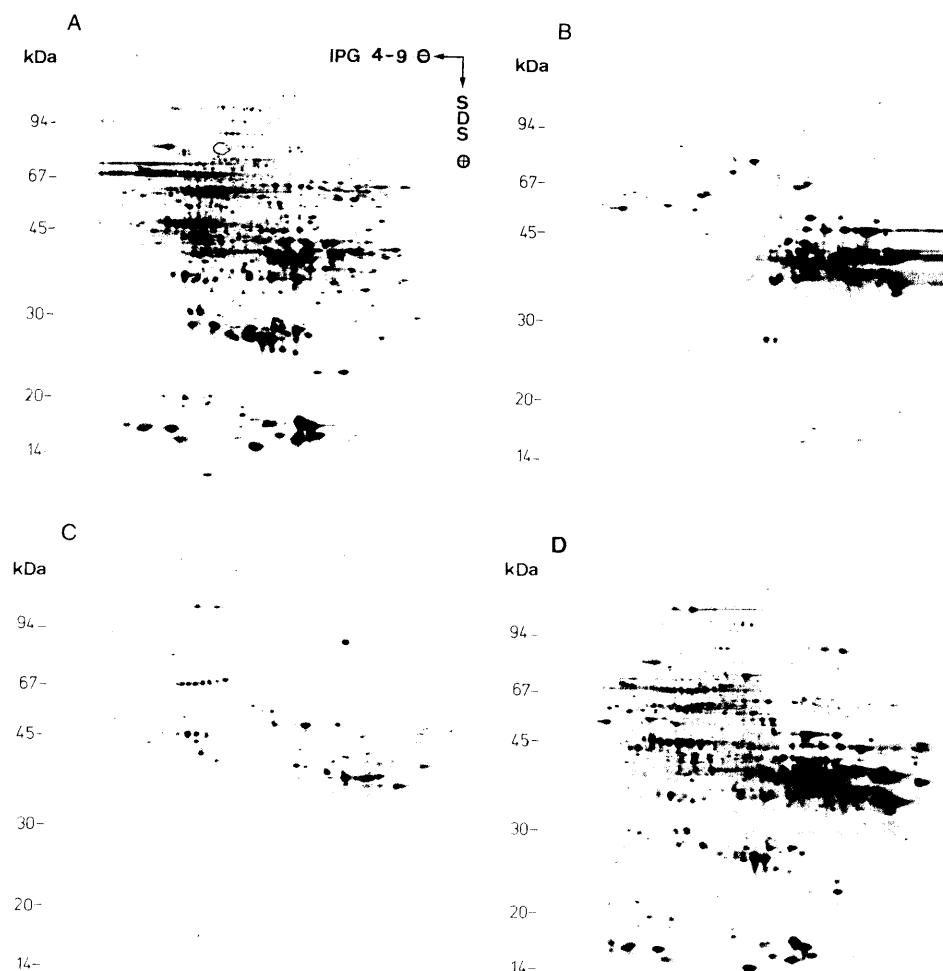


Figure 5. IPG-Dalt [12] of wheat grain proteins. First dimension: IEF with IPG 4–9. Second dimension: SDS-PAGE, 10–15% T. (A)–(C): Protein fractions obtained by sequential extraction of whole-meal flour. (A) Salt-soluble proteins (albumins/globulins). (B) 75% ethanol-soluble proteins (gliadins). (C) Urea/DTT-soluble proteins (glutenins). (D) Total proteins, extracted with urea/DTT/Triton X-100. Silver stain according to [16]. Sample: cultivar “Fregatt”.

dins are not visible. There are at least four explanations for this phenomenon: (i) The Ω -gliadins were not bound tightly enough and were therefore washed off the membrane during the incubation and washing steps. (ii) The detection system (HRP-labeled antibodies) was not sensitive enough to detect the Ω -gliadins since these proteins are present in wheat seed in lower quantities than the α -, β - and γ -gliadins. (iii) The Ω -gliadins were not present in the antigen preparation which had been used for the immunization of the rabbit. (iv) The three-dimen-

sional structure of the proteins was altered by the SDS/DTT treatment, and a considerable amount of the antibody binding sites (the conformational epitopes) has disappeared; these polypeptides are therefore poorly recognized by the antibodies. Whatever the mechanism for the apparent loss of the Ω -gliadins, we conclude that at least the α -, β - and γ -gliadins were bound to the PVDF-membrane and were recognized by the rabbit IgG antibodies. In analogy, these polypeptides should also bind IgE antibodies from the patients' sera – provided

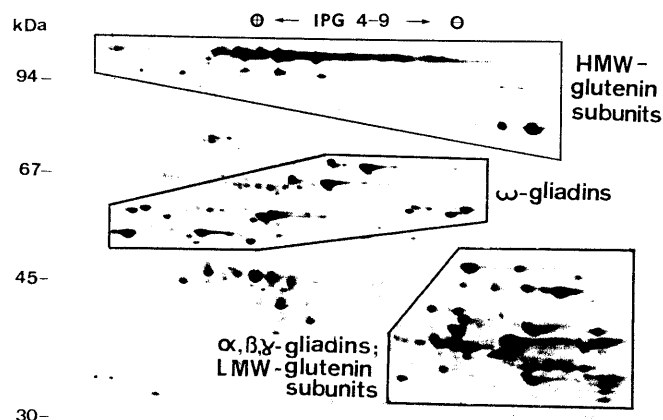


Figure 6. IPG-Dalt [12] of gliadin and glutenin proteins. IEF and SDS gels as in Fig. 5. Silver stain according to Oakley *et al.* [17]. Sample: cultivar "Fregatt".

that the sera contain IgE antibodies which are directed against these proteins.

3.2 Characterization of wheat seed proteins binding IgE from sera of allergic persons

The IgE binding pattern of the different protein fractions upon SDS-PAGE is presented in Fig. 8. It is evident that the IgE antibodies of the patients' sera were reactive to proteins of all three solubility fractions. However, most IgE binding activity was clearly observed with the albumin/globulin protein fraction (Fig. 9a and Table 2). The major albumin (or globulin) allergen is an M_r 27000 polypeptide. Quantitative evaluation showed that up to 60% of the radioactive uptake of all albumin and globulin allergens, and up to 50% of the radioactive uptake of all wheat allergens are allocated to this allergen (Fig. 9b). However, there are relatively large cultivar-specific differences: Whereas almost 50% of the total bound radioactivity is taken up by this particular polypeptide in cultivars 1, 3, 5 and 6, this percentage is considerably lower in cultivars 2 and 4 (13 and 17%, respectively) (Fig. 9b).

A more detailed characterization of this allergen by IPG-Dalt revealed that it is not a single polypeptide spot but forms an "IEF-train", consisting of five polypeptide spots which differ in their pI in about 0.5 pI units each (Fig. 10). This pI heterogeneity may be caused by differences in the primary structure (exchange of amino acids and/or proteolytic cleavage of terminal amino acids), or post-translational modifications such as different levels of carbamylation, deamidation and/or phosphorylation. Differential glycosylation is also possible since some minor Con A-binding polypeptides were detected in the M_r 25000–30000 region of the gel in our previous experiments (see Fig. 3 and Fig. 7c). Other major albumin/globulin allergens are an M_r 18000, a 20000 and a 35000 allergen (Fig. 8). IPG-Dalt of these proteins revealed that these allergens are not single polypeptides either, but are composed of several polypeptide spots which exhibit a relatively acidic pI around pH 4.5 (Fig. 10 and

Table 1. Relative molecular masses and isoelectric points of the major Con A-binding glycoproteins as determined by IPG-Dalt

M_r	pI
70000–80000	6.5–7.5
55000–65000	6.0–8.0
40000–45000	6.5–8.5
35000–40000	4.0–6.0
15000–30000	5.0–7.5

Table 2. Specific IgE binding to the main wheat protein fractions of seven cultivars, determined by densitometric evaluation of the autoradiogram presented in Fig. 8

% Uptake ^a	Minimum	Maximum	Mean
Protein fraction			
Albumin/globulin	69.0	81.0	76.4
Gliadin	7.8	17.4	12.7
Glutenin	8.2	15.0	10.9

^a Radioactive uptake of the individual solubility fractions as percentage of the total uptake

Fig. 11). Besides these major allergens, additional IgE binding polypeptides which are located in the M_r range above 40000 were observed (Figs. 8 and 12). These allergens were further characterized by IPG-Dalt and immunoblotting. Prolonged exposure time (up to six weeks) revealed a number of minor IgE-binding polypeptides, the most prominent of which are 55000–65000 glycoproteins (not shown). A map of the IgE-binding polypeptides is presented in Fig. 11.

Apart from the albumins and globulins, IgE of the patients also binds to α -, β - and γ -gliadins ($M_r \approx 35000$ –45000) and to the glutenins (Fig. 8). Although less marked than with albumins, there are also cultivar-specific differences in radioactive uptake in these protein fractions. For example, the gliadins of cultivar 1 bind a much higher amount of IgE than do the gliadins of cultivar 3 (Fig. 9c). In contrast to the α -, β - and γ -gliadins, no IgE binding was observed with the Ω -gliadins. This may be due to reasons similar to those discussed above for IgG binding (Section 3.1.2).

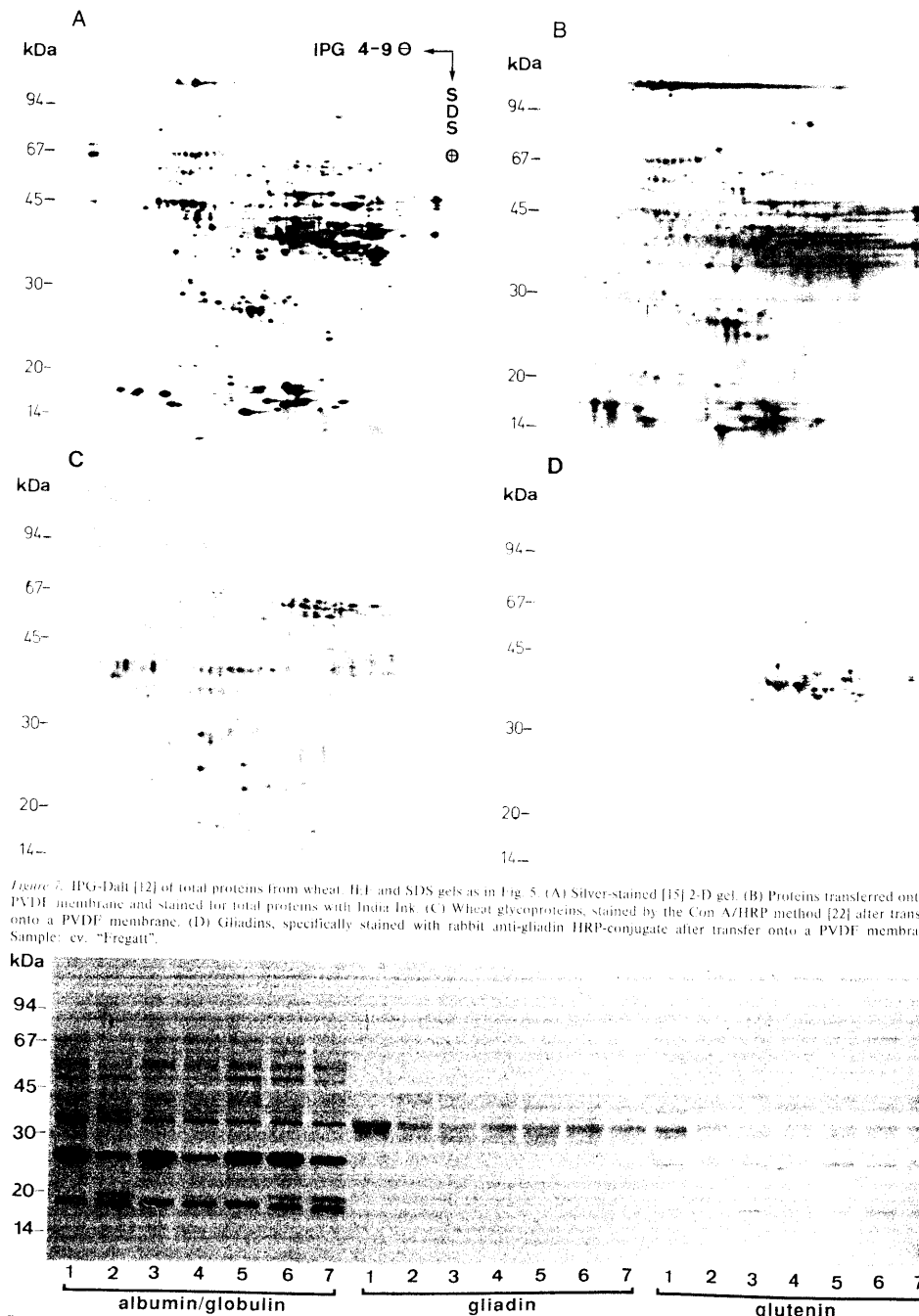


Figure 7. IPG-Dalt [12] of total proteins from wheat. IEF and SDS gels as in Fig. 5. (A) Silver-stained [15] 2-D gel. (B) Proteins transferred onto a PVDF membrane and stained for total proteins with India Ink. (C) Wheat glycoproteins, stained by the Con A/HRP method [22] after transfer onto a PVDF membrane. (D) Gliadins, specifically stained with rabbit anti-gliadin HRP-conjugate after transfer onto a PVDF membrane. Sample: cv. "Fregatt".

Figure 8. Detection of IgE binding proteins (*i.e.*, allergens) after SDS-PAGE (10–15% T) and blotting onto a PVDF membrane. The membrane was incubated with the pooled serum from four asthmatic bakers. Bound IgE was visualized with ^{125}I -labeled anti-human IgE, followed by autoradiography (4 weeks exposure time). Samples as in Fig. 2.

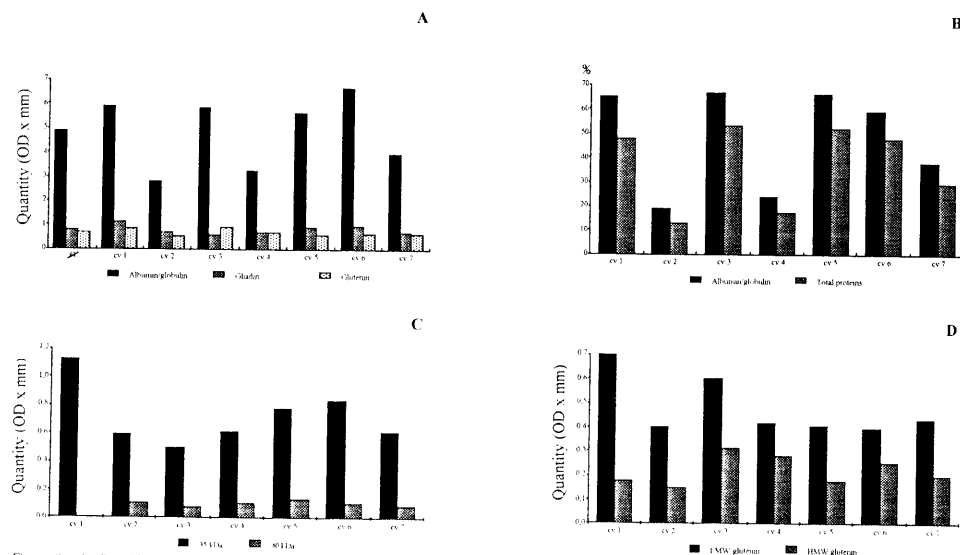


Figure 9. (A) Specific binding of IgE antibodies to the three main protein fractions (albumin/globulin, gliadin and glutenin) of seven wheat cultivars. (B) Specific IgE binding to the M_r 27000 albumin/globulin polypeptides extracted from whole-meal flour of seven different wheat cultivars. Vertical axis indicates values as percent radioactive uptake of the total uptake of the albumin/globulin and of the total wheat allergens, respectively. (C) Specific IgE binding to gliadin polypeptides extracted from whole-meal flour of seven wheat cultivars. (D) Specific IgE binding to LMW (M_r 80000-110000) and HMW (M_r 35000-50000) glutenin subunits extracted from whole-meal flour of seven wheat cultivars. The allergenicity of the fractions was determined by densitometric evaluation of the autoradiogram presented in Fig. 8.

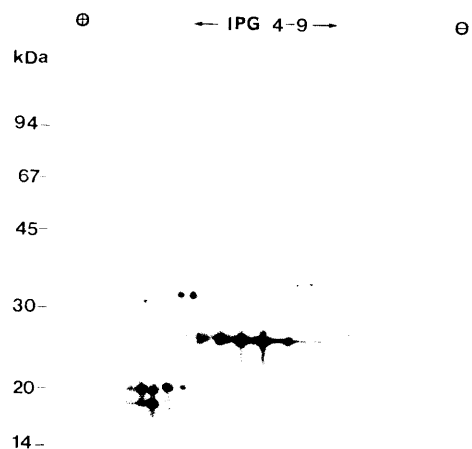


Figure 10. Detection of IgE binding proteins (allergens) after IPG-Dalt [12] and blotting onto a PVDF membrane. IEF and SDS gels as in Fig. 5. Sample: Total proteins from wheat variety 'Fregati'. Bound IgE was visualized as described in Fig. 8. Two weeks exposure time.

Glutenins and gliadins bind IgE in the same order of magnitude, but albumins and/or globulins bind much more IgE (Fig. 8, Fig. 9a and Table 2). The radioactive uptake of the LMW glutenin subunits ($M_r \approx 35000-45000$) is about twice as high than was bound by the HMW subunits ($M_r \approx 90000$) (Fig. 9d). Besides the LMW ($M_r \approx 35000-45000$) and the HMW ($M_r \approx 80000-110000$) glutenin subunits, additional IgE binding zones are observed within the glutenin fraction (e.g. the M_r 18000 and 27000 bands). However, these allergens are albumin/globulin impurities as can easily be seen from a comparison with the IgE binding zones of the albumin/globulin fraction (see Fig. 8, left and right side).

Although the major IgE binding proteins are located in the albumin/globulin protein fraction, the total number of these allergens ($n < 30$) is quite small in comparison to the total number of albumins and globulins detected by silver staining ($n \gg 100$; see Figs. 5a and 10). In contrast, most of the gliadins and glutenins which can be visualized by silver staining also bind the IgE from the serum of the allergic bakers (see Figs. 2 and 8). This behavior can probably be explained by the biochemical nature of the different protein classes: The albumins and globulins are a heterogeneous mixture of polypeptides and are related only distantly, if at all, whereas most of the gliadins and glutenins, derived from a common ancestor by gene duplication, are closely related, and therefore exhibit similar or identical epitopes. Hence, if a particular IgE antibody binds to one gliadin (or glutenin), it will probably also bind to a number of related

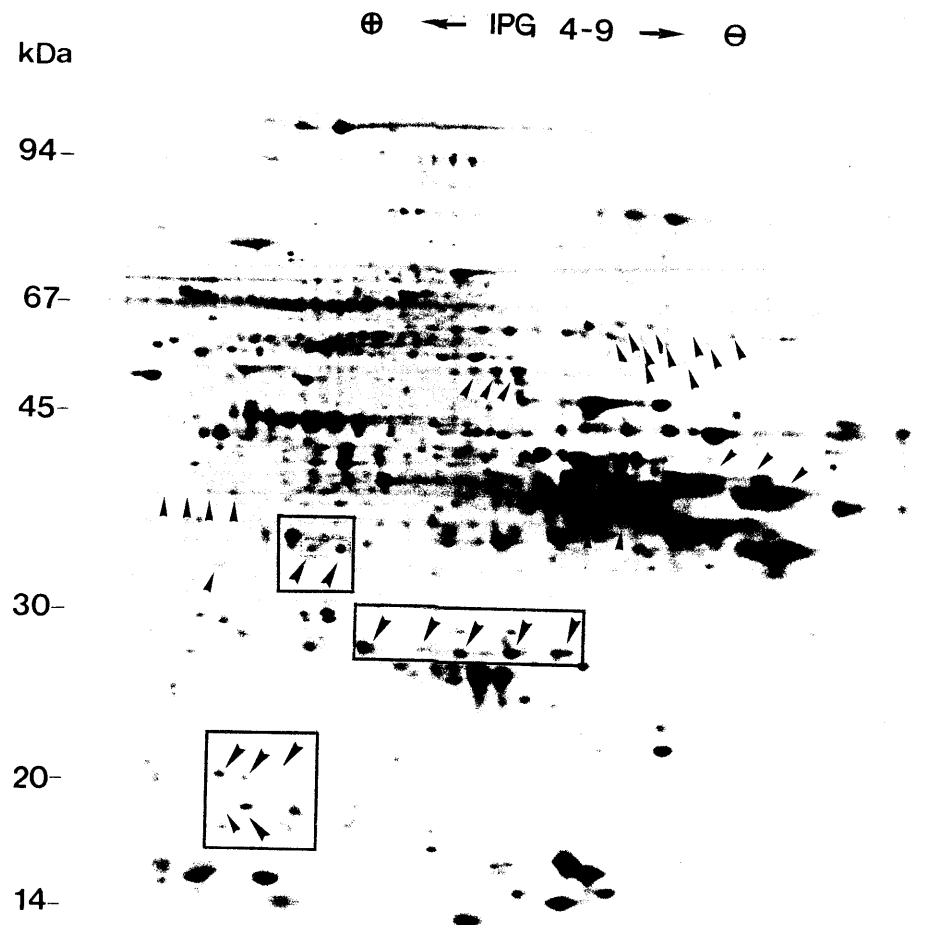


Figure 11. Localization of wheat flour allergens in the 2-D polypeptide map. IgE binding polypeptides are labeled with arrows, and major allergens are enclosed by frames. IPG-Dalt [12] of total proteins from wheat whole-meal flour. IEF and SDS gels as described in Fig. 5. Silver stain [16]. Sample: cultivar 'Fregati'.

ones. Therefore, a small number of specific IgE antibody species should be sufficient to recognize almost all gliadins (or glutenins) present.

Whereas the serum of the four bakers allergic to flour contained IgE antibodies which bound to polypeptides of all three solubility fractions, the serum pool obtained from the healthy donors did not show antibodies of this isotype that bind to any of the wheat seed proteins (results not shown).

The results of the immunoblotting experiments can be summarized as follows: (i) All three solubility fractions contain proteins which bind IgE from the sera of allergic bakers. (ii) The highest percentage of IgE binding was observed with certain albumin and/or globulin polypeptides, whereas the gliadins and glutenins showed considerably less radioactive uptake. (iii) SDS-PAGE revealed that the protein which bound the highest percentage of IgE from the sera of the allergic bakers is an M_r 27000 albumin (or globulin). More detailed investigations

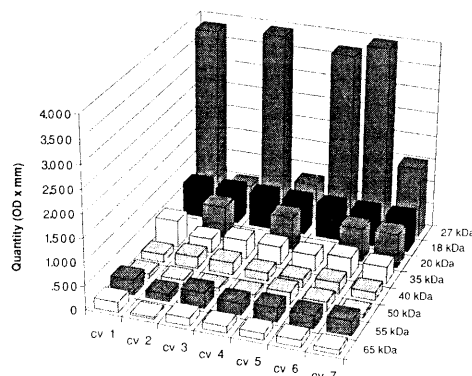


Figure 12. Specific IgE binding to individual albumin/globulin polypeptides extracted from whole-meal flour of seven wheat cultivars. The allergenicity of the polypeptides was determined by densitometric evaluation of the autoradiogram presented in Fig. 8.

using I-PG-Dalt showed that this allergen is not a single polypeptide, but consists of several polypeptides differing in *pI*. This charge heterogeneity may be due to slight differences in their primary structures, or to posttranslational modifications such as glycosylation. (iv) Most albumins and globulins which bind to IgE antibodies exhibit "charge-trains" upon I-PG-Dalt. Several of these allergens are Con A-binding glycoproteins (e.g. the *M*₅₅₀₀₀–65000 glycoproteins). (v) Quantitative studies have revealed that the amount of IgE bound by particular allergens shows considerable cultivar-specific differences.

In general, the results of our study are for the most part consistent with those of Baldo, Wrigley and co-workers [2–6]. Baldo *et al.* [2] showed that the major IgE binding proteins for the majority of all patients are located in the albumin fraction, especially in the *M*₅₅₀₀₀–65000 range between 15000 and 35000 [4]. In addition, they demonstrated that the glutenins also express a high degree of allergenicity for a number of patients [3]. Deviating from their findings, we were not able to fully confirm the importance of the glutenins. However, the discrepancy in IgE binding of the individual protein classes observed by Baldo *et al.* [2] and by us may be due to the different experimental conditions. Baldo *et al.* had almost exclusively used non-denaturing protein separation techniques in order to preserve the native state and the three-dimensional conformation of the allergens. In our experiments the three-dimensional conformation of the proteins was altered due to the SDS/DTT treatment. Probably, Baldo *et al.* [2] studied IgE antibodies primarily directed against conformational epitopes, whereas we studied IgE antibodies directed against sequential antigen binding sites.

4 Concluding remarks

One interesting aspect of our study is the detection of particular allergens (such as the *M*₂₇₀₀₀ albumin allergen) which bind different amounts of IgE from allergic bakers, depending on the source (*i.e.*, wheat cul-

tivar) from which these allergens were obtained. We have not been able to find the reason for this phenomenon. One possible explanation is that these allergens are present in lower amounts in certain cultivars than in others. A second explanation is that the allergens extracted from different cultivars differ slightly in amino acid composition and/or post-translational modifications, and therefore exhibit different epitopes. Further analyses have to be performed to evaluate this problem in more detail. To find out whether differences between the primary structures of these proteins exist, we plan to separate wheat proteins with high-resolution I-PG-Dalt and transfer the separated proteins onto a PVDF membrane. Then those polypeptide spots which have been shown to bind IgE will be cut out and subjected to peptide mapping. Alternatively, the amino acid sequences of these polypeptides can be determined by gas-phase amino acid sequencing. Whatever the results of these studies, better knowledge of the molecular structure of these allergens may be beneficial in many ways. It could, e.g., be used for breeding new, "less allergenic" varieties which contain either lower amounts of these allergens or modified non-allergenic proteins.

Received March 8, 1993

5 References

- [1] Thiel, H. and Kallweit, C., *Allergologie* 1984, 7, 407–414.
- [2] Baldo, B. A. and Wrigley, C. W., *Clinical Allergy* 1978, 8, 109–124.
- [3] Walsh, B. J., Wrigley, C. W., Musk, A. W. and Baldo, B. A., *J. Allergy Clin. Immunol.* 1985, 76, 23–28.
- [4] Baldo, B. A., Sutton, R. and Wrigley, C. W., *Prog. Allergy* 1982, 30, 1–66.
- [5] Sutton, R., Wrigley, C. W. and Baldo, B. A., *J. Immunol. Methods* 1982, 52, 183–194.
- [6] Sutton, R., Skeritt, J. H., Baldo, B. A. and Wrigley, C. W., *Clinical Allergy* 1984, 11, 93–107.
- [7] Osborne, T. B., *The Proteins of the Wheat Kernel*, Carnegie Inst. Washington Publ. 84, Judd and Detweiler, Washington 1907.
- [8] Görg, A., Postel, W., Günther, S. and Weser, J., in: Dunn, M. J. (Ed.) *Electrophoresis '86*, VCH Verlagsgesellschaft, Weinheim 1986, pp. 435–449.
- [9] Görg, A., Postel, W., Westmeier, R., Granazza, F. and Righetti, P. G., *J. Biochem. Biophys. Methods* 1980, 3, 273–284.
- [10] Weiss, W., Postel, W. and Görg, A., *Electrophoresis* 1991, 12, 330–337.
- [11] Laemmli, U. K., *Nature* 1970, 227, 680–685.
- [12] Görg, A., Postel, W. and Günther, S., *Electrophoresis* 1988, 9, 531–546.
- [13] Görg, A., Postel, W., Baumer, M. and Weiss, W., *Electrophoresis* 1992, 13, 192–201.
- [14] Görg, A., Postel, W., Friedrich, C., Kuick, R., Strahler, J. R. and Hanash, S. M., *Electrophoresis* 1991, 12, 653–658.
- [15] Blum, H., Beier, H. and Gross, H. J., *Electrophoresis* 1987, 8, 93–99.
- [16] Heukeshoven, J. and Dernick, R., *Electrophoresis* 1988, 9, 28–32.
- [17] Oakley, B. R., Kirsh, D. R. and Morris, N. R., *Anal. Biochem.* 1980, 105, 161–162.
- [18] Kyhse-Andersen, J., *J. Biochem. Biophys. Methods* 1984, 10, 203–209.
- [19] Towbin, H., Staehelin, T. and Gordon, J., *Proc. Natl. Acad. Sci. USA* 1979, 76, 4350–4354.
- [20] Hancock, K. and Tsang, V. C. W., *Anal. Biochem.* 1983, 133, 157–162.
- [21] Domingo, A. and Marco, R., *Anal. Biochem.* 1989, 182, 176–181.
- [22] Hawkes, R., *Anal. Biochem.* 1982, 123, 143–146.
- [23] Weiss, W., Postel, W. and Görg, A., *Electrophoresis* 1991, 12, 323–330.
- [24] Thiel, H. and Kallweit, C., *Allergologie* 1984, 7, 407–414.
- [25] Baldo, B. A. and Wrigley, C. W., *Clinical Allergy* 1978, 8, 109–124.
- [26] Walsh, B. J., Wrigley, C. W., Musk, A. W. and Baldo, B. A., *J. Allergy Clin. Immunol.* 1985, 76, 23–28.
- [27] Baldo, B. A., Sutton, R. and Wrigley, C. W., *Prog. Allergy* 1982, 30, 1–66.
- [28] Sutton, R., Wrigley, C. W. and Baldo, B. A., *J. Immunol. Methods* 1982, 52, 183–194.
- [29] Sutton, R., Skeritt, J. H., Baldo, B. A. and Wrigley, C. W., *Clinical Allergy* 1984, 11, 93–107.
- [30] Osborne, T. B., *The Proteins of the Wheat Kernel*, Carnegie Inst. Washington Publ. 84, Judd and Detweiler, Washington 1907.
- [31] Görg, A., Postel, W., Günther, S. and Weser, J., in: Dunn, M. J. (Ed.) *Electrophoresis '86*, VCH Verlagsgesellschaft, Weinheim 1986, pp. 435–449.
- [32] Görg, A., Postel, W., Westmeier, R., Granazza, F. and Righetti, P. G., *J. Biochem. Biophys. Methods* 1980, 3, 273–284.
- [33] Weiss, W., Postel, W. and Görg, A., *Electrophoresis* 1991, 12, 330–337.
- [34] Laemmli, U. K., *Nature* 1970, 227, 680–685.
- [35] Görg, A., Postel, W. and Günther, S., *Electrophoresis* 1988, 9, 531–546.
- [36] Görg, A., Postel, W., Baumer, M. and Weiss, W., *Electrophoresis* 1992, 13, 192–201.
- [37] Görg, A., Postel, W., Friedrich, C., Kuick, R., Strahler, J. R. and Hanash, S. M., *Electrophoresis* 1991, 12, 653–658.
- [38] Blum, H., Beier, H. and Gross, H. J., *Electrophoresis* 1987, 8, 93–99.
- [39] Heukeshoven, J. and Dernick, R., *Electrophoresis* 1988, 9, 28–32.
- [40] Oakley, B. R., Kirsh, D. R. and Morris, N. R., *Anal. Biochem.* 1980, 105, 161–162.
- [41] Kyhse-Andersen, J., *J. Biochem. Biophys. Methods* 1984, 10, 203–209.
- [42] Towbin, H., Staehelin, T. and Gordon, J., *Proc. Natl. Acad. Sci. USA* 1979, 76, 4350–4354.
- [43] Hancock, K. and Tsang, V. C. W., *Anal. Biochem.* 1983, 133, 157–162.
- [44] Domingo, A. and Marco, R., *Anal. Biochem.* 1989, 182, 176–181.
- [45] Hawkes, R., *Anal. Biochem.* 1982, 123, 143–146.
- [46] Weiss, W., Postel, W. and Görg, A., *Electrophoresis* 1991, 12, 323–330.

Heide Schnabl
Torsten Klockenbring
Michael Huhn
Margot Schulz

Institut für Landwirtschaftliche
Botanik, Universität Bonn

Purification of phosphoenolpyruvate carboxylase subunits and isoforms from *Vicia faba* L. by preparative gel electrophoresis and their detection by enzyme-linked immunosorbent assay

A purification procedure which yields nearly homogenous subunits of stomatal phosphoenolpyruvate carboxylase from epidermal strips of *Vicia faba* L. is reported. Preparative gel electrophoresis was found to be the most suitable technique for subunit purification. Denatured subunits of the stomatal enzyme in the eluate were immunologically detected by enzyme-linked immunosorbent assay (ELISA) tests. The enzyme preparation meets all requirements for its use in antibody production.

Gas exchange and transpiration of higher plants are regulated by modulations of stomatal apertures. Stomatal opening and closing depend on alterations in the increase or decrease of the stomatal cell turgor due to modulations of ion flux rates and the concentration of osmotically active substances. In particular, potassium ions and malate are known to be important factors in stomatal movements [1, 2]. During stomatal opening, malate functions as a counter-ion to incoming K⁺-ions and therefore stabilizes the cytosolic pH [3, 4]. Similar to crassulacean acid metabolism, the first step in malate production is the irreversible β -carboxylation of phosphoenolpyruvate catalyzed by phosphoenolpyruvate carboxylase (PEPCase, EC 4.1.1.31). The product oxaloacetic acid is subsequently reduced to malate by malic enzyme. A strong correlation between the *K_m* of stomatal PEPCase and the K⁺-induced swelling of guard cell protoplasts has been described by Kottmeier and Schnabl [5]. Furthermore, the *K_m* is modulated by cytosolic pH, malate concentration and additional effectors such as glucose-6-phosphate. Inhibition and activation of stomatal PEPCase are most important events in the regulation of stomatal movements.

PEPCase is widely distributed in higher plants with tissue- and cell-specific functions, reflected by different isozymes and isoforms of the enzyme [6, 7, 8]. PEPCase is only a minor constituent of total protein. The low amounts of different isoforms in these plants and their major localization in a single cell type, the guard cell, cause severe difficulties during enzyme purification. Using only strips of the abaxial epidermis, an excessive contamination with PEPCase isoforms from the mesophyll can be avoided. This results in a reduction of suitable plant material, which is about 100-fold lower compared to the material used for a successful purification of some other plant PEPCases. As a consequence, the number of feasible conventional purification steps is limited. A recently developed purification procedure including ammonium sulfate precipitation, gel filtration and chromatography on diethylaminoethyl (DEAE)-cellulose yielded an 80-fold enrichment of stomatal

PEPCase, but contamination by copurified proteins was about 50% (Denecke, personal communication). An efficient purification of the enzyme by preparative sodium dodecyl sulfate (SDS)-gel electrophoresis, however, is based on this procedure.

Epidermal strips of *V. faba* were homogenized by grinding in extraction buffer (100 mM Tris/HCl, pH 7.5; 2 mM EDTA; 20 mM sodium ascorbate; 5 mM dithioerythritol; 1 mM MgCl₂; 10 mM KCl; 10% v/v glycerol), the resulting homogenate was filtered through a nylon net (20 μ m), and the filtrate was centrifuged (22000 g, 45 min, 4°C). The supernatant was used for ammonium sulfate precipitation (33–70%), and precipitates were pelleted by centrifugation (44000 g, 15 min, 4°C). The final pellet was dissolved in 2 mL extraction buffer and the protein concentrate applied onto a Sephacryl S-400 HR column (Pharmacia). Gel filtration resulted in a three- to fivefold enrichment of PEPCase activity: For preparative gel electrophoresis, 0.9 mL (1.56 $\pm$ 0.1 mg protein containing 2.13 $\pm$ 0.14 U PEPCase activity) of the active fractions, concentrated by ultrafiltration after molecular sieve chromatography, were mixed (1:1) with SDS sample buffer and boiled for 5 min.

Preparative polyacrylamide gel electrophoresis (PAGE) was performed with Prep Cell 491 (Bio-Rad). Gels, cast 6 cm high, were used for protein separation. A total monomer concentration of 9% and a cross-linker concentration of 0.5% were found to guarantee optimal separating conditions. During overnight polymerization, a constant temperature (22°C) was maintained by the central cooling finger. A stacking gel, 2 cm high, was polymerized on the separating gel. After sample loading, a constant current of 40 mA was supplied. Constant cooling of the gel was achieved by pumping running buffer (0.38 M glycine, 0.05 M Tris, 0.1% SDS, and 0.1% EDTA) via the central cooling finger. The protein mixture was separated by vertical migration through the cylindrical gel and elution of the bands was provided by an elution chamber. The eluate was monitored for protein content by a UV-detector and then fractionated. Table 1 lists the set of conditions used for preparative SDS-PAGE.

Fractionated proteins were tested for the presence of PEPCase by indirect enzyme-linked immunosorbent assay (ELISA), according to a procedure described in [9].

Correspondence: Prof. Dr. H. Schnabl, Institut für Landwirtschaftliche Botanik, Meckenheimer Allee 176, D-53115 Bonn, Germany

Abbreviations: ELISA, enzyme-linked immunosorbent assay; PEPCase, phosphoenolpyruvate carboxylase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate