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Sequence analysis of wheat grain allergens separated by two-dimensional electrophoresis with immobilized pH gradients

Micropreparative two-dimensional (2-D) gel electrophoresis with immobilized pH gradients (4–8) in the first dimension (IPG-DALT) was optimized for the separation of salt-soluble wheat grain proteins associated with bakers' asthma disease. The resolved polypeptides were electroblotted onto a polyvinylidene difluoride (PVDF) membrane and incubated with the pooled sera from four asthmatic bakers. Bound IgE was demonstrated by alkaline phosphatase conjugated anti-human IgE. Major IgE binding was detected in the 27 kDa, 37 kDa and, to a lesser extent, in the 14–18 kDa area of the 2-D immunoblots, respectively. Since the main purpose of our study was to determine the N-terminal amino acid sequences of the major wheat grain allergens, N-terminal sequencing was performed for six out of a total of eleven major allergens located in the 27 kDa area, for one out of two 37 kDa allergens, and for two out of four 14–18 kDa allergens. Our results revealed that two of the 27 kDa polypeptides are clearly related to several Acyl-CoA oxidase variants of barley and rice, whereas no significant homologies were found for the remaining four 27 kDa allergens analyzed. The N-terminus of the 37 kDa allergen appeared to be blocked so that no sequence information was obtained, while the two 14–18 kDa allergens analyzed were identified as members of the wheat α -amylase-inhibitor family.

1 Introduction

Two-dimensional polyacrylamide gel electrophoresis with immobilized pH gradients (IPG-DALT) [1] has a unique capacity for the resolution of complex mixtures of proteins. We are employing this technique to identify wheat grain allergens involved in bakers' asthma [2]. Similarly, Varjonen *et al.* [3] characterized soluble wheat flour extracts by one-dimensional immunoblotting with serum samples of 40 allergic persons. In accordance with our results, the major allergens have been localized in the 26 kDa protein region. Once the relevant proteins have been identified by electrophoresis and immunoblotting, further characterization of these allergens would be desirable in order to develop better diagnostic tools, or to prevent this disease by only processing flours obtained from "hypoallergenic" cultivars. Recently, methods for the direct chemical characterization of proteins separated by 2-D PAGE have become available [4]. The proteins are electroblotted from the 2-D PAGE gel onto an inert support matrix which can be directly applied to an automated protein sequencer. This technique allows the direct determination of amino acid sequences of proteins of interest. Because of its improved reproducibility [5] and higher protein loading capacity in comparison to traditional 2-D electrophoresis techniques [6], IPG-DALT is the method of choice for

micropreparative isolation of proteins from 2-D gels. In the present paper, IPG-DALT was optimized for the micropreparative isolation of high sample loads of wheat seed proteins. Our objective was to exploit the micropreparative capacity of IPG-DALT for the characterization of IgE binding proteins by amino acid microsequence analysis.

2 Materials and methods

Wheat seeds of the cultivar Fregatt (450 mg) were crushed in a mortar with a pestle. The flour was then transferred to an Eppendorf centrifuge tube. Salt-soluble wheat proteins were extracted with 1.0 mL of buffer (0.8% w/v Pharmalyte pH 3–10) for 30 min (4°C) with occasional vortexing. Afterwards, the sample was centrifuged for 1 h at 40000 g. The clear supernatant was removed, diluted with an identical volume of solubilization buffer (9 M urea, 0.8% w/v carrier ampholytes, pH 3–10, 1% dithiothreitol and 2% CHAPS) and stored at –78°C until use. IPG-DALT was carried out according to Görg *et al.* [1, 7] with minor modifications. IPG gels with linear gradients pH 4–8 (4%T, 4%C, size 250 × 180 × 0.5 mm³) were cast as described [8, 9]. 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, side by side, onto the cooling plate of a flatbed focusing chamber. A sample volume of 100 µL was applied near the anode with the help of sample application cups (Pharmacia DryStrip kit) placed onto the surface of the IPG gel strips. When 2-D PAGE was followed by electroblotting and microsequencing, about 500 µg of protein was loaded onto each IPG gel strip; for analytical runs 60–80 µg was applied. IEF was performed for 60000 Vh

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

Keywords: Bakers' asthma / Wheat grain allergens / Two-dimensional polyacrylamide gel electrophoresis / Immobilized pH gradients / Protein sequencing

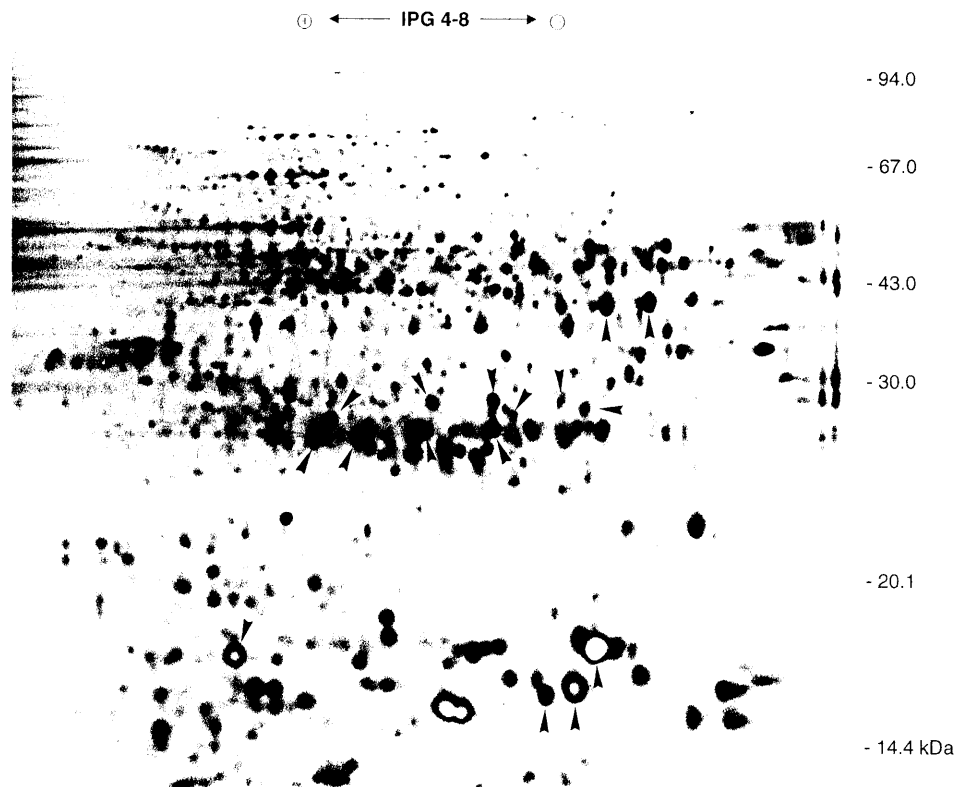


Figure 1. IPG-DALT of salt-soluble wheat seed proteins. First dimension: IPG 4–8. Second dimension: SDS-PAGE, 13% T. Silver stain. Major wheat grain allergens identified by 2-D immunoblotting are labeled with arrows.

at 20°C [10] for both analytical and micropreparative runs. Following IEF, the IPG gel strips were immediately stored in a plastic bag at –78°C. Prior to SDS-electrophoresis, the IPG gel strips were equilibrated for 2×10 min in 2×10 mL equilibration solution (0.05 M Tris-HCl buffer, pH 8.8, containing 6 M urea, 30% w/v glycerol, 2% w/v SDS, and a trace of bromophenol blue). DTT (1% w/v) was added to the first equilibration solution and iodoacetamide, 250 mM, to the second. Second-dimensional SDS-PAGE (13% T, 2.6% T) was carried out simultaneously on 10 slab gels ($210 \times 185 \times 1.0$ mm³) in a DALT tank [11]. Proteins of analytical 2-D gels were silver stained according to Blum *et al.* [12], whereas micropreparative 2-D gels were equilibrated in 50 mM Tris base/50 mM boric acid, pH 8.5 (blotting buffer) for 10 min [13] and electroblotted onto PVDF-membranes (Fluoro Trans[®]) at 1 mA/cm² for 1 1/2 h. For immunostaining, the 2-D blots were incubated with pooled sera of four asthmatic bakers, and IgE binding was demonstrated by alkaline phosphatase conjugated anti-human IgE. Next, the major IgE binding polypeptides were subjected to N-terminal amino acid sequence anal-

ysis. For this, the blotted proteins were visualized with Coomassie Brilliant Blue R-250 stain (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 directly into the New Blot cartridge[™] from Applied Biosystems (ABI) fitted to an ABI Model 477A Pulsed-Liquid Protein Sequencer equipped with an on-line phenylthiohydantoin (PTH) amino acid analyzer (Model 120A). Routinely, ten to fifteen Edman degradation cycles were performed for each spot. The partial protein sequences were compared for homologies to already known proteins using the BLAST algorithm [14].

3 Results and discussion

Micropreparative IPG-DALT was optimized for the separation of the albumin/globulin protein fraction of whole meal flour, since these polypeptides exhibited the highest percentage of IgE binding (~75%) compared to



Figure 2. Salt-soluble wheat seed proteins separated by IPG-DALT (cf. Fig. 1) and electroblotted onto a PVDF membrane. Coomassie stain. Major wheat grain allergens identified by 2-D immunoblotting are labeled with arrows and N-terminal sequenced allergens are indicated by numbers.

the gliadin and glutenin protein fraction, respectively [2]. Figure 1 shows the silver-stained 2-D pattern of salt-soluble wheat seed proteins (analytical gel) and Fig. 2 illustrates the Coomassie Brilliant Blue-stained albumin/globulins of a micropreparative 2-D gel electroblotted onto a PVDF membrane. Both the analytical and the micropreparative 2-D analyses were highly reproducible and showed excellent resolved spots without streaking. The 2-D gels and 2-D blots were digitized and subjected to computer analysis using the PDQUEST software package, resulting in the detection of about 670 polypeptide spots on the silver-stained gel. The most abundant 150 spots were also detectable on the Coomassie-stained 2-D blots. Immunoblotting experiments revealed that eleven of the major IgE binding polypeptides were located in the 27 kDa and two in the 37 kDa region of the 2-D immunoblots, respectively. Furthermore, four protein spots belonging to the 14–18 kDa region exhibited significant levels of IgE reactivity (see Fig. 3). While some of these low molecular mass allergens (14–18 kDa) may be members of the α -amylase inhibitor family [15–18],

nothing is known about the nature of the allergens located in both the 27 kDa and 37 kDa gel region. In the present paper, six out of eleven polypeptides (numbered 1–6 in Fig. 2) exhibiting strong IgE reactivity in the 27 kDa area were excised from the 2-D blots and subjected to N-terminal amino acid sequence analysis. The results of N-terminal sequencing are presented in Table 1. While no significant homologies with previously described proteins were found for protein spots 1, 4, 5 and 6, polypeptides 2 and 3 are obviously related to several Acyl-CoA oxidase variants of barley and rice. The N-terminal amino acid sequences of the remaining 27 kDa allergens could not be determined because the amount of protein present on the 2-D blots was probably not sufficient for sequence analysis.

Again, no sequence information on allergen 9 (37 kDa area) could be gained, probably because of a blocked amino terminus. In order to better characterize and identify this allergen, deblocking of the N-terminal amino acid and/or internal sequence analyses will have to be



Figure 3. Salt-soluble wheat seed allergens separated by IPG-DALT (cf. Fig. 1) and electroblotted onto a PVDF membrane. Immunostaining with patients' sera and secondary alkaline phosphatase labeled anti-human IgE.

performed. Internal sequences may also yield better homologies with proteins already described.

The N-terminal amino acid sequences of the two analyzed low molecular mass (14–18 kDa) allergens (spots 7 and 8 in Fig. 2) revealed a very high degree of homology with wheat monomeric α -amylase inhibitor 1 (WMAI-1). This finding is in accordance with previously published reports from other authors [15–18].

4 Concluding remarks

Our knowledge on the molecular characteristics of nutritive and/or inhalative allergens has been extended enormously during the past few years. In particular, the application of electrophoretic separation and blotting techniques enabled the identification and characterization of allergens, as well as did techniques for the determination of the primary and tertiary structures of particular allergens.

However, the techniques applied until now for the purification and isolation of allergens, e.g. salt precipitation or

column chromatography, are relatively ineffective and/or time-consuming and labor-intensive. On the other hand, with the technique presented in this paper, it is possible to isolate simultaneously dozens of different allergens for amino acid sequence analysis in a very short period of time.

The major objective of our study was to isolate some of the major wheat flour allergens by means of micro-preparative IPG-Dalt and protein blotting, and to identify and characterize these allergens by N-terminal amino acid sequence analysis. Our results revealed that those allergens which could be identified by protein database searching are either enzymes or enzyme inhibitors. Whether there exists a relationship between the enzymatic and/or enzyme-inhibitory activity, and the allergenicity of proteins, is not clear at the moment. However, several authors suppose that allergens which exhibit enzymatic [19] or enzyme-inhibitory activity may more easily penetrate mucous membrane barriers and trigger allergic reactions [20, 21]. Our present results seem to support this hypothesis; however, further investigations are required to fully answer this question.

Table 1. N-terminal sequence analysis of IgE binding polypeptides (indicated by arrows in Fig. 2 and 3) involved in bakers' asthma^{a)}

Polypeptide No.	N-terminal amino acid sequence	Homology	SWISS-PROT accession number
1	GDVATGRLpE	none	
2	PGLAIGDTVANLEV	Acyl-CoA oxidase	HVB1SC_1
3	PGLTIGEAVPNL	Acyl-CoA oxidase	HVB1SC_1
4	GDVAXGRGSEKVVHgap	none	-
5	DGLsKQDNVP	none	-
6	KRKFIYXXNss	none	-
7	SPGWMYPTQAAIV	α -amylase inhibitor	P01085
8	SPGWMXYPGQAFQV	α -amylase inhibitor	P01085
9	(blocked)	-	-

a) Gaps in the sequence are denoted by X and residues which are unclear are written in small letters.

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