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ISOLATION AND PURIFICATION OF INDIVIDUAL GLIADIN PROTEINS BY PREPARATIVE ACID POLYACRYLAMIDE GEL ELECTROPHORESIS (A-PAGE) FOR ALLERGENIC RESEARCH*

Jacek Waga, Jerzy Zientarski

Department of Cereals Breeding and Quality Evaluation, Plant Breeding and Acclimatisation Institute, Cracow

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Gliadins are a large and complex group of proteins, consisting of dozens to hundreds of unique polypeptides in any wheat cultivar. Individual gliadins differ in molecular weight, isoelectric point, and amino acid sequences, making their analyses especially difficult. We here used preparative polyacrylamide gel electrophoresis in acidic conditions to fractionate gliadins of the cultivar Fraza. Resulting fractions were then analysed by ELISA to test their immunoreactive properties with antigliadin polyclonal antibodies. Preparative electrophoresis yielded 60 aliquots, of which 15 contained single proteins. Separated gliadins differed in immunoreactive properties: antigliadin antibodies bound stronger to α and β gliadins, while the immunoenzymatic reaction with γ and ω-gliadins was much weaker.

INTRODUCTION

Wheat gliadin proteins are important structural elements of the gluten network, considerably affecting flour baking quality and having an important role in human nutrition. However, to persons sensitive to gluten, gliadins are strong allergens, and for those suffering from celiac disease, gliadins are toxic. This is a serious problem related to food safety and quality.

In recent years, researchers have attempted to explain the biochemical and molecular bases of these unfavourable properties, and to develop strategies for their limitation or elimination [Mc Mowat, 2003; Waga, 2004]. In such research, it is important to select adequate methods which enable correct evaluation of gliadin allergenicity [Ebo & Stevens, 2001]. The most popular methods are ELISA and immunoblotting after electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS PAGE) [Battais *et al.*, 2003; Lehto *et al.*, 2003]. These applications provide important information for both the medical sciences and for cereal chemistry. Results can evaluate disease development by determining the content of specific antigliadin antibodies in patients' serum. These methods also show immunoaffinity of allergenic protein fractions to serum antibodies, and reveal the relationship between structural properties and allergenicity.

Numerous studies confirm the allergenicity of the gliadin complex [Vainio *et al.*, 1983; Varjonen *et al.*, 1995, 1997; Maruyama *et al.*, 1998]. However, few studies have analyzed allergenic properties of individual gliadin proteins. Many gliadins differ in physicochemical properties, as shown by electrophoresis and HPLC [Bietz & Waga, 1997; Waga & Węgrzyn, 2000]. In any cultivar, one-dimensional electrophoresis may separate several dozens of unique gliadins, while two-dimensional electrophoresis may reveal several hundreds of gluten proteins differing in molecular weight, isoelectric point, and amino acid sequence [Shewry & Lookhart, 2003].

Due to considerable polymorphism of gliadins, electrophoresis is commonly used to identify wheat cultivars and lines [Bietz & Huebner, 1994]. This differentiation is possible because of the way that gliadins are inherited. They are coded by six *loci* on chromosomes of the 1st and 6th homeological groups [Gianibelli *et al.*, 2001]. Each locus consists of several strongly linked genes (the so-called "gene clusters") which form a series of multiple alleles. Each locus may have several or even several dozens of variants. Individual alleles control synthesis of one to six gliadins, revealed as groups of bands called protein blocks upon electrophoresis [Sozinov & Poperella, 1980].

Gliadin polymorphism probably also relates to and differentiates allergenic properties of wheat. The ω-5 group of gliadins and 65 kDa γ-gliadins are important allergens in wheat-dependent exercise induced anaphylaxis, while a 14 kDa α-gliadin induces atopic dermatitis [Palosuo *et al.*, 1999; 2001a,b; Varjonen *et al.*, 2000]. It is also well-known that A-gliadins (a specific group of α-gliadins coded by the chromosome *6A*) are the most toxic in celiac disease [Kasarda, 1994]. These facts suggest that biochemical and immunological analyses of purified gliadins could contribute to better knowledge of allergenic properties of the whole complex.

We thus need good methods to isolate and analyze individual gliadins. One of such methods is preparative electrophoresis in an acidic medium [Howes & Kosmolak, 1982; Rumbo *et al.*, 1999, 2000], which enables the collection of isolated native

Author's address for correspondence: dr Jacek Waga, Department of Cereals Breeding and Quality Evaluation, Plant Breeding and Acclimatisation Institute, ul. Zawiła 4, 30-423 Kraków, Poland; tel.: (48 12) 266 57 00; fax: (48 12) 266 59 48; e-mail: zhwaga@cyf-kr.edu.pl

proteins. In contrast, gliadins isolated by electrophoresis in the presence of sodium dodecyl sulfate, which eliminates most non-covalent protein-protein interactions, can have altered allergenic properties [Simonato *et al.*, 2001].

In this study, we fractionated the gliadin complex by a modified preparative PAGE method in acidic medium, and assessed the isolated fractions by ELISA to determine whether they differed in immunoreactive properties.

MATERIAL AND METHODS

Extraction of gliadins. Gliadins of the winter wheat cultivar Fraza were extracted from flour with 70% ethanol (1:10 w/v). Test-tubes containing flour plus ethanol were gently shaken overnight at room temperature. Extracts were then centrifuged for 10 min at 12.500 × *g*. Supernatant was then used for analytic and preparative electrophoreses and for ELISA.

Preparative electrophoresis (A PAGE). Preparative acid polyacrylamide gel electrophoresis (A-PAGE) in lactate aluminium buffer (pH=3.1) was used to isolate and purify gliadin fractions. This method was based on the analytical method of Bushuk & Zillman [1978]. Separations were performed in a Model 491 PrepCell (BioRad, USA). A preparative column (diameter 37 mm, height 10 cm) was filled with polyacrylamide gel, in which the total concentration of monomers (T) was 8% (w/v), and the concentration of crosslinker (C) was 0.29% (w/v). The strongly exothermic polymerization required intensive cooling of the column during gel preparation. For separations, 6 mL of a mixture of gliadin extract and saturated sucrose solution in lactate aluminium buffer was overlaid on the gel surface. Separations were carried out at a constant voltage (U=500 V) and current (I=50 mA). Sixty 7-mL fractions containing gliadins were collected beginning five hours after the start of electrophoresis using pH 3.1 aluminium lactate buffer as an eluent. The separation was completed 12 h after the onset of fraction collection.

Analytical electrophoresis (A PAGE). The purification of gliadin fractions from preparative electrophoresis was evaluated by modified analytical A-PAGE in aluminium lactate buffer, pH=3.1 [Bushuk & Zillman, 1978]. Separated aliquots (120 μL) mixed with a sucrose solution (as in preparative electrophoresis) were introduced into wells in gel slabs composed of T=8% (w/v) and C=0.29% (w/v). Separations were done in a Desaphor electrophoretic chamber (Desaga GmbH, Germany) for about 4 h at a constant voltage (U=500 V) and current (I=80 mA). Gels were stained in Coomassie Brilliant Blue (0.026%), methanol (17%) and trichloroacetic acid (5%) solution overnight, and destained in distilled water.

ELISA. Immunoreactive properties of gliadin fractions were determined by the "direct ELISA" method. For analysis, 16 aliquots were selected, representing α, β, γ and ω-gliadins, at a protein concentration of 500 μg/mL. Wells of the microtitration plate were coated overnight at 4°C with an aliquot solution (100 μL) diluted with 0.05 mol/L carbonate buffer, pH=9.6. Each sample was analysed in eight replications. The next day, the plates were blocked with a 1% solution of foetal calf serum (FCS) for 2 h at room temperature, and then incubated with a conjugate (a solution of rabbit polyclonal antigliadin antibodies linked with horseradish peroxidase [AGA-HRP – Sigma Co., USA], diluted at 1:1000) for 1.5 h at 37°C. After each stage, the plates were rinsed with a buffered sodium chloride solution containing 0.05% Tween 20 (PBST). The substrate used for HRP was "ready to use" orthophenylenediamine (OPD) (Sigma Co., USA), dissolved in distilled water according to manufacturer's instructions. After 20 min, the reaction was stopped by addition of 0.5 mol/L H_2SO_4. The optical density (OD) of the immunoenzymatic reaction product was read with an ELISA test reader (Opsys MR, Thermolab System, USA). Variations in OD values among fractions were accepted as the indicators of gliadin immunoreactive properties.

RESULTS AND DISCUSSION

The gliadin A PAGE pattern of the wheat cultivar Fraza consists of 20 bands coded by chromosomes *1A, 1B, 1D. 6A, 6B* and *6D* (Figure 1). Groups of bands forming gliadin blocks were described by symbols used to identify winter wheats according to the catalogue of the Plant Breeding and Acclimatization Institute (PBAI) in Cracow [Waga, 2000]. Identified blocks were noted as electrophoretic formulae, which for the cultivar Fraza is:

Gli A1 – 1, *Gli B1* – 1, *Gli D1* – 1.1, *Gli A2* – 1.1, *Gli B2* – 1, *Gli D2* – 2.1

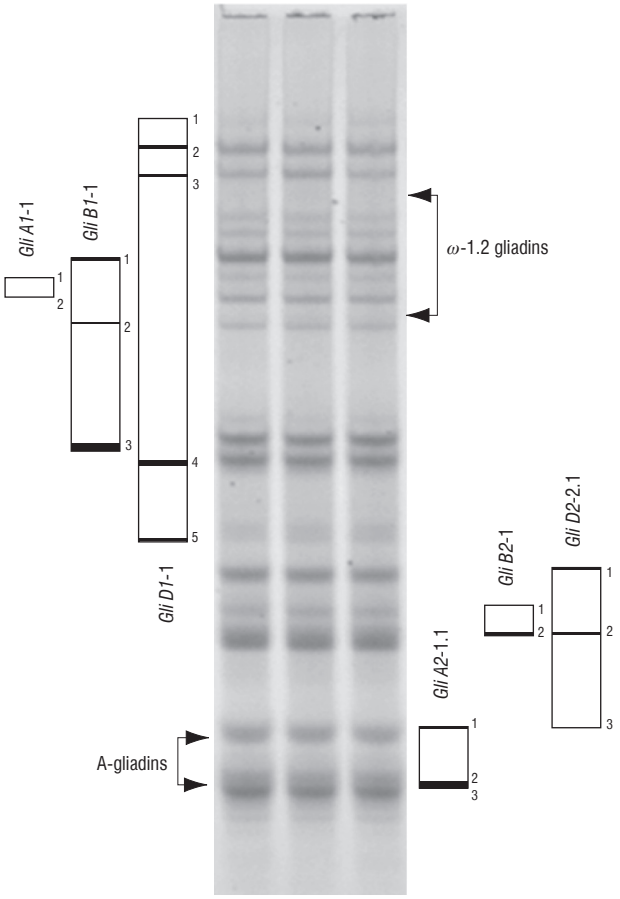

FIGURE 1. Gliadin protein blocks of winter wheat cultivar Fraza. Each band among the block is described by the Roman number. The most toxic proteins in celiac, so called A-gliadins, as well as ω-1.2 gliadins are marked by the arrows.

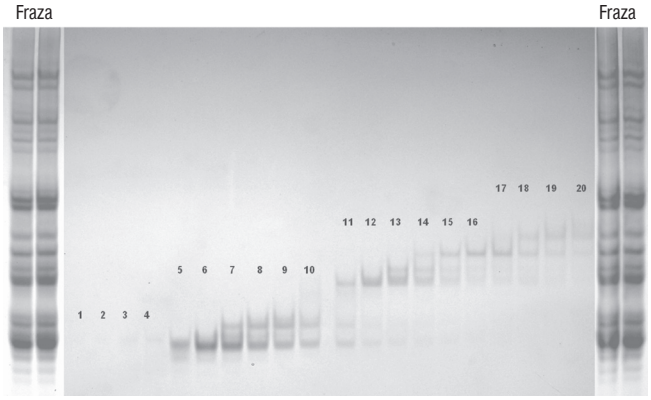

FIGURE 2a. Electrphoregrams of gliadin proteins of the winter wheat cultivar Fraza separated by preparative A PAGE electrophoresis, aliquots 1–20.

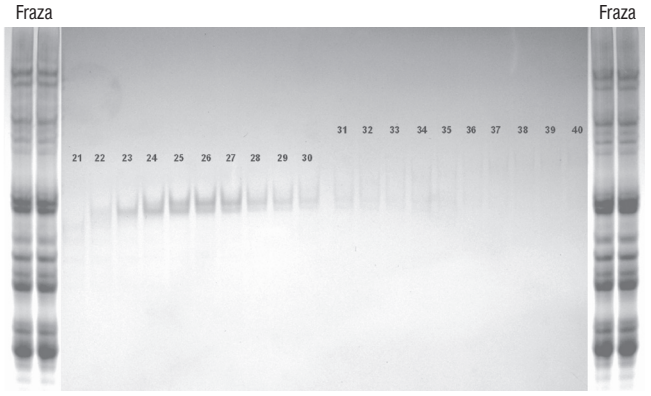

FIGURE 2b. Electrphoregrams of gliadin proteins of the winter wheat cultivar Fraza separated by preparative A PAGE electrophoresis, aliquots 21–40.

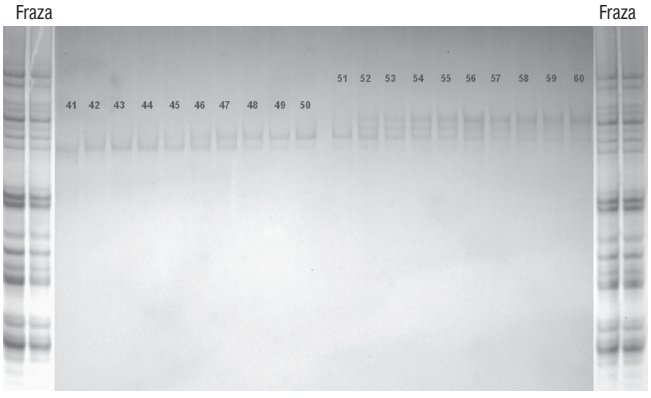

FIGURE 2c. Electrphoregrams of gliadin proteins of the winter wheat cultivar Fraza separated by preparative A PAGE electrophoresis, aliquots 41–60.

In this formula, *Gli* denotes gliadin; *A1, B1, D1, A2, B2* and *D2* are the symbols of the chromosomes *1A, 1B, 1D, 6A 6B* and *6D*; and the number following the chromosome symbol is the block catalogue number.

Fraza gliadin was fractionated by preparative A-PAGE into 60 aliquots, each containing 1–3 gliadin bands as revealed by analytical A-PAGE (Figure 2 a, b, c). Results reveal that preparative electrophoresis separated all Fraza gliadins except for the highest molecular weight ω-gliadins. Purification of these proteins requires small changes in methodology, such as reducing the gel column length, as shown by Rumbo *et al*. [1999].

Aliquots 5–10 contain the highest-mobility α-gliadins, coded by chromosomes *6A* and *6D*. In Fraza, α-gliadins contain A-gliadin (block *Gli A2*-1.1, bands 1, 2 and 3). Preparative A PAGE made it possible to isolate one (No. 2) of the two closely located bands (No. 2 and 3) of this block (aliquots 5 and 6). They were separated from the third A-gliadin band (No. 1). It interferes to the other band from the α group which, being coded by chromosome *6D,* was not an A-gliadin (Figure 2a).

Aliquots 11–21 from preparative A PAGE include β gliadins. Two aliquots (12 and 16) contained individual gliadins coded by chromosomes *6B* and *6D.*

In Fraza, γ-gliadins consist of two bands having medium electrophoretic mobilities; one (of lower mobility) is coded by chromosome *1B*, while the other (of higher mobility) is coded by chromosome *1D.* Gamma gliadins were found in aliquots 22–35, with near-homogeneous polypeptides in aliquots 23, 24 and 30.

Aliquots 35–40 contained no proteins, in accordance with analytical A PAGE of Fraza (Figure 2b), which shows no gliadin bands in a board zone between γ- and ω-gliadins.

Aliquots 40–60 contained four proteins corresponding to lower mobility ω-gliadins, marked with the symbol ω-1.2 [Wieser, 1991]. Two of the four bands in the ω-1.2 group (block *Gli B1*-1, bands 1 and 2) are coded by chromosome *1B,* and two (block *Gli A1*-1, bands 1 and 2) by chromosome *1A.* Nearly pure proteins occurred in aliquots 43–46 and 51––54 (Figure 2c).

A total of 15 aliquots from preparative A PAGE contained nearly homogeneous gliadin proteins. These aliquots were numbers 6, 12, 16, 23, 29, 30, 41, 42, 48, 49, 50, 51, 58, 59 and 60. Other aliquots contained two to three proteins.

ELISA test results showed good OD repeatability for the analysed samples: the coefficient of variability ranged from

TABLE 1. Characterization of chosen gliadin fractions analysed with the ELISA.

Fraction No.	Gliadin group	Gliadin block (blocks)	Band No. (s)	OD (at 490 nm)	CV (%)
5	α	A2-1.1	2, 3	3.353	4.19
6	α	A2-1.1	2	3.454	0.93
9	α	A2-1.1/D2-2.1	1, 3	3.461	1.16
10	α	A2-1.1	1, 3	3.502	0.34
12	β	B2-1/D2-2.1	2, 2	2.495	2.56
16	β	D2-2.1	1	2.435	1.07
23	γ	D1-1	4	1.844	5.49
25	γ	D1-1/B1-1	4, 3	1.177	2.52
29	γ	B1-1	3	1.026	8.47
30	γ	B1-1	3	0.851	3.29
41	ω	B1-1	2	1.346	2.06
42	ω	B1-1	2	1.326	1.35
48	ω	A1-1	2	1.041	1.15
50	ω	A1-1	2	1.083	0.94
58	ω	B1-1	1	1.541	0.93
59	ω	B1-1	1	1.581	0.37

OD – optical density (mean values from eight repetion); CV (%) – coefficient of variability for OD (%)

0.34% to 5.49% (except for sample 29, were it was 8.47%) (Table 1). Considerable differences among OD values for the analysed fractions occurred, indicating differences in gliadins' immunoreactivity. Highest OD values were found for α and β gliadins; the highest value was for 3 α-gliadins coded by chromosomes *6A* and *6D* (aliquot 10). Values for γ- and ω-gliadins were much lower, the lowest being for a γ-gliadin of medium mobility coded by chromosome *1D*.

Isolation and purification of gliadins is much more difficult than that of many other protein groups, posing serious problems to cereal chemists. This difficulty is due to gliadins' high level of complexity, polymorphism, and unique solubility characteristics [Wieser, 1991]. Moreover, gliadins may interact with each other and with other gluten and non-gluten proteins and other grain components during dough formation, further increasing their complexity [Bietz, 1979]. Different solvents may also affect aggregation [Weegels *et al.,* 1995]. Thus, purifying individual gliadins to study their functional properties is a complicated task, also affecting research on gliadins' allergenicity.

In this study, gliadins from the wheat cultivar Fraza were fractionated by preparative A PAGE. While this application is not commonly used to purify gliadins, it offers the possibility of obtaining native proteins, since structure and functionality have not been significantly altered by extraction and separation conditions. Rumbo *et al.* [1999] obtained groups of α, β, γ and ω-gliadins using apparatus and separation method much the same in our research. Howes &Kosmolak [1982] isolated one gliadin ("band 50") from the cultivar Marquis-K in sufficient quantity for biochemical characterization. However, their procedure was more complex, including an initial separation by size exclusion chromatography on Sephacryl G200. Our research yielded 15 aliquots containing near-homogeneous gliadins, suggesting, that the modified preparative separation method we used is an effective way of isolating gliadins for further analyses.

ELISA showed different immunoreactive properties for purified gliadins; this characteristic is potentially related to allergenicity and toxicity. Since protein content of all analysed fractions was 500 μg/mL, the possibility that differences in reactivity were due to quantitative factors was eliminated. The resulting differences in reactivity among protein fractions were considerable – immunoreactivity for α and β-gliadins was much higher than for γ and ω gliadins. These observations are consistent with findings of studies on gliadin toxicity in celiac disease, which show that low-molecular weight α-gliadins are most toxic [Kasarda, 1994]. This suggests the existence of a possible relationship between immunoreactivity and toxicity of separated gliadins. Further studies of more purified gliadins from different wheat genotypes will make it possible to confirm this relationship.

CONCLUSIONS

1. Preparative acid electrophoresis (A PAGE) enabled the isolation of several highly purified gliadins, including A-gliadin, the protein especially toxic in celiac disease.

2. Purified gliadins differ in immunoreactivity. α and β gliadins bind more strongly to antigliadin antibodies than γ and ω-gliadins.

3. Results show that preparative A-PAGE is an effective method for purifying gliadins for further studies on their immunoreactive and allergenic properties.

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IZOLOWANIE I OCZYSZCZANIE ALERGIZUJĄCYCH BIAŁEK GLIADYNOWYCH METODĄ ELEKTROFOREZY PREPARATYWNEJ (A-PAGE)

Jacek Waga, Jerzy Zientarski

Zakład Oceny Jakości i Metod Hodowli Zbóż, Instytut Hodowli i Aklimatyzacji Roślin, Kraków

Gliadyny stanowią rozbudowany kompleks białkowy, który – zależnie od odmiany pszenicy – tworzy od kilkudziesięciu do kilkuset alergizujących monomerów. Poszczególne monomery różnią się masą cząsteczkową, punktem izoelektrycznym, a przede wszystkim sekwencjami aminokwasów, co znacznie utrudnienia analizę właściwości alergogennych. W niniejszej pracy wykorzystano zmodyfikowaną metodę elektroforezy preparatywnej na żelu poliakryloamidowym w środowisku kwaśnym do rozdziału gliadyn pszenicy ozimej Fraza. Oczyszczone białka analizowano następnie pod względem właściwości immunoreaktywnych testem ELISA w reakcji z poliklonalnymi przeciwciałami antygliadynowymi. Uzyskano 60 frakcji, z których 15 zawierało pojedyncze monomery. Stwierdzono zróżnicowanie immunoreaktywności rozdzielonych białek – przeciwciała antygliadynowe silnie wiązały gliadyny α i β natomiast reaktywność γ i ω gliadyn była znacznie niższa.