A Modified Polyacrylamide Gel Method for Assessing the Extraction of Gliadins

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Several methods for the extraction of wheat prolamines have been compared for their rapidity, ease, yield, and the quality of the extracted product (purity, total nitrogen). A polyacrylamide gel electrophoresis method has been developed for the control and analysis of gliadins.

Prolamines were first defined by Osborne (1907) and can be extracted by relatively concentrated alcoholic solutions. Among the various extraction methods proposed for wheat gliadins, column percolation is the oldest (Huebner and Rothfus, 1968; Ninot et al., 1969; Charbonnier, 1973). These long and fastidious methods are now seldom used. Methods for routine identification of wheat varieties are simple and rapid, the extract being used directly without any precipitation or lyophilization (Autran, 1975). However, these steps become necessary when more detailed studies on gliadins are carried out.

Charbonnier (1970, 1982) reported that the best results were obtained by stirring the ground wheat in 70% (v/v) ethanol after lipid removal or in 75% (v/v) ethanol without prior lipid removal. Patey and Evans (1973) and Autran et al. (1979) added a dialysis step at the end of extraction and monitored the extracted products by electrophoresis.

Starch gel electrophoresis, the initial method developed, was used for a long time both for varietal identification (Woychick et al., 1961; Autran, 1975; Autran and Bourdet, 1975; Branlard and Rousset, 1980; Clydesdale et al., 1982) and for more detailed biochemical studies (Charbonnier, 1982). The numerous disadvantages of this method have been discussed by Autran (1984).

Polyacrylamide gel electrophoresis (PAGE) is being used more often (Bushuk and Zillman, 1978; Autran et al., 1979; Tkachuk and Metlish, 1980; Wrigley et al., 1982; Khan, 1982; Khan et al., 1983, 1985). The major disadvantage of this method is the toxicity of acrylamide, but it has many compensating advantages in comparison to starch gel (Autran, 1984). Thus, the gel is easy to prepare, migration times are shorter, and resolution and reproducibility are better. Several problems remain with most existing methods, especially the lack of standardization of the material used and a number of analytical problems.

The aim of this present work was to compare the methods used for the extraction and control of gliadins. A modified electrophoretic method is proposed.

MATERIALS AND METHODS

Plant Materials. Wheat seeds (Cornette) were obtained from M. Desprez (Cappelle, France). Wheat flour (Festival) was obtained from Grands Moulins de Paris (France).

Chemicals: polyvinylpyrrolidone (PVP), Hopkin and Williams Searle Co., London, England, at high concentration in water; acrylamide and *N,N'*-methylenebisacrylamide, Eastman Kodak Co., Rochester, NY; Coomassie brillant blue R-250, Fluka, Buchs, Switzerland; other products, Prolabo, Paris, France.

Extraction Procedures. Wheat prolamines (ground seeds and flour) were extracted without lipid removal as described by Charbonnier (1982) with 75% (v/v) ethanol at 25 °C or 50% (v/v) ethanol or 25% (v/v) 2-chloroethanol. Wheat seeds and flour were also extracted with the method described by Autran et al. (1979). The final product was dissolved in 0.01 M acetic acid and was dialyzed thoroughly against distilled water. The prolamines obtained were freeze-dried, sometimes after a concentration step in PVP (for the dialyzed products), and stored at −20 °C. Total nitrogen was assayed by the Kjeldahl method. Protein digestion was made in H_2SO_4 with Se–Cu catalyst (40 g of $CuSO_4$, 11.2 g of SeO_2). Assays were performed in Parnas and Wagner apparatus: distilled NH_3 was assayed by acid titrimetry.

Procedure for Vertical Polyacrylamide Gel Electrophoresis (PAGE). *I. Aluminum Lactate Buffer (pH 3.1) (Bushuk and Zillman, 1978; Autran, 1984).* Gel solution: 75 g of acrylamide, 3.75 g of *N,N'*-methylenebisacrylamide, 1.0–1.5 g of ascorbic acid, 0.025 g of ferrous sulfate, aluminum lactate buffer (pH 3.1) q.s. 1000 mL. This solution can be stored at 4 °C for a long time but must be filtered before use. Catalyst: 3% H_2O_2 in water freshly prepared and filtered before use. Aluminum lactate buffer: stock solution of 50 g of aluminum lactate/L of distilled water, stored at 4 °C, and filtered before use. The buffer was prepared by diluting 250 mL of stock solution with 500 mL of distilled water and adjusting to pH 3.1 with lactic acid. The solution was labile and must be used immediately after preparation.

Sample dissolution: aluminum lactate buffer (pH 3.1)/60% glycerol/98% ethanol (2/1/1, v/v/v).

Stain: stock solution of 5 g of Coomassie brillant blue R-250, 500 mL of methanol, 100 mL of glacial acetic acid, and 400 mL of distilled water, stored at room temperature. Staining solution discarded after use: 5 mL of stock solution, 40 mL of methanol, 10 mL of glacial acetic acid, 45 mL of distilled water. Destaining solution: 200 mL of methanol, 100 mL of glacial acetic acid, 680 mL of distilled water, 20 mL of glycerol. The destaining solution was stored at room temperature and could be reused after filtering through activated vegetal charcoal.

Procedure. Two glass-plates (18 × 20 cm) were carefully washed with detergent, then rinsed out successively with water, distilled water, and ethanol, dried, and cleaned with acetone. The two plates and a U-gasket (silicon, 1.5-mm thick × 5-mm width) were clamped together to form a mold that was put into crushed ice. Gel and 3% H_2O_2 must be cooled to avoid a quick polymerization. Then, 3% H_2O_2 was added to the gel (30 μL for 80 mL), and the solution was quickly put into the mold. A slot former was placed at the top of the gel between the two

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Table I. Composition of White and Yellow Fractions Extracted following Autran et al. (1979) from Festival Flour

	white	yellow
C, %	49.21	57.88
H, %	7.03	8.37
O, %	25.79	21.44
N, %	15.72	9.49
S, %	1.35	0.63
fatty acid	trace	trace
sugar	trace	trace
color	0	flavonol?

plates. After polymerization, slot former and U-gasket were drawn out and two lateral gaskets were placed. Two pieces of scotch kept the plates together. Methyl green and methyl green coupled with albumin were used to follow the migration.

Conditions: Pharmacia GE 2/4 LS tank; cooling system with temperature at the start 15 °C and 25 °C at the end of migration; LKB 2103 voltmeter–amperemeter, 500 V, 100-mA current generator. Electrophoresis lasted 4.5–5 h, and gels were stained for 1.5–2 h and destained for 24–48 h. The gel was finally rinsed with distilled water and could be stored in the same liquid for several days.

II. Sodium Lactate Buffer (pH 3.2) (Bushuk and Zillman, 1978; Tkachuk and Metlish, 1980; Huebner and Bietz, 1984; Maier and Wagner, 1980). Gel: 60 g of acrylamide, 3 g of *N,N'*-methylenebisacrylamide, 0.017 g of ferrous sulfate, 0.53 g of ascorbic acid dissolved in 1 L of sodium lactate buffer (pH 3.2). The solution was stored at 4 °C and filtered before use. Catalyst: 1.7 g of ammonium persulfate dissolved in 100 mL of distilled water. It was freshly prepared, filtered before use, and used in the proportion of 1 mL/100 mL of gel. Sodium lactate buffer (Mac Gibbon and Cross, 1982): 0.85 g of sodium hydroxide dissolved in 200 mL of distilled water. The pH was adjusted to 3.1 with lactic acid and the solution was diluted to 5000 mL with distilled water (pH 3.2). The buffer was freshly prepared before use and could not be reused.

Sample dissolution: sodium lactate buffer (pH 3.2)/60% glycerol/98% ethanol (2/1/1, v/v/v). Staining and destaining were as in the first method.

Procedure. It was not necessary to cool the plates or the gel; 700 V was obtained (100 mA at the beginning and 80 mA at the end of migration). Migration time was 2 h.

III. Gel Scanning. After staining and destaining, the gels could be scanned at 560 nm (Shimadzu CS-930 scanner).

RESULTS AND DISCUSSION

The products obtained after extraction and lyophilization with the technique of Charbonnier (1982) had several different characteristics: 25% (v/v) 2-chloroethanol yielded a powder comparable to the commercial products (yellowish, fine particle size, good brittleness); 50% (v/v) ethanol gave a paler product with large particles and good brittleness; 75% (v/v) ethanol gave products with intermediate characteristics. When the method of Autran et al. (1979) was used, two fractions were obtained, one yellow the other white. These two fractions were separated when the extracted product was dissolved in 0.01 M acetic acid. The yellow fraction, compact and not very hygroscopic after lyophilization, remained insoluble or precipitated in the dialysis tubes. This fraction was separated by centrifugation. The white fraction, soluble in 0.01 M acetic acid, flocculated and was very hygroscopic after lyophilizing. Both fractions were analyzed (Table I). It was found that the white fraction contained twice as much sulfur, more nitrogen, and a bit less carbon than the yellow fraction. Differences of the other elements were not very significant. These elementary microanalyses were carried out under inert gas by the Central Analysis Service (CNRS, Solaize, Rhône, France). C, H, and O were evaluated by IR after combustion, N by catharometry, and S by coulometry. Both fractions contained traces of fatty acids and sugars. The color in the yellow fraction was probably due to flavonol. Autran et al. (1979) observed that a small part of crude extracted gliadin precipitated during dialysis. The precipitate, which accounted for less than 5% of the total extract and was eliminated by centrifugation, was not analyzed in that work. To our knowledge, there is no other published report on this phenomenon.

In the present case, the precipitate accounted for 30% of the extracted product. This was initially attributed to the temperature difference between the dialysis water (4 °C) and the water added when the bath was changed (20–25 °C), but the situation was unchanged by the use of chilled water.

Table II lists the yields of the different extractions (mean of three) and total nitrogen (mean of four assays). Yields are expressed as grams of lyophilized product obtained per 100 g of flour or ground seeds. There were no main yield differences between the four extractions with 75% (v/v) ethanol (1.43–1.5 g/100 g). Lower yields were obtained with 50% (v/v) ethanol followed by 25% (v/v) 2-chloroethanol.

Charbonnier (1982) examined the factors that could influence the extraction of gliadins. Different alcohols were used, at varying concentrations and different temperatures. It was concluded that the best compromise between yield and purity of the extracted products was obtained by extracting with 75% (v/v) ethanol without prior lipid removal either at room temperature or at higher temperature. The crude gliadins obtained nevertheless still contained glutenins and albumins. We carried out our extractions at room temperature without lipid removal by using two extraction solutions besides 75% (v/v) ethanol for the purposes of comparison (2-chloroethanol was not tested by Charbonnier). The resulting yields (Table II) confirm that, among the three solutions used, the best yield was obtained with 75% (v/v) ethanol.

Total nitrogen is expressed per 100 g of dry weight of product. Gliadin extracted from Festival flour with 75% (v/v) ethanol according to Charbonnier (1982) was the richest in total nitrogen. The next richest were commercial gliadins (whose extraction procedure is not known) and then the gliadin extracted from Festival flour with 75% (v/v) ethanol according to Autran et al. (1979) (white fraction). The two yellow fractions extracted with 75% (v/v) ethanol from Cappelle seeds and Festival flour (Autran et al., 1979) were those containing the least total nitrogen. The levels in the other extraction products were intermediate.

Crude lyophilized gliadin is generally described as a white or very slightly yellow powder containing 17–17.5% total nitrogen (Autran et al., 1979; Charbonnier, 1973, 1982). Among the gliadins extracted, none included both these characters: they were all relatively yellow or beige, except the white fraction obtained with the method of Autran et al. (1979) after solubilization in 0.01 M acetic acid and dialysis. The only gliadin whose nitrogen content was equivalent to the theoretical value was that extracted from Festival flour with 75% (v/v) ethanol according to Charbonnier (1982). The nitrogen content in the white fraction was slightly lower than the theoretical value, but this was the only extracted product whose characteristics approached the definition of crude gliadins.

Crude gliadin may include impurities, such as albumins, glutenins, and occasionally lipids. Their proportions are variable as a function of the extraction tech-

Table II. Extraction Yield of Gliadins and Nitrogen Content of Different Extracts

			freeze-dried gliadin, g/100 g flour		total N, g/100 g freeze-dried gliadin
commercial products		Sigma			15.98
		Roth			16.15
extraction following Charbonnier (1982)	Cappelle seeds	50% (v/v) ethanol	2.72		15.30
		25% (v/v) 2-chloroethanol	1.50		14.68
		75% (v/v) ethanol	2.86		12.28
	Festival flour	75% (v/v) ethanol	3.00		17.05
extraction following Autran et al. (1979)	Cappelle seeds	75% (v/v) ethanol	white fraction	2.40	12.91
			yellow fraction	0.60	9.39
			total	3.00	
	Festival flour	75% (v/v) ethanol	white fraction	2.20	15.77
			yellow fraction	0.76	9.49
			total	2.96	

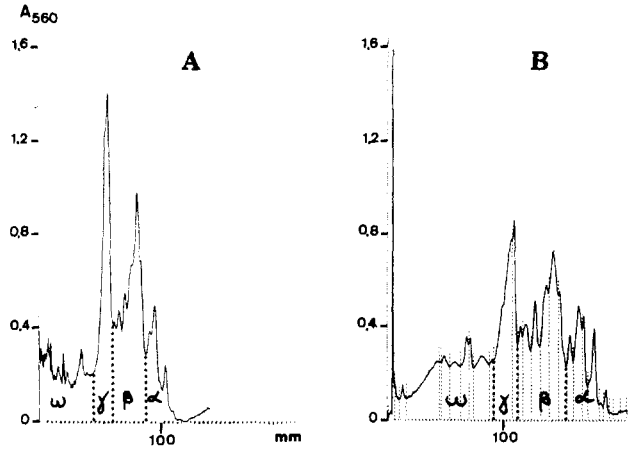

Figure 1. Scanner recordings of the electrophoregram of crude gliadin extracted from wheat flour with 75% (v/v) ethanol according to Charbonnier (1982): (A) PAGE in aluminum lactate buffer (pH 3.1); (B) PAGE in sodium lactate buffer (pH 3.2).

nique used (alcohol concentration, prior lipid removal, extraction temperature), the variety of wheat used, and the form in which it is used (flour or ground seeds). The percentage nitrogen can thus vary considerably, probably explaining the differences between the values obtained and the theoretical level. It should be noted in general that when flour is the starting material, the total nitrogen content of the gliadins is higher than when ground seeds are used.

Polyacrylamide gel electrophoresis in aluminum lactate buffer, recommended by Bushuk and Zillman (1978) and by Autran (1984), gave poor results in spite of attempts to modify the gel preparation (Figure 1A). The profiles obtained were not sharp and were incomplete. This method was difficult and long, requiring long migration times, and gave poor reproducibility. The main problems were above all due to the catalyst and the buffer (instability of the catalyst and difficulty in assay, electrolysis of the buffer with alumina deposit on the upper electrode, migration prevented or disturbed). In addition, scanner recordings could not be interpreted either qualitatively or quantitatively.

This technique was thus modified to a considerable extent. Part of the gel composition was taken from Tkachuk and Metlish (1980) (proportions of acrylamide and *N*,*N*-methylenebisacrylamide). The catalyst (H_2O_2) was replaced by the one indicated by Huebner and Bietz (1984) (ammonium persulfate), and the ascorbic acid and ferrous sulfate concentrations used were those recommended by Maier and Wagner (1980). The quality of the gel obtained was much higher, and it was easier to prepare. Khan (1982) and Khan et al. (1983, 1985) recommended replacing the buffer aluminum lactate by sodium lactate and bringing the pH to 3.2. This buffer

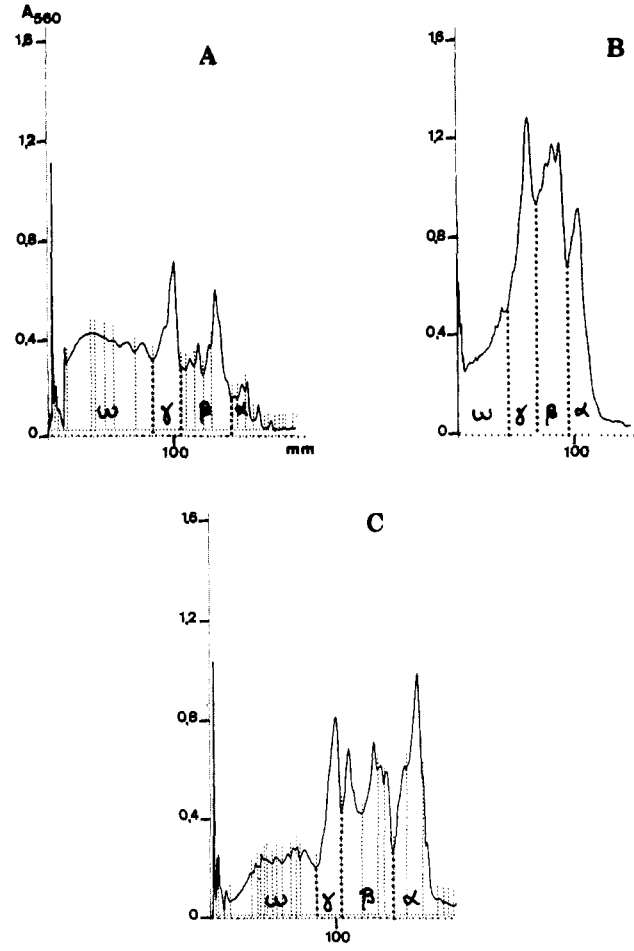

Figure 2. Scanner recordings of electrophoregrams: (A) crude gliadin extracted from wheat flour with 25% (v/v) 2-chloroethanol according to Charbonnier (1982), PAGE in sodium lactate buffer (pH 3.2); (B) commercial gliadin (Sigma), PAGE in sodium lactate buffer (pH 3.2); (C) white fraction extracted from wheat flour with 75% (v/v) ethanol according to Autran et al. (1979), PAGE in sodium lactate buffer (pH 3.2).

was used, prepared as described by Mac Gibbon and Cross (1982). It was thus possible to increase the voltage to 500–700 V and to reduce migration times by half. The scanner recordings are given in Figures 1B and 2A–C.

Gliadin extracted with 50% (v/v) ethanol results in trailing all along the profile. Gliadins extracted with 25% (v/v) 2-chloroethanol gave the same type of profile as did commercial gliadin (Sigma) (Figure 2A,B). In the case of the latter two products, most of the ω fraction was missing. The profile of gliadin extracted with 75% (v/v) ethanol according to Charbonnier (1982) included all the fractions, with less trailing (Figure 1B). The profile of the white fraction obtained when extraction was

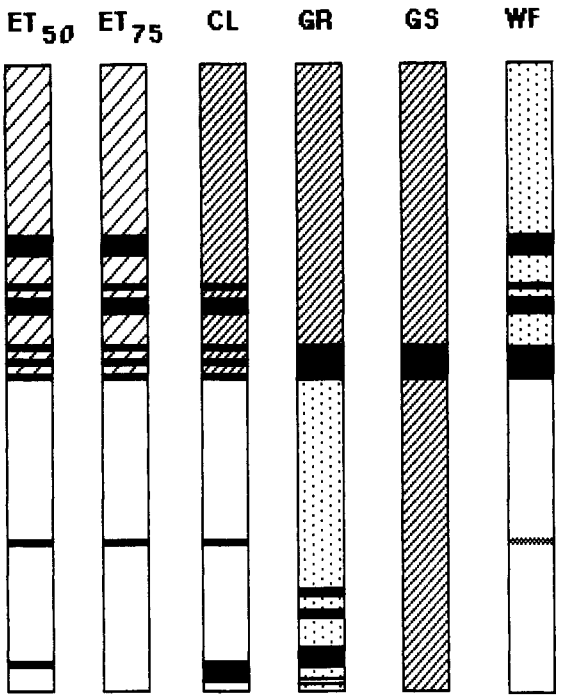

Figure 3. Reconstructed electrophoregram of crude gliadins extracted from wheat flour. PAGE in sodium lactate buffer (pH 3.2), migration time 0.5 h. Key: Et_{50}, 50% (v/v) ethanol (Charbonnier, 1982); Et_{75}, 75% (v/v) ethanol (Charbonnier, 1982); CL, 25% (v/v) 2-chloroethanol (Charbonnier, 1982); GR, commercial gliadin (Roth); GS, commercial gliadin (Sigma); WF, white fraction, 75% (v/v) ethanol (Autran et al., 1979).

done with 75% (v/v) ethanol according to Autran et al. (1979) had the slightest trailing (Figure 2C). Compared to the white fraction, the profile of the yellow fraction showed that it contained a small quantity of gliadins. Figure 3 shows the impurities, especially albumins, contained in the gliadins extracted with the different techniques. All crude gliadins contained albumins; however, the purest pattern was the white fraction.

Each technique was repeated 10 times, and in comparison to the method using aluminum lactate buffer (pH 3.1) the profiles obtained with our modifications are much sharper. Resolution is good, and the method is perfectly reproducible. Scanner recordings of the electrophoretic profiles lead to a better visualization of the results on slabs. They are only qualitative as a result of the even minimal imperfections in the gel and the sensitivity of the instrument. In addition, staining intensity can vary from one gel to another but nonetheless gives an idea of the relative proportions of the different fractions.

CONCLUSION

The present results show that gliadins are best extracted with 75% (v/v) ethanol at room temperature and without prior lipid removal from the flour or ground seeds. It is better to use flour than ground seeds and to use the extraction method of Autran et al. (1979). Polyacrylamide gel electrophoresis in sodium lactate buffer (pH 3.2) is an easy technique to use, is reproducible, and enables rapid control after extraction.

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