An Improved Method for Standardizing Polyacrylamide Gel Electrophoresis of Wheat Gliadin Proteins[1]

G. L. LOOKHART,[2] B. L. JONES,[2] S. B. HALL,[3] and K. F. FINNEY[2]

Cereal Chem. 59(3):178-181

ABSTRACT

Because different methodologies for polyacrylamide gel electrophoresis (PAGE) analysis precluded direct comparisons of results, we developed a standardized method for PAGE analysis of wheat gliadin extracts. Commercially available equipment and reagents were used. The standardized method should reduce the deleterious effects of the variables (purity and concentration of aluminum lactate, the type of gel former, and the method of gel preparation) and enhance the day-to-day reproducibility of the gliadin separations. The method was adjusted to allow maximum resolution of the gliadin protein bands.

Much research has been directed toward finding a method to identify wheat cultivars. The three most promising methods all involve electrophoresis (Autran and Bourdet 1975, Bushuk and Zillman 1978, Wrigley and Shepherd 1974). However, interlaboratory variations in electrophoresis methodology, equipment, and results make comparison of electrophoretic patterns obtained by various investigators difficult. Leaders of the above three research groups recently stated in a preliminary report (Autran et al 1979) that the polyacrylamide gel electrophoresis (PAGE) method of Bushuk and Zillman (1978) was most suitable as an international standard. Its major faults include a gel former that is not commercially available and must be handmade in each laboratory and a nonoptimal protein band resolution.

A study group of the International Association for Cereal Chemistry is now collaborating to develop a standard electrophoretic method for identifying cereal varieties. The PAGE method described here uses only commercially available reagents and equipment and gives more reproducible separation of wheat gliadin proteins than do methods now used. This improved electrophoresis method uses an updated combination of the aluminum lactate and lactic acid buffer first described by Jones et al (1959) and the acrylamide polymerization technique of Jordan and Raymond (1969).

MATERIALS AND METHODS

Reagents

Acrylamide, *N,N'*-methylene-bis-acrylamide, ascorbic acid, Coomassie brilliant blue R-250 (CB), and methyl green were obtained from Sigma Chemical Company; lactic acid (USP grade) and ferrous sulfate heptahydrate (AR grade), from Mallinckrodt Chemicals; and LC grade ethanol, from Burdick and Jackson Laboratories. Hydrogen peroxide (3% practical grade) was purchased in small plastic bottles from a local pharmacy. Trichloroacetic acid (TCA) was obtained from MCB Chemicals. Water was purified by passage, in series, through a membrane filter, a charcoal filter, and two mixed-bed ion exchange filters.

[1]Cooperative investigations, Agricultural Research Service, U.S. Department of Agriculture, and the Department of Grain Science and Industry, Kansas State University. Contribution 81-251-J, Department of Grain Science and Industry, Kansas Agricultural Experiment Station, Manhattan 66506.

Portions of this paper were presented at the AACC 65th Annual Meeting, Chicago, IL, September 1980.

Mention of firm names or trade products does not constitute endorsement by the USDA over others not mentioned.

[2]Research chemists, U.S. Grain Marketing Research Laboratory, Agricultural Research Service, USDA, Manhattan, KS 66502.

[3]Research assistant, Department of Grain Science and Industry, Kansas State University, Manhattan 66506.

Three aluminum lactate samples were examined, one from Pfaltz and Bauer and two (with different lot numbers) from K & K Laboratories.

Apparatus

The electrophoresis apparatus was an E-C Apparatus Corporation No. 6052 vertical gel former (180 × 120 × 6 mm). It has large buffer reservoirs (2,000 ml) and cooling plates that allow relatively good maintenance of constant temperature. The fixed cooling plates ensure that the gel thickness does not change between runs or laboratories, and they reduce gel-surface variations that occur when acrylamide polymerizes in the presence of air. The gel solution was poured while the apparatus was maintained at a slight incline (about 3°) from the horizontal position. After the gel polymerized, the apparatus was placed in a vertical position for operation. Because the slots in the gel were vertical and the protein extract solution was denser than the tank buffer, protein extracts concentrated in the bottom of the slots. The entire apparatus is relatively inexpensive and easy to use.

Grinding of Wheat

Whole kernel wheat samples (3.0 g) were ground in a Udy cyclone mill fitted with a 1-mm sieve (Bushuk and Zillman 1978).

Gliadin Extraction

Ground wheat (0.25 g) was extracted with 750 μl of 70% aqueous ethanol by mixing on a vortex mixer for 20 sec in a stoppered test tube. The tube was allowed to stand at room temperature for 1 hr and then centrifuged at 4,550 × *g* for 10 min. The supernatant was transferred to a 2-ml screwcap vial, and five drops of glycerin were added to increase the density of the protein solution. A drop of 10% methyl green solution was added as a tracking dye and to show how the sample layered in the slots.

Gliadin extracts were stored at 5°C in sealed vials for several months with no noticeable change in the electrophoregrams.

Preparation of the Gel

The electrophoresis apparatus was snapped together and positioned about 3° with the horizontal so the gel could be poured. Peroxide catalyst (1.25 ml of a 3% solution) was quickly added to 250 ml of gel solution (Table I) previously cooled to 1°C. The mixture was swirled briskly for 1–2 sec and poured into the gel former. An eight-place slot former was quickly positioned, and the gel polymerized in about 30 sec after the catalyst was added. After the gel had set for 2 min, the slot former was removed, the apparatus was moved to a vertical position, the buffer reservoirs were filled, and coolant was circulated through the system at 21°C.

TABLE I
Recipes for Gel and Tank Buffer Solutions

Solution[a]	Amount Required
Gel, ml	1,000
Acrylamide, g	60.0
N,N'-Methylene-bis-acrylamide, g	3.0
Ascorbic acid, g	1.0
$FeSO_4 \cdot 7H_2O$, g	0.04
Aluminum lactate, g	2.50
Lactic acid, ml	4.50 (to pH 3.1)
Catalyst	
3% Hydrogen peroxide, ml	1.25
Tank buffer, ml	2,000
Aluminum lactate, g	2.50
Lactic acid, ml	3.70 (to pH 3.1)

[a] Amounts of gel, catalyst, and tank buffer solutions used were 250, 1.25, and 2,000 ml per gel, respectively.

Sample Application

Aliquots (20-μl) of gliadin extracts were carefully placed in the eight gel slots with a disposable-tip microliter syringe. The dense extracts sank through the buffer and formed a thin band on the bottoms of the slots. Gliadins extracted from the cultivar Marquis were placed in slots 1, 4, and 8. That facilitated gel slot identification, ensured that the "unknown" samples were not subjected to "edge effects," and gave the Marquis bands as standards with which to calculate the relative mobilities of the cultivar bands. Five different unknown samples were introduced into the other slots on each gel.

Electrophoresis Conditions

The anode of the power supply (Hewlett-Packard 6521A) was connected to the upper buffer, which was in contact with the slotted end of the gel, and the cathode was connected with the lower buffer. Voltage was maintained at 290 V (about 16 V/cm), which resulted in a current flow of 70–72 mA. Electrophoresis was continued for 5.25 hr.

Staining

Following electrophoresis, the power supply was turned off, the high-voltage cables were disconnected for freedom of movement, the tank buffer was poured off, the two-piece gel former was unsnapped, and the portion of the apparatus containing the upper buffer tank was removed. The gel was loosened from the apparatus by forcing water under the gel with the aid of a Teflon-coated spatula. The gel, removed by sliding a plexiglass sheet under it, was placed in a polyethylene staining tray (Tupperware 29 × 18 × 5-cm food saver dish). Each gliadin-containing gel was stained for 15 hr with a solution consisting of 18 ml of an ethanol solution of 1% CB diluted with 300 ml of 12% TCA. An additional 5 ml of 1% CB was

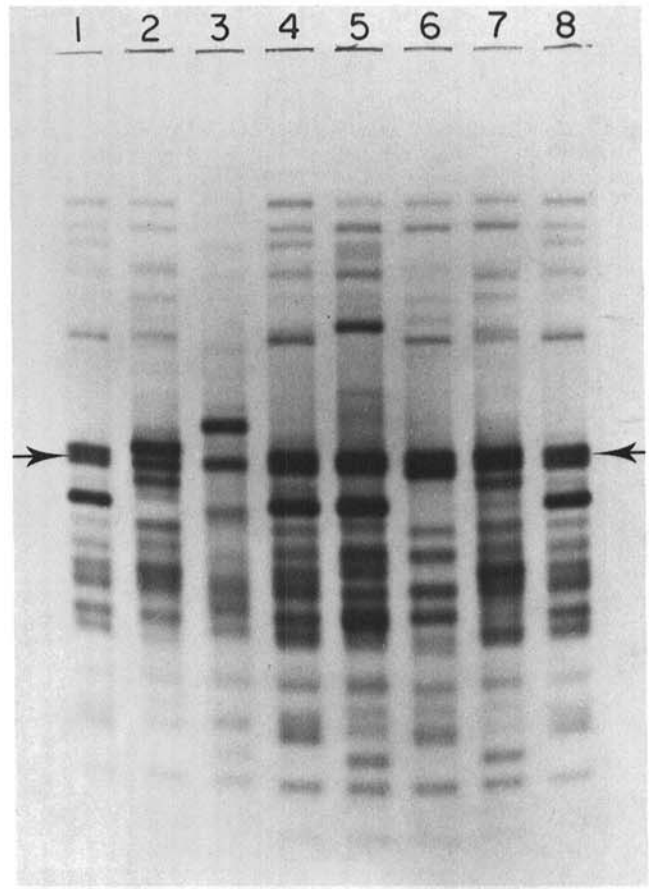

Fig. 1. Gel electrophoregrams of gliadins extracted from representative wheats: **1, 4,** and **8,** Marquis; **2,** Palo Duro; **3,** Protor; **5,** Kharkov; **6,** TAM 101; and **7,** Waldron. Arrow shows Marquis doublet.

then added; the gels were stained another 24 hr, then destained 5 hr with 300 ml of 12% TCA.

Photography

After destaining, the gels were placed on clear glass plates and rinsed with distilled water. They were then laid on a frosted glass surface of a "light box," and illuminated from below with two 15-W daylight fluorescent lamps. The gels were photographed with Kodak Ektapan 4162-thick film (4 × 5 in.), which was developed with HC-110 developer using dilution B (Kodak film instruction sheet), producing 10 × 12.5-cm (4 × 5-in.) negative. From that, a 12.5 × 17.5-cm positive (reversal negative) was produced with Kodalith Ortho film, type 3, No. 2556, developed with Kodak D-19 developer. The developing and exposure times were adjusted to give optimum contrast. The final positive film, in which the protein bands appear black against a transparent background, was made the same size as the gel. The positives were scanned with a Kratos SD 3000 spectrodensitometer at 550 nm.

RESULTS AND DISCUSSION

Typical Results

Typical electrophoregrams of gliadins extracted from some American wheat cultivars are shown in Fig. 1. Gliadins extracted from ground whole wheat, ground degermed wheat, and flour gave identical electrophoregrams. Resolution was assumed to be satisfactory when the Marquis doublet shown by the arrow in Fig. 1 was resolved. Electrophoretic patterns for most wheat varieties were unique, but some very closely related cultivars yielded very similar or identical electrophoregrams. Varietal differences were revealed by the presence or absence of particular bands in the electrophoregrams. The gliadin electrophoretic patterns of specific cultivars and wheat classes are discussed by Jones et al (1982).

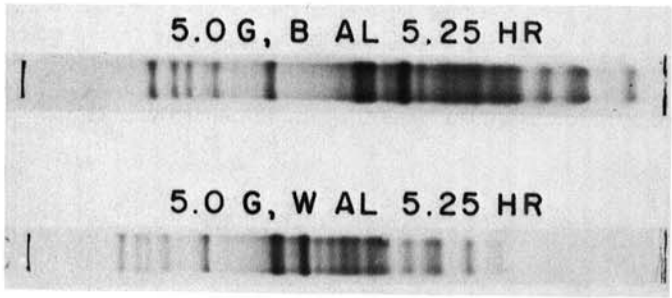

Fig. 2. Effect of purity of aluminum lactate (**AL**) on Marquis gliadin band separation. **B,** = brown (impure) AL, **W** = white (purer) AL. AL concentration of tank buffer = 5.0 g/2,000 ml; electrophoresis time = 5.25 hr.

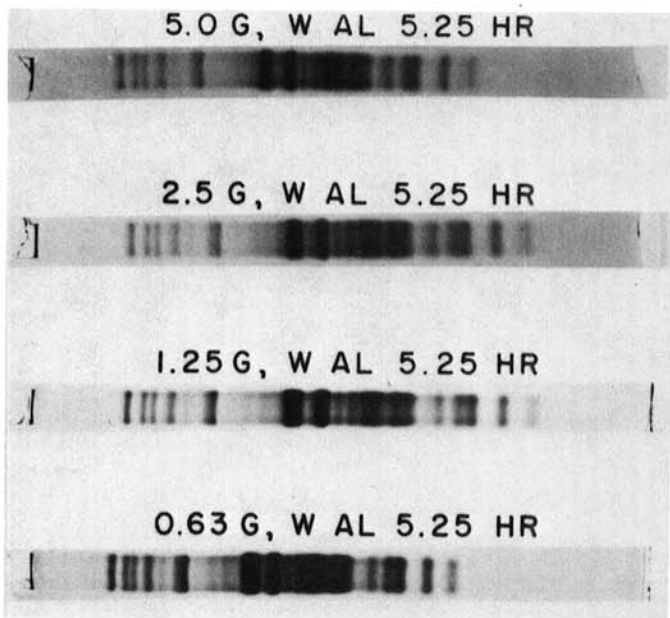

Fig. 3. Electrophoretic separation of Marquis gliadin bands for 5.0, 2.5, 1.25, and 0.63 g of white aluminum lactate (**W AL**) per 2,000 ml of tank buffer. Electrophoresis time = 5.25 hr.

Variables Affecting Resolution

When the gel preparation methods, the voltage, and the temperature were kept constant, the aluminum lactate purity and concentration, the electrophoresis time, and the apparatus all affected the electrophoregrams.

White crystalline aluminum lactate (WAL) obtained from K & K Chemicals (lot 36122A) gave a cleaner background and better band resolution (Fig. 2) than did the brownish-green aluminum lactate from P & B Chemicals or from K & K (lot 32118A). The concentration of aluminum lactate in the buffer also affected band resolution (Fig. 3); the best definition of the gliadin bands was found when WAL concentrations of 1.25–2.50 g per 2,000 ml of tank buffer solution was used.

Figure 4 shows the effect of electrophoresis time on gliadin band separation. A run time of 5.25 hr gave optimal results; the Marquis doublet was well resolved by then, other gliadin bands only slightly separated at 4.0 hr were somewhat better resolved, and the faster-moving bands sometimes found in other varieties had not run off the gel. Relative mobilities can be calculated more precisely when the bands are separated over a longer distance. When gels were run 7.0 hr, some of the gliadins migrated off the end of the gel.

Advantages of the Method

Uriel (1966) suggested that to deliver optimal electrophoretic separations, an electrophoresis apparatus should have three attributes: 1) ability to maintain the gel at a uniform thickness throughout, 2) the same material in contact with all of the gel solution during polymerization, because gel strength may be affected by its surroundings, and 3) a cover for the gel before and during electrophoresis to keep the gel surface from drying out. In addition, the gel should be maintained at a constant temperature slightly below room temperature for ease in temperature maintenance and to reduce the gel-to-room temperature gradient. Also, pressure on the gel should be held constant while the gel is poured and during electrophoresis.

The vertical gel former apparatus described here meets all of those requirements and is a standard model commercially available around the world. Also, the protein samples are placed in slots on top of the gel and settle to the bottoms of the slots to form a thin layer, so that proteins enter the gel as thin bands.

Related Considerations

Standardization of the electrophoresis procedure was a primary objective of this study. The pH measurements of aluminum lactate

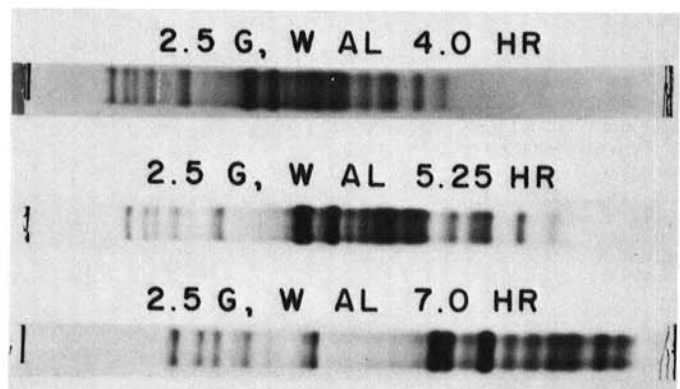

Fig. 4. Effect of length of electrophoresis time on the separation of Marquis gliadin bands. Tank buffer (2,000 ml) contained 2.5 g of white aluminum lactate (**W AL**). Electrophoresis times were 4.0, 5.25, and 7.0 hr.

solutions tend to drift over a period of time. Therefore, the volume of lactic acid required to adjust the pH of the aluminum lactate buffer and gel solutions to pH 3.1 was determined on five separate days. They averaged 3.70 and 4.50 ml, respectively. Thereafter, when buffer and gel solutions were made up, 3.70 and 4.50 ml of lactic acid, respectively, were added, and therefore, measurement of the pH of the solutions each time was not necessary.

The purity of the aluminum lactate samples from K & K Chemicals and Pfaltz and Bauer Chemicals is currently being investigated. We found that recrystallizing colored aluminum lactate samples made them white, so the color probably stems from some contaminant. Atomic absorption spectroscopy showed that the colored material contained three times the silicon, four times the iron, six times the sodium, 15 times the calcium, 60 times the copper, and 100 times the barium in the white material.[4]

The method described here has been used to identify the 80 most commonly grown wheat varieties in the United States (Jones et al 1980) and to examine the gliadins of triticales and their wheat and rye parents (Chung et al 1980). A catalog of the gliadin electrophoregrams of the 88 most commonly grown U.S. wheat varieties is being prepared.

ACKNOWLEDGMENT

We thank Michael Klinker for the photography.

[4]Lookhart and Jones, unpublished data.

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[Received March 2, 1981. Accepted October 23, 1981]