

Polyacrylamide Gel Electrophoresis for Wheat Variety Identification: Effect of Variables on Gel Properties¹

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ABSTRACT

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A polyacrylamide gel electrophoresis procedure was investigated to determine its suitability for wheat variety identification by analysis of gliadin proteins. Relative amounts of the gel catalysts (ascorbic acid, ferrous sulfate, and hydrogen peroxide) had profound effects on gel polymerization time, pore size, firmness, and stickiness. Gels of varying

firmness were obtained by varying relative amounts of catalysts. Gel firmness and pore size affected resolution of the gliadin proteins. Scanning electron microscopy showed differences in the physical structure of gels of different firmnesses.

Polyacrylamide gel electrophoresis (PAGE) of gliadin proteins has been investigated as a method for wheat variety identification (Bushuk and Zillman 1978, du Cross and Wrigley 1979, Khan 1982, Khan et al 1983, Lookhart et al 1982, Tkachuk and Metlish 1980, Wrigley et al 1982). Bushuk and Zillman (1978) used a modified version of the procedure of Jordan and Raymond (1969) in which three catalysts—ascorbic acid, ferrous sulfate, and hydrogen peroxide—are used for polymerization. Other researchers (Tkachuk and Metlish 1980, Lookhart et al 1982) subsequently also modified the procedure of Jordan and Raymond (1969) by using different amounts of catalysts. However, of those researchers who modified this original PAGE procedure, only Khan et al (1983) gave any reason for doing so: a slightly firmer gel, giving better resolution of gliadin, could be obtained by reducing the amount of hydrogen peroxide originally used by Bushuk and Zillman (1978).

This study, therefore, investigated in detail the effects of ascorbic acid, ferrous sulfate, and hydrogen peroxide on gel firmness and resolution of gliadin proteins to provide information useful for interlaboratory standardization of PAGE for wheat variety identification and wheat quality research.

MATERIALS AND METHODS

Wheat Samples

Grain samples of the hard red spring wheats Marquis, Coteau, and Len, and the durum wheats Rugby and Lloyd, and the hard red winter wheats Agate and Roughrider were obtained from the Seed Stock Project, Department of Agronomy, North Dakota State University, Fargo.

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Grain samples were ground on a Udy cyclone mill (Bushuk and Zillman 1978) or with a mortar and pestle. Extraction was performed by adding 1.0 ml of 70% aqueous ethanol to 0.5 g of meal in a 15-ml Corex glass centrifuge tube, and vortexing the suspension. The sample was then vortexed every 10 min for 1 hr at room temperature, and then centrifuged at $20,000 \times g$ for 15 min at 20°C. The supernatant was decanted and diluted with 0.5 ml of sodium lactate pH 3.1 buffer (Bushuk et al 1981) containing 50% (w/v) sucrose and 3% (w/v) methyl green tracking dye.

Our PAGE procedure was based on the procedure of Jordan and Raymond (1969) as modified by Bushuk and Zillman (1978), except that 2.5 g/L of Na lactate adjusted to pH 3.1 with lactic acid (Bushuk et al 1981) was used instead of Al lactate pH 3.1 (Bushuk

and Zillman 1978). A gel containing 6% (w/v) acrylamide with 0.3% (w/v) bisacrylamide (cross-linker) was used. For a firm gel we used 0.024% (w/v) ascorbic acid, 2×10^{-2} % (w/v) ferrous sulfate-heptahydrate, and 0.1% (v/v) of 3% hydrogen peroxide (purchased from a local pharmacy or prepared from a 30% stock solution). Gels of varying firmness were prepared by varying the amounts of ascorbic acid, ferrous sulfate, and hydrogen peroxide.

The E-C 470 AX vertical gel apparatus with the E-C 435T1.5 slot former (E-C Corporation, St. Petersburg, FL) was used to make 3-mm-thick gels. Acrylamide and bisacrylamide were not recrystallized as in previous experiments (Khan et al 1983). Gels were electrophoresed at 50 mA until the tracking dye was about 0.5 cm from the end of the gels. Gels were stained, destained, and photographed according to Bushuk and Zillman (1978) and Khan et al (1983). Chemicals and reagents used were obtained from the same sources as described previously (Khan et al 1983).

Firmness Measurements

Solutions of ascorbic acid and ferrous sulfate were prepared in Na lactate pH 3.1 buffer containing 6% (w/v) acrylamide and 0.3% (w/v) bisacrylamide. An appropriate volume of each solution, containing required amounts of catalysts, was then added to the gel solution, which was diluted to volume with Na lactate pH 3.1 buffer containing 6% (w/v) acrylamide and 0.3% (w/v) bisacrylamide. Upon addition of hydrogen peroxide, gel solutions (15 ml) were polymerized in 25-ml glass beakers for 15 min. After polymerization, gels were carefully removed and placed on the platform of an Instron 1000 Universal Testing Instrument (Instron Corporation, Canton, MA). Gel firmness was measured by compression to 25% deformation of the cylindrical (2.0-cm diameter, 2.4-cm high) gels with a 1-kg weight attached to a 10-lb weight beam. Deformation speed was 20 mm/min, and the compression anvil was 57.5 mm high. The force (gcm) to compress each gel was determined from a curve of a chart recorder (full scale, 0 to 1 kg; chart speed, 20 mm/min). Each determination was made on six identically prepared gels.

Scanning Electron Microscopy (SEM)

Pieces of gels (about 1 cm long \times 0.5 cm wide \times 0.2 cm thick) were placed in small plastic weighing boats, put into a petri dish and covered, frozen overnight at -15°C , and lyophilized for at least 48 hr. Only gel pieces showing no apparent deformation were chosen for electron microscopy. Gel pieces were mounted on stubs, sputter-coated with gold or gold-palladium, and viewed with a JEOL JSM-35 scanning electron microscope at 8–10 kV.

Statistical Analysis

The Statistical Analysis System (SAS 1982) and response surface methodology (Myers 1976) were used for statistical analyses of firmness data.

RESULTS AND DISCUSSION

Effect of Catalyst Type and Level on Gel Firmness

Table I compares the original PAGE formulation of Jordan and

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Raymond (1969) with the modified formulations of other researchers. Most researchers used 0.0025% ferrous sulfate, but Lookhart et al (1982) used 0.004%. Most researchers used 0.10% ascorbic acid, but Tkachuk and Metlish (1980) used a lower amount (0.02%). Hydrogen peroxide levels used were most variable, ranging from 0.135% (Tkachuk and Metlish 1980) to 0.5% (Lookhart et al 1982). No reasons were given for most modifications, but Khan et al (1983) found that a hydrogen peroxide concentration lower than that used by Bushuk and Zillman (1978) gave a firmer gel.

For firmness testing, gels were prepared using various combinations of the three catalysts. Six levels of ascorbic acid were used (0.008, 0.016, 0.024, 0.032, 0.040, and 0.08%, w/v) along with four levels of ferrous sulfate (0.0002, 0.0004, 0.002, and 0.004%, w/v) and hydrogen peroxide (0.1, 0.2, 0.3, and 0.4%, v/v). These

catalyst concentrations covered the ranges used previously by researchers as listed in Table I.

Table II shows firmness values obtained from our experiments. At fixed ferrous sulfate and hydrogen peroxide concentrations, increasing the ascorbic acid concentration resulted in a rapid increase in gel firmness, then a gradual decrease. At fixed ascorbic acid and hydrogen peroxide concentrations, increasing the ferrous sulfate concentration generally resulted in a decrease in gel firmness; this decrease was more marked at higher ascorbic acid concentrations. At fixed ascorbic acid and ferrous sulfate concentrations, increasing the hydrogen peroxide concentration did not significantly affect gel firmness. The firmest gels generally occurred at ascorbic acid concentrations (w/v) of 0.024 to 0.032% and ferrous sulfate concentrations (w/v) of 0.0002 to 0.0004%, but were largely independent of H₂O₂ concentrations (Table II). Statistical analyses of the firmness data also showed that ferrous sulfate had the greatest effect on gel firmness followed by ascorbic acid and hydrogen peroxide.

Firmness values were also determined for gels as prepared by other researchers. Soft gels were obtained using formulations of Lookhart et al (1982) (firmness, 140 gcm) and Bushuk and Zillman (1978) (firmness, 182 gcm), who used high amounts of all three catalysts. The formulation of Tkachuk and Metlish (1980), which used lower levels of ascorbic acid and hydrogen peroxide, gave a firmer gel (404 gcm). These firmness values are in agreement with our results reported in Table II.

We also examined the effect of gel catalysts on polymerization time (Table III). Ferrous sulfate had the greatest effect, since doubling the amount of ferrous sulfate reduced polymerization time from 10 to 5 min (formulations A and B), whereas it took four times as much hydrogen peroxide to reduce polymerization time to 5 min (formulations A and C). Ascorbic acid had no apparent effect

TABLE I
Comparison of the Ingredient Levels of Various Polyacrylamide Gel Electrophoresis Formulations

	Jordan and Raymond (1969) (%)	Bushuk and Zillman (1978) (%)	Tkachuk and Metlish (1980) (%)	Lookhart et al (1982) (%)	Khan et al (1983) (%)
Gel system (w/v)	12.0	6.0	6.0	6.0	6.0
Catalyst system					
FeSO ₄ ·7H ₂ O (w/v)	0.0025	0.0025	0.0025	0.004	0.0025
Ascorbic acid (w/v)	0.10	0.10	0.020	0.10	0.10
H ₂ O ₂ (3% solution), (v/v)	0.30	0.50	0.135	0.50	0.25

TABLE II
Firmness Values of 6% (w/v) Polyacrylamide Gels Made from Combinations of Gel Catalysts

% Ascorbic Acid (w/v)	Firmness Values (gcm)							
	% FeSO ₄ (w/v)				% FeSO ₄ (w/v)			
	0.0002	0.0004	0.002	0.004	0.0002	0.0004	0.002	0.004
	0.10% (v/v) H ₂ O ₂ ^a				0.20% (v/v) H ₂ O ₂ ^a			
0.008	230	180	380	250	212	172	242	324
0.016	428	448	418	408	386	390	372	400
0.032	354	400	258	122	456	460	298	184
0.024	494	460	280	130	488	412	364	374
0.032	354	400	258	122	456	460	298	186
0.040	362	408	256	122	450	444	250	166
0.080	304	400	204	84	424	392	236	140
	0.30% (v/v) H ₂ O ₂ ^a				0.40% (v/v) H ₂ O ₂ ^a			
0.008	214	144	308	340	250	196	218	260
0.016	402	348	258	292
0.024	418	406	352	326	498	366	262	242
0.032	498	392	304	280	464	402	264	214
0.040	454	370	210	190
0.080	428	368	212	110	402	364	174	102

^a 3% solution.

TABLE III
Effect of Gel Catalysts on Onset of Polymerization of Firm and Soft Polyacrylamide Gels

	Gel Formulations				
	A (%)	B (%)	C (%)	D (%)	E ^a (%)
Acrylamide (w/v)	6.0	6.0	6.0	6.0	6.0
Bisacrylamide (w/v)	0.3	0.3	0.3	0.3	0.3
FeSO ₄ ·7H ₂ O (w/v)	2 × 10 ⁻⁴	4 × 10 ⁻⁴	2 × 10 ⁻⁴	2 × 10 ⁻⁴	2.5 × 10 ⁻²
Ascorbic acid (w/v)	0.024	0.024	0.024	0.08	0.10
H ₂ O ₂ (3% solution, v/v)	0.10	0.10	0.40	0.10	0.50
Firmness value (gcm)	494	460	498	304	182
Polymerization time	10 min	5 min	5 min	10 min	1 min

^a Bushuk and Zillman (1978).

on polymerization time (formulations A and D). When both ferrous sulfate and hydrogen peroxide are at high levels (formulation E, Bushuk and Zillman 1978), polymerization is almost instantaneous. Also, yellow-green gels are obtained using high ferrous sulfate and hydrogen peroxide levels (Bushuk and Zillman 1978, Lookhart et al 1982) and if ascorbic acid level is low (Tkachuk and Metlish 1980). When long polymerization times are used (formulations A, B, C and D, Table III), there is some syneresis when the gel solution polymerizes in the E-C 470 apparatus. Unpolymerized solution should be poured off before removing the slot former.

SEM of Polyacrylamide Gels

To observe physical differences between firm and soft gels, freeze-dried pieces of gels were viewed with the scanning electron microscope (Figure 1). A firm gel (formulation A of Table III) showed more uniform pores and thicker walls than a soft gel (formulation E of Table III), which contained apparently larger pores covered with many thin strands; as a result, firm hydrated gels can be lifted and suspended without breaking, whereas soft gels break immediately when lifted.

Electrophoretic Separations on Firm and Soft Gels

Figure 2 shows a typical separation on a firm gel (formulation A of Table III) of gliadin proteins of the hard red spring wheat variety Marquis, the reference cultivar often used for wheat variety identification (Bushuk and Zillman 1978). Patterns 1, 2, and 3 contained 4, 6, and 10 μ l of gliadin extract. Usually 6 μ l of extract is applied: this sample volume contains enough protein for clear separation of the doublet bands and for visualization of the rest of the Marquis pattern (Figure 2, pattern 2). If the doublet is clearly resolved, a separation is considered acceptable; if not, the PAGE

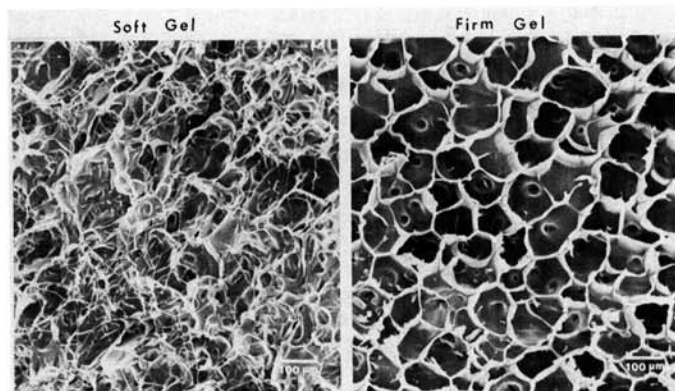


Fig. 1. Scanning electron micrographs of soft (formulation E of Table III) and firm (formulation A of Table III) gels.

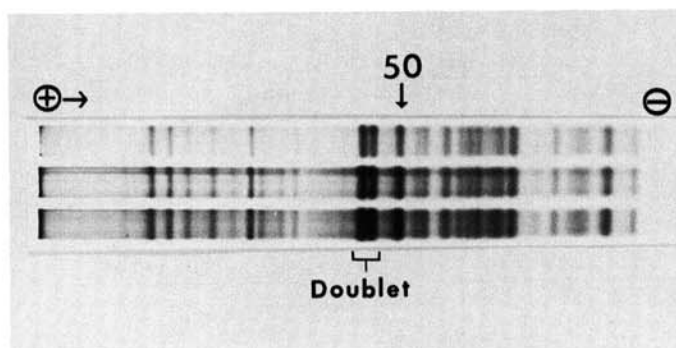


Fig. 2. Polyacrylamide gel electrophoresis of the gliadin protein extract of the hard red spring wheat variety Marquis on a 3-mm-thick firm gel (formulation A of Table III). Slots 1, 2, and 3 contained 4, 6, and 10 μ l extract, respectively. Duration of electrophoresis was 4 hr at 50 mA constant current.

procedure must be modified to improve resolution of the doublet, while maintaining or improving resolution of other bands.

Figure 3 compares separation of gliadin proteins of different wheat classes on firm (formulation A, Table III) and soft (Bushuk and Zillman 1978) gels. Several differences in resolution are apparent. In patterns 1 and 8, the Marquis doublet (first arrow from the positive electrode) is resolved on the firm gel but not in the soft gel, even when using a longer electrophoresis time. A faint band occurs between the doublet and band 50 (second arrow from the positive electrode, pattern 1), however, on the soft gel but not on the firm gel. In pattern 2, ω -gliadin bands (indicated by arrows) are more clearly separated on the soft than the firm gel. In pattern 3, three γ -gliadin bands appear on the firm gel (arrows), whereas only two appear on the soft gel. In pattern 4, two ω -gliadins (arrow) are better resolved on the firm gel, whereas only one band appears in the same region on the soft gel. Also in pattern 4 some β - and γ -gliadins (arrows) are more clearly resolved on the firm gel. In pattern 5, two bands in the β -region (arrow) are resolved on the firm gel, but only one appears on the soft gel. In pattern 6, two γ -bands (arrow) are better resolved on the firm gel, whereas some α -bands (arrows) resolve better on the soft gel. However, in pattern 7 of Roughrider a number of bands in the α - and β -regions (arrows) are better separated on the soft gel. The bands on the firm gel, however, are much sharper than on the soft gel.

Effect of Cross-Linking on Resolution

Because SEM (Figure 1) showed apparently slightly larger pore structure in the soft gel, and because resolution of α -gliadins was generally better on soft gels (Figure 3), we decided to make a gel having slightly larger pores, hoping to improve separation, especially of α -gliadins. Consequently, the bisacrylamide concentration (formulation A of Table III) was reduced from 0.3%

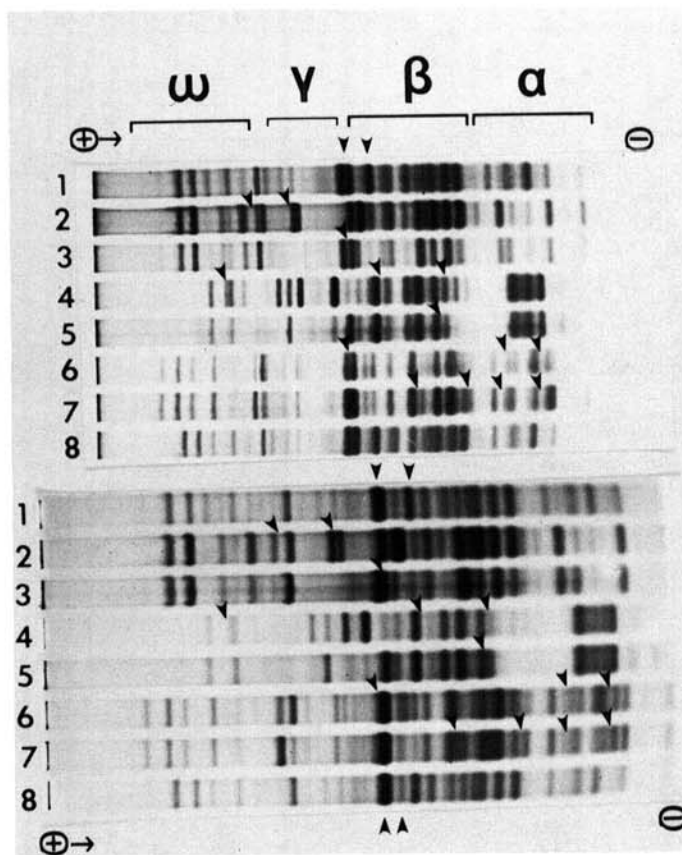


Fig. 3. Polyacrylamide gel electrophoresis on firm (top) and soft (bottom) gels of gliadin protein extracts (6 μ l each) of the following wheat varieties: 1 and 8, Marquis; 2, Coteau; 3, Len; 4, Rugby; F, Lloyd; 6, Agate; 7, Roughrider. Duration of electrophoresis (at 50 mA) of the firm gel was 3 hr 35 min, and for the soft gel was 3 hr 50 min.

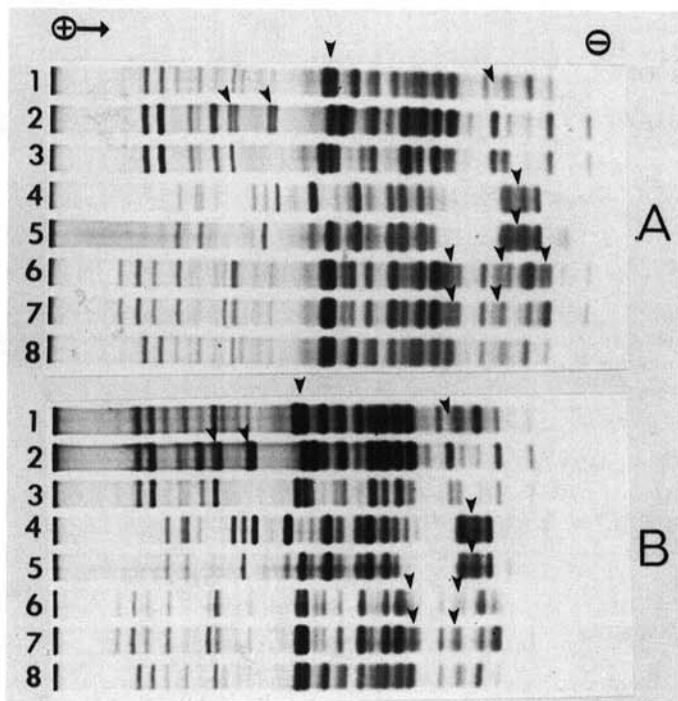


Fig. 4. Polyacrylamide gel electrophoresis of gliadin proteins (6 μ l extracts) on gels containing 0.25% (w/v) bisacrylamide (gel A) or 0.30% (w/v) bisacrylamide (gel B). Varieties are as in Figure 3. Duration of electrophoresis for both gels was 3 hr 35 min at 50 mA.

to 0.25% (w/v) while all other ingredient levels were kept the same to retain as much firmness as possible. Firm gels made with 0.25% (gel A) and 0.3% (Gel B) (w/v) bisacrylamide (Figure 4) showed some improvement in separation on gel A, especially of α - and β -gliadins (arrows in the patterns of Marquis, Rugby, Lloyd, Agate, and Roughrider) and also in ω -gliadins of Coteau (arrows); the Marquis doublet (pattern 12, first arrow from the positive electrode) is still resolved.

Effect of Gel Thickness

Figure 5 compares the separation of Marquis gliadins on 3- and 6-mm-thick firm gels. Resolution and separation are better on the thinner gel. Thin firm gels are also easy to handle while soft thin gels break easily. Consequently, we routinely use 3-mm gels with the E-C 470 apparatus.

GENERAL DISCUSSION

Standardization of PAGE is important for interlaboratory wheat variety identification and also for research in bread and pasta-making quality. Many variables may affect PAGE performance. This study has shown that gel catalysts (ascorbic acid, ferrous sulfate, and hydrogen peroxide) affect gel firmness, thereby affecting resolution of gliadins. By varying the relative amounts of these catalysts, gels of varying firmness could be obtained (Table II). It was also shown that high-mobility α -gliadins separate better on soft than on firm gels. Gels of different firmness (Table II) can thus be selected to best separate particular mixtures of proteins.

Firm gels have several advantages over soft gels. Firm gels are easier to work with; even 6% gels as thin as 0.75 mm can be easily handled. Also, firm gels are not sticky, as are soft gels made with high levels of hydrogen peroxide. Resolution of most gliadins, especially in the β - and γ -regions, is better on firm than on soft gels. This improved separation may be especially useful for differentiating varieties, because the β - and γ -gliadin PAGE regions contain the most bands. Furthermore, because firm gels (A

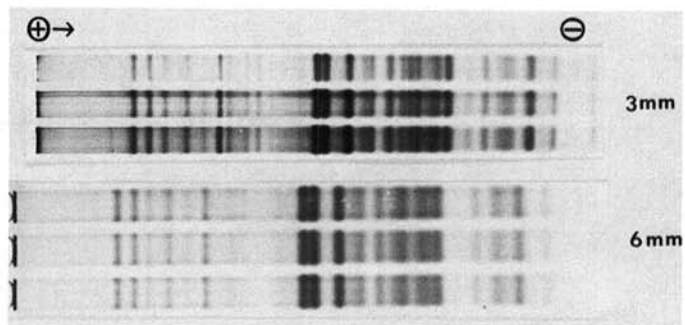


Fig. 5. PAGE of Marquis gliadins on 3- and 6-mm thick firm gels. Duration of electrophoresis for the 3-mm gel was 4 hr at 50 mA, and for the 6 mm gel was 6 hr 30 min at 75 mA.

to D of Table III) take longer to polymerize than do soft gels (formulation E of Table III), there is more time to carry out manipulations associated with the procedure and apparatus. In contrast, polyacrylamide gels used by Bushuk and Zillman (1978) (formulation E of Table III) polymerize in about one minute.

To formulate firm gels that give better separations of gliadins, we have used much lower levels of catalysts than in the formulations that result in soft gels and poorly resolved gliadins. Even though the results of Figure 4 indicate that pore differences may also influence gliadin separations, it is also possible that gliadins may interact with high levels of catalysts in soft gels, thus leading to poorer resolution.

Thinner gels may also better resolve and separate gliadin proteins (Figure 5) as well as reduce run times. Research is continuing in our laboratory on variables such as gel thickness, types of buffers and their pH and ionic strengths, temperature effects, and apparatus design in an overall effort to optimize resolution and rapidity of gliadin PAGE for wheat variety identification.

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