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HIGH-RESOLUTION TWO-DIMENSIONAL ELECTROPHORESIS OF WHEAT PROTEINS[a]

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SUMMARY

A high-resolution two-dimensional electrophoretic method for separating wheat endosperm proteins is described. Non-equilibrium pH gradient electrophoresis (NEPHGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were used to examine proteins in non-fractionated extracts of wheat flours. NEPHGE was conducted in 100-μl capillary tubes at 3000 V, for a total of 4800 V h. The second dimension, SDS-PAGE, was performed as a slab mini-gel (70 × 80 × 1.0 mm) with constant application of 200 V. Image analysis of the two-dimensional separations of three wheat varieties showed approximately 35 and 41% homology of Centurk 78 and Rodeo varieties, respectively, when compared to a reference (Scout 66).

INTRODUCTION

Protein quality has long been considered to be of primary importance to the breadmaking potential of a wheat flour[1,2]. Cohesive and extensive functional properties of a dough are attributed to gliadins (prolamins), while elasticity is provided by glutenins (glutelins)[3]. Gliadins[4], glutenins[5] and acetic acid insoluble glutenins[6], among other components, have been reported to control loaf volume. Recently, Chakraborty and Khan[7] published a comparison study of three[5,8–10] different wheat protein fractionation procedures based on solubility. After baking reconstituted flours from two of the fractionation procedures[5,8,9], Chakraborty and Khan[7] concluded that fractions containing more glutenin proteins improved loaf volume response.

Many researchers including Jackson *et al.*[11] and Brown and Flavell[12] agree that the Osborne[13] solubility classes overlap; therefore, Jackson *et al.*[11] and Payne *et al.*[14]

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separated proteins using gel filtration chromatography in combination with differential solubility extractions, or solubility tests of gel filtration fractions. Reduced glutenins were classified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) into high relative molecular mass (M_r) "A", and low M_r "B", "C" and "D"[15,11] subunits. Jackson *et al.*[11] and Payne *et al.*[16] prefer to describe classical glutenin as aggregated, and classical gliadin as non-aggregated, prolamins in dissociating media.

The method presented here utilizes two-dimensional (2D) electrophoresis to examine the various proteins present in wheat flour, and to determine relative amounts of individual proteins. Other researchers have previously used 2D electrophoretic procedures for wheat protein analysis. Brown and co-workers[17,18], Brown and Flavell[12], Holt *et al.*[19] and Payne and co-workers[14,16] used modifications of the O'Farrell[20] and O'Farrell *et al.*[21] 2D electrophoresis methods to better understand chromosomal location of the storage protein genes. SDS ranging from 0.5%[12,17,18] to 2%[19] was included in the extracting solvent to solubilize all protein classes, and a minimum of 80%[19] of the endosperm protein was extracted as determined by the colorimetric method of Bramhall *et al.*[22]. Holt *et al.*[19] used both isoelectric focusing (IEF) and non-equilibrium pH gradient electrophoresis (NEPHGE) techniques to separate alkaline and acidic proteins. Payne *et al.*[14] presented a combination of two gels, one with NEPHGE–SDS-PAGE and one with isoelectric focusing (IEF)–SDS-PAGE, on which the locations of the Sephadex/Osborne classified protein groups were outlined.

Lei and Reeck[23] used both NEPHGE–SDS-PAGE and IEF–SDS-PAGE to reexamine the Osborne[13] classification of protein based on solubility. They concluded the same proteins were present in the gliadin and in the glutenin fractions and that differences between the two fractions were in the relative quantities of individual proteins present.

In the work presented here, proteins were directly analyzed without fractionation into their respective Osborne[13] classes. Improvements over other electrophoretic methods include decreased analysis time, smaller amounts of sample and ampholytes required, and use of image analysis for comparative and quantitative evaluation of wheat varieties.

EXPERIMENTAL

Chemicals and reagents

All chemicals used were reagent grade or highest purity.available. Acrylamide and other stock solutions used for electrophoresis were stored at 4°C and discarded after a maximum of 60 days. Acrylamide, N,N′-methylenebisacrylamide and urea were ultrapure grade purchased from Schwartz/Mann Biotech (Division of ICN, Cleveland, OH, U.S.A.). Pharmalyte brand of ampholytes (pH 5.0–8.0 and pH 8.0–10.5), N′,N′,N′,N′tetramethylethylenediamine (TEMED), 2-mercaptoethanol (MCE), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, U.S.A.). Urea-containing solutions were stored at −70°C.

Centurk 78, Rodeo, Scout 66 and Chinese Spring wheat varieties were provided by Paul Mattern (Department of Agronomy, University of Nebraska, Lincoln, NE,

U.S.A.). The protein contents (Kjeldahl N × 5.7, dry basis) of Centurk 78, Rodeo and Scout 66 were 14.00, 12.67 and 14.20%, respectively. Chinese Spring, a variety widely evaluated by other researchers[14,24], was used to compare the separation obtained using our electrophoretic procedure to those previously published. Scout 66, a commercial hard red winter (HRW) variety that has been widely grow in the central U.S.A. for several years, was selected as a reference wheat for quantitative comparison of gels. Centurk 78 and Rodeo, other commercial HRW varieties selected for comparison, were grown at several Nebraska locations and samples from each location were combined to minimize environmentally induced differences. These two study varieties were chosen based on their differences in baking quality.

Sample preparation

All wheat varieties were milled on a Buhler mill (Uzwil, Switzerland) to obtain a straight grade flour. In a 1.5-ml microcentrifuge tube 0.02 g CHAPS, 0.0003 g DTT and 1.05 ml of 9.0 *M* deionized urea were mixed. Urea was deionized by passing through a column of Amberlite MB-3 mixed bed ion-exchange resin just prior to use in sample preparation. An amount of flour sufficient to provide 6 mg protein (based on Kjeldahl analysis) was added. The contents of the centrifuge tube were gently stirred overnight with a magnetic stir bar at 4°C, then stored at −70°C until used in NEPHGE. Ampholytes (5 μl per 100 μl of sample) were added just prior to NEPHGE. Final ampholyte concentration was 2% and consisted of a mixture of 50% pH 5.0–8.0 and 50% pH 8.0–10.5.

Protein measurement

Flour protein was determined on 1-g flour samples by the Kjeldahl method (AACC Method 46-12)[25]. To estimate the amount of protein solubilized, 1 g of Centurk 78 flour was extracted with a solution containing 0.40 g CHAPS, 0.006 g DTT and 21 ml 9.0 *M* urea. The preparation was stirred gently overnight at 4°C, then centrifuged at 11 000 *g* for 15 min. Urea was removed from the samples by extensive dialysis against 0.1 m*M* $NaHCO_3$ and the sample was lyophilized prior to analysis by Kjeldahl.

Non-equilibrium pH gradient electrophoresis

NEPHGE was performed using a modification of the O'Farrell[20] method. This first separation incorporated high field strengths (approximately 300 V/cm) and small diameter (100 mm diameter, 100 μl volume) capillary tubes as previously described by Zeece *et al.*[26]. NEPHGE gels contained 4% acrylamide (bisacrylamide–acrylamide, 1:38), 2.0% CHAPS and 2.0% ampholytes (composed of 50% pH 5.0–8.0 and 50% pH 8.0–10.5) in 9.0 *M* aqueous urea. Prefocusing of gels prior to sample addition did not improve resolution and was therefore eliminated. Samples (10 μl) were loaded into the capillary tubes, covered with 10 μl of overlay solution containing 2.5% ampholytes (50% pH 5.0–8.0 and 50% pH 8.0–10.5) in 9.0 *M* urea and placed in the modified disc gel electrophoresis apparatus described by Zeece *et al.*[26]. The cathode (lower chamber) was filled with freshly made and degassed 0.01 *M* sodium hydroxide to a height sufficient to cover the gels, and maintained at 20°C with a water bath. The anode (upper chamber) contained 0.01 *M* orthophosphoric acid. NEPHGE separation was carried out using a programmable power supply (ISCO, Lincoln, NE, U.S.A.) to

TABLE I

FIRST DIMENSION POWER PROGRAM FOR SEPARATION OF WHEAT PROTEINS BY NEPHGE IN CAPILLARY TUBES

V	*mA*[a]	*W*[a]	*V h*[b]
500	1.0	1.0	200
1500	1.0	2.0	800
2000	1.0	2.0	800
3000	1.0	2.0	3000[c]

[a] Maximum limit setting.

[b] Volt-hours (V h) represent the product of volts × time and were used by the program to determine automatic crossover points to the next higher voltage, as well as the end point.

[c] Total V h used in this separation was 4800.

change volts, amperes and watts automatically at selected V h (volt-hour) crossover and end points, as shown in Table I. Immediately upon completion of the run, capillary tubes were removed from the apparatus and stored at −20°C until used for SDS-PAGE separation. These tubes were used within five days of freezing.

NEPHGE gels were thawed (approximately 3 min), removed from the capillary tube and placed into a small trough containing 5.0 ml SDS equilibration buffer [consisting of 5% (v/v) MCE, 10% (w/v) glycerol, 0.0625 *M* Tris–HCl at pH 6.8, and 2.3% (w/v) SDS], then allowed to equilibrate for 15 min.

The second dimension separation (SDS-PAGE) was carried out in a mini-gel format (70 × 80 × 1.0 mm, Biorad Labs., Richmond, CA, U.S.A.) using a constant voltage mode as previously described[26]. The SDS-PAGE system was essentially that developed by Laemmli[27], and consisted of a 4% stacking gel over a 12% separating gel using a bisacrylamide–acrylamide ratio of 1:50. SDS-PAGE was conducted at 200 V (constant voltage) until a prestained molecular weight marker (lactic dehydrogenase, M_r = 36000) reached 1.6 cm from the bottom of the gel (approximately 55 min). Gels were stained with 0.1% Coomassie Blue R250 in 5% methanol–10% acetic acid for 1 h with constant agitation. Gels were destained in several changes of 5% acetic acid–7.5% methanol overnight, again with constant agitation.

Image analysis

Image analysis was performed using a Visage 110 [BioImage (Kodak), Ann Arbor, MI, U.S.A.) machine vision image analyzer. Images were acquired by a solid-state CCD array 512 × 512, 8-bit video camera, providing a resolution of approximately 0.17 mm/pixel. Analysis was performed using BioImage EQ 2D electrophoresis pattern analysis software in a Sun 110/3 supermicrocomputer. The software captured the gel image and constructed a data base, referred to as the spot list, in which the *x*/*y* location, area and shape of each spot were reported. In addition, the integrated intensity (absorbance × mm^2) was calculated for each spot. To reduce variation introduced by inconsistencies in sample loading and/or staining, integrated intensities were summed and the percent integrated intensity reported. The computer generated image was recorded using a Gould 6320 colorwriter, with each spot plotted as a regular ellipse. Three gels from each variety were prepared, and it was necessary for a spot to occur on two of three gels in order for it to be evaluated. Rodeo and

Centurk 78 varieties were compared to Scout 66 which was selected as a reference. Spatial differences between a study and reference gel were corrected by a computer algorithm after manually identifying a limited number of spots that appeared on both gels. A computer program then identified all spots on a study gel that had an equivalent spot on the reference gel, based on the x/y location of the spot centers. The program identified spots where corrected x/y locations occurred in a 9×9 pixel square surrounding the center pixel of a reference spot.

Isoelectric focusing conditions

To evaluate the extraction procedure and for interlaboratory comparison purposes, Chinese Spring was also analyzed using equilibrium IEF in the first dimension according to previously described extraction and electrophoresis conditions, with the following modifications. In the first dimension gel, 50% pH 3–10, 25% pH 4–6.5, 12.5% pH 5–8 and 12.5% pH 8–10.5 ampholytes were used. Gels were run to equilibrium, a total of 6000 V h with 1000 V maximum.

RESULTS AND DISCUSSION

Extracted proteins were not fractionated prior to electrophoresis. It was estimated that 70–80% of the protein, as measured by the previously described Kjeldahl method, was extacted from the wheat flours. The procedure avoided the use of SDS-containing buffers which could after the pI of polypeptides if incompletely removed.[24]

Comparison of previously published methods for 2D electrophoretic separation of wheat proteins with the method presented here has revealed several important differences. First, the NEPHGE step used small diameter (approximately 1.0 mm), thin walled tubes. This allowed application of higher field strengths (approximately 300 V/cm) than were possible with the larger tubes used in other studies[17,18,23,28]. The higher field strength was tolerated without adverse effects because of faster heat dissipation. Secondly, by applying up to 3000 V, only 2.3 h were required to obtain a total of 4800 V h. In order to achieve equivalent separations, other NEPHGE methods cited[19,23,28] required 4 h (500 V; 2000 V h), 7–8 h (1000 V max.; 6000–7000 V h) and 14 000 V h respectively. Thirdly, the use of smaller SDS-PAGE gels ($70 \times 80 \times 1.0$ mm) for the second dimension resulted in much shorter run times (55 min as compared to 3–22 h) and more rapid staining–destaining[19,28]. The thinner SDS-PAGE gels were also of benefit to silver staining procedures (results not shown). Finally, the smaller scale system described here also required less sample, and allowed us to obtain quantitative results. While small diameter tubes have been used before in isoelectric focusing applications[29,30], few others have also incorporated the use of high field strengths to concomitantly improve resolution and decrease separation time.

The variety Chinese Spring has been extensively studied by 2D electrophoresis[14,28] and was therefore included in this study for comparative purposes. Payne *et al.*[14] separated approximately 100 polypeptides from Chinese Spring by using both IEF and NEPHGE in the first dimension and composing a collage of the two final gels. Using only a NEPHGE technique in the first dimension, Anderson *et al.*[28] identified approximately 80 polypeptides, including ten low-molecular-weight peptides not analyzed by Payne *et al.*[14]. The results presented here showing more than 25

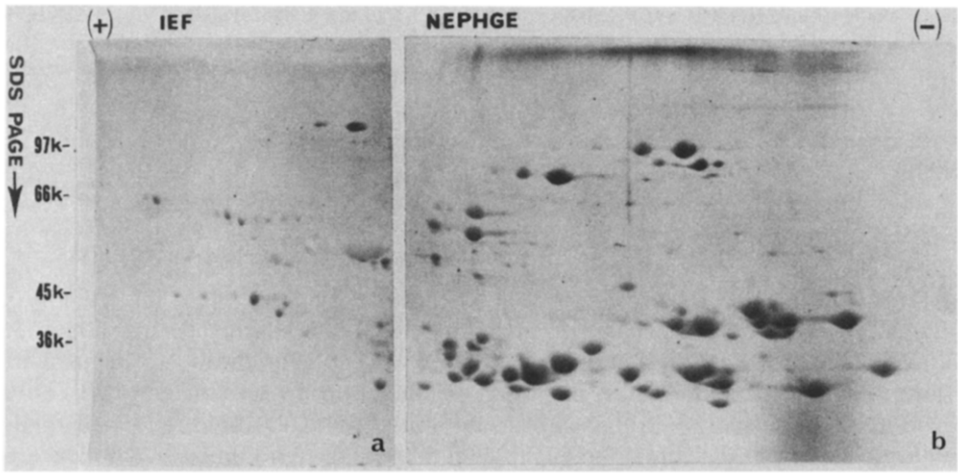

Fig. 1. Two-dimensional electrophoretic separation of Chinese Spring wheat proteins. IEF–SDS-PAGE separation in panel a. NEPHGE–SDS-PAGE separation of 44 μg total protein in panel b. Electrophoretic separations were conducted as described in the Experimental section. Positions of M_r markers are indicated at the left and are: phosphorylase B, 97 000; bovine serum albumin, 66 000; ovalbumin, 45 000; and lactic dehydrogenase, 36 000.

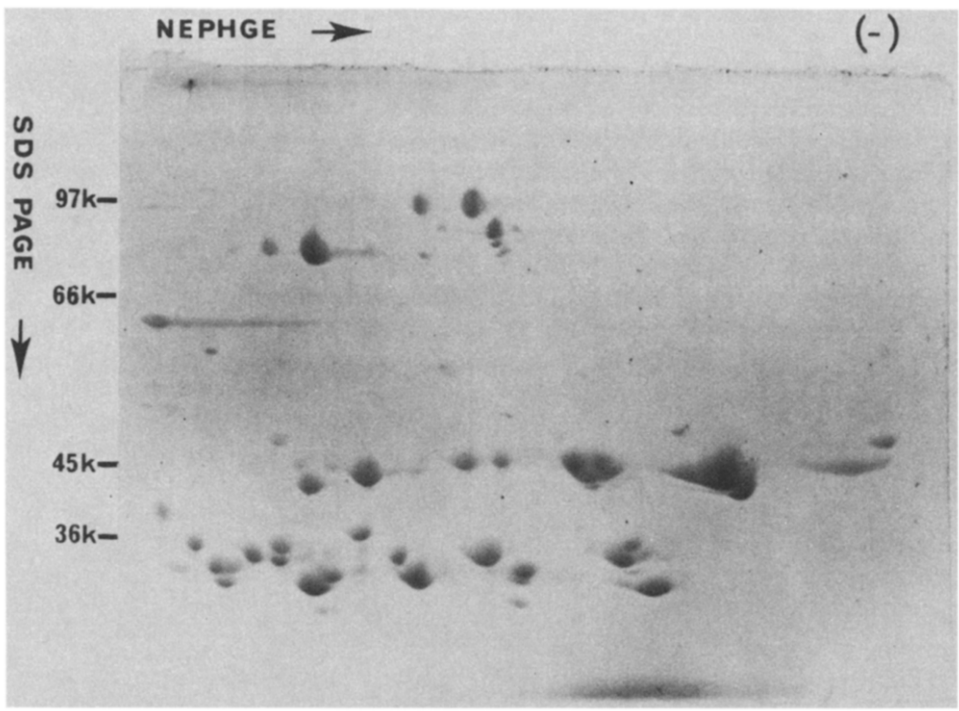

Fig. 2. NEPHGE–SDS-PAGE separation of Centurk 78 wheat endosperm proteins (44 μg).

polypeptides in the IEF separation (Fig. 1a) and an additional 74 in the NEPHGE separation (Fig. 1b) are thus in good agreement with these investigators.

Improvement in the resolution of individual Chinese Spring polypeptides within certain regions of the gel was observed in the results reported here (Fig. 1a and b), compared to those of others[14,28]. Groups of high M_r spots tentatively designated as high M_r glutenins and albumins, appear to have been more completely resolved. There is greater horizontal (IEF and NEPHGE) separation of individual polypeptides and less streaking of unresolved components.

Figs. 2–4 contain representative gels of the 2D electrophoretic separation of endosperm proteins extracted from the commercial HRW varieties Centurk 78, Rodeo and Scout 66. In these separations, NEPHGE was chosen as the sole first dimension method. While recognizing that not all endosperm polypeptides would be resolved, this compromise was accepted because it was not practical to compose a collage of two separations when using image analysis. In addition, the NEPHGE method was found to be very effective at separating most of the endosperm components. Fig. 5 contains the computer generated image from analysis of the Scout 66 gel shown in Fig. 4. This variety was used as the standard to which the others were compared, and the system of spot numbering is shown in Fig. 5. For the three varieties studied, the percent integrated intensity values obtained from image analysis of these spots are presented in Table II. Spots in Centurk 78 and Rodeo for which there were no corresponding spots in Scout 66 were not reported. The total number of spots for Centurk 78, Rodeo and Scout 66 was 59, 80 and 99, respectively.

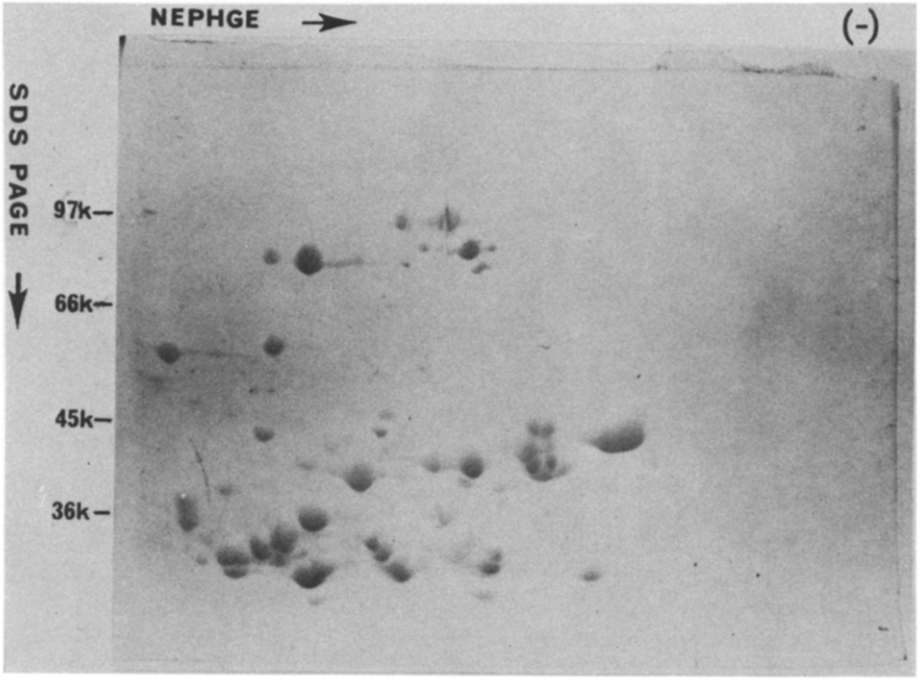

Fig. 3. NEPHGE–SDS-PAGE separation of Rodeo wheat endosperm proteins (44 μg).

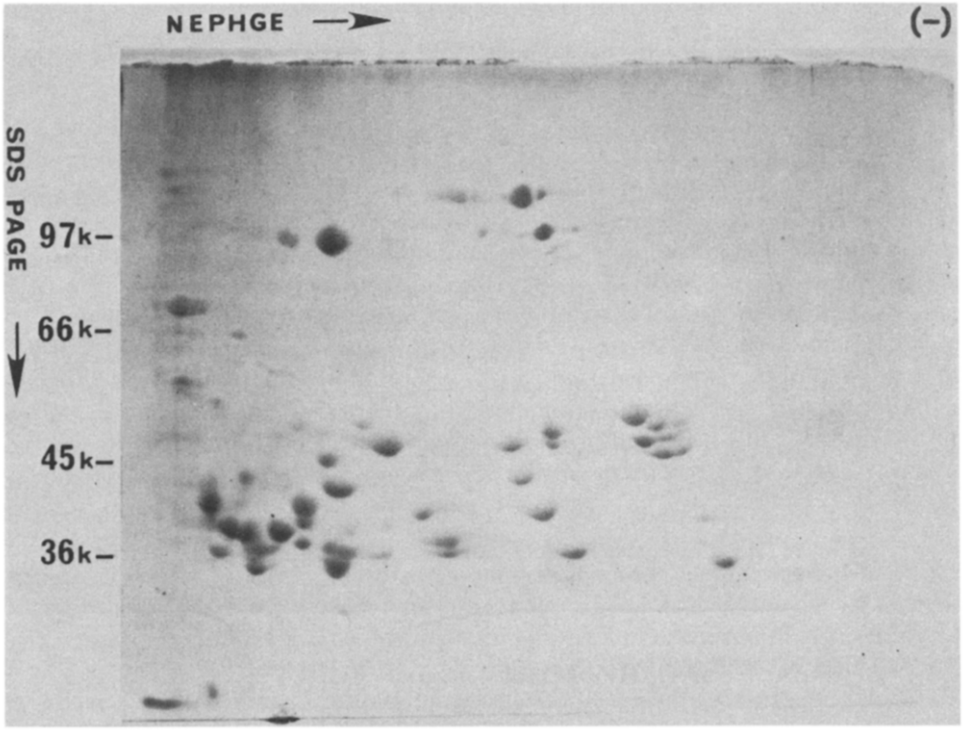

Fig. 4. NEPHGE–SDS-PAGE separation of Scout 66 wheat endosperm proteins (44 μg).

Centurk 78 was found to have 35 spots in common with Scout 66 (Table II), while Rodeo had 41 spots in common with Scout 66. The 2D separation patterns of all three commercial HRW varieties showed some common features. The apparent M_r of polypeptides in the results presented here (Figs. 1–4) varied from 25 000 to 100 000. There was a group of 7–14 polypeptides with M_r values in the range of 70 000 to 100 000, corresponding to spots 4–10 and 12–17 in Table II. The components migrating in this 70 000–100 000 range appear to be high M_r glutenins, as similar separation patterns have been obtained by others[14]. However, it must be emphasized that identification of these components as high M_r glutenins is only tentative, as comparison of relative mobilities with purified glutenins has not yet been performed. The apparent M_r range found here for these polypeptides is not in agreement with Ng and Bushuk[31], who reported high M_r glutenins in the range of 92 000 to 146 000. No components with M_r values greater than 105 000 were found in our study. The most likely explanation for this discrepancy is the demonstrated variability of SDS-PAGE determined M_r values for wheat proteins[32–34]. Apparent M_r values have been shown to vary with acrylamide concentration, as high acrylamide concentrations in the separating gel result in lower apparent M_r values[33]. The acrylamide concentration used in SDS-PAGE separations shown here was 12% (bisacrylamide–acrylamide, 1:50).

There was a second group of polypeptides in the 45 000 to 66 000 M_r range. These

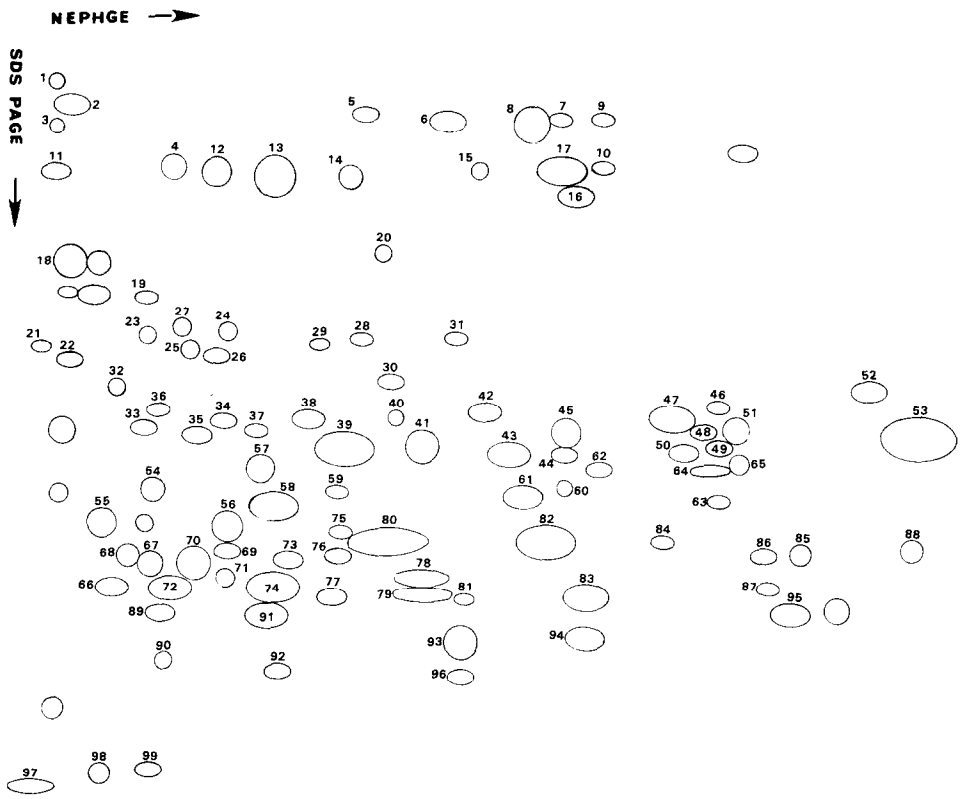

Fig. 5. Computer generated plot from image analysis of NEPHGE–SDS-PAGE gel containing Scout 66 wheat endosperm proteins. The image analysis program assigned numbers (1–99) to each of the spots as indicated.

components were concentrated in the relatively more acidic region of the gel (left side) and corresponded to spots 18–38 in Table II. Finally, a large group of polypeptides was found in the 20 000 to 45 000 M_r range. This region contained the greatest variability and was composed of 30–50 components. This region corresponds approximately to spot numbers 39–99 in Table II, from which it can be seen that there was also a large amount of variability in the percent integrated intensities of many components.

The four wheat varieties examined by 2D electrophoresis show considerable variation in the profile of constitutive polypeptides, which may reflect their quality as bread-making wheats. These varieties range in quality from very poor (Chinese Spring) to good (Centurk 78). The electrophoretic procedure described here will be of use in analyzing specific polypeptides for their influence on functional qualities of wheat flours. The presence of specific polypeptides and their relative amounts may be of significance to loaf volume, mixing time and other quality characteristics. An investigation to examine the correlation of the endosperm components with these baking quality parameters is currently underway. This procedure may also be of use in genetic studies of wheat varieties.

TABLE II

MEAN PERCENT INTEGRATED INTENSITIES OF INDIVIDUAL SPOTS IN THE VARIETIES RODEO AND CENTURK 78 THAT MATCH THOSE IN THE VARIETY SCOUT 66

Numbers *1–99* indicate the spot number in the electropherogram (see Fig. 5). Integrated intensity values are the mean of three gels.

Variety									
				1	*2*	*3*	*4*	*5*	*6*
Scout 66				0.382	0.254	0.190	0.085	0.192	1.107
Centurk 78				0.000	0.000	0.000	0.000	0.000	1.411
Rodeo				0.000	0.038	0.000	0.000	0.020	1.001
	7	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*
Scout 66	0.629	3.427	0.115	0.290	0.143	0.679	4.280	0.176	0.320
Centurk 78	0.000	3.635	0.000	0.146	0.000	0.000	4.133	0.000	0.155
Rodeo	0.000	2.850	0.057	0.178	0.000	0.538	4.383	0.000	0.174
	16	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*
Scout 66	0.321	1.856	1.530	0.160	0.033	0.147	0.398	0.018	0.088
Centurk 78	0.295	1.836	1.097	0.000	0.000	0.000	0.078	0.000	0.000
Rodeo	0.463	2.353	0.065	0.000	0.000	0.000	0.304	0.000	0.000
	25	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*
Scout 66	0.066	0.073	0.043	0.069	0.034	0.259	0.106	0.202	0.082
Centurk 78	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.143	0.000
Rodeo	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.089	0.000
	34	*35*	*36*	*37*	*38*	*39*	*40*	*41*	*42*
Scout 66	0.278	0.167	0.049	0.284	0.623	4.081	0.281	0.316	0.227
Centurk 78	0.279	0.000	0.000	0.205	0.000	4.840	0.000	0.000	0.000
Rodeo	0.000	0.000	0.000	0.000	0.206	0.000	0.298	0.000	0.000
	43	*44*	*45*	*46*	*47*	*48*	*49*	*50*	*51*
Scout 66	2.547	0.895	2.997	0.390	3.631	1.183	0.695	1.542	1.077
Centurk 78	2.187	0.627	0.000	0.000	0.000	0.000	1.669	3.628	0.000
Rodeo	1.486	0.000	0.000	0.000	2.423	0.728	0.779	1.253	0.000
	52	*53*	*54*	*55*	*56*	*57*	*58*	*59*	*60*
Scout 66	0.229	1.341	0.476	2.477	2.816	1.240	2.840	0.150	0.158
Centurk 78	0.000	0.000	0.000	1.164	0.000	2.122	0.000	0.000	0.000
Rodeo	0.000	9.657	0.633	2.218	0.000	0.000	4.676	0.000	0.448
	61	*62*	*63*	*64*	*65*	*66*	*67*	*68*	*69*
Scout 66	2.388	0.422	0.173	1.463	0.783	0.893	1.816	1.627	0.930
Centurk 78	0.000	0.000	0.000	0.369	1.134	0.732	0.000	1.584	2.112
Rodeo	0.000	0.000	0.000	0.987	0.959	0.554	0.000	0.478	4.377
	70	*71*	*72*	*73*	*74*	*75*	*76*	*77*	*78*
Scout 66	3.266	0.873	1.592	0.664	2.973	0.196	0.318	0.970	5.060
Centurk 78	2.193	1.147	3.569	0.000	0.000	0.000	0.000	0.000	0.000
Rodeo	2.431	0.603	4.727	0.000	0.000	0.000	0.596	0.000	0.000
	79	*80*	*81*	*82*	*83*	*84*	*85*	*86*	*87*
Scout 66	1.843	1.995	0.410	4.277	2.973	0.121	0.292	0.923	0.216
Centurk 78	0.000	0.000	0.000	0.000	3.556	0.000	0.191	0.633	0.000
Rodeo	0.000	0.000	0.000	0.000	1.872	0.000	0.000	0.000	0.000
	88	*89*	*90*	*91*	*92*	*93*	*94*	*95*	*96*
Scout 66	0.168	1.092	0.117	3.132	0.429	0.222	0.878	2.501	0.164
Centurk 78	0.000	0.940	0.000	7.863	0.527	0.000	0.693	3.298	0.000
Rodeo	0.000	0.825	0.000	6.099	0.488	0.000	0.888	1.370	0.000
	97	*98*	*99*						
Scout 66	1.886	0.737	0.049						
Centurk 78	0.000	0.000	0.000						
Rodeo	0.000	0.000	0.000						

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