Composition of Acid- and Alkali-Extracted Barley Proteins as Revealed by Isoelectric Focusing[1]

A. M. EL-NEGOUMY, C. W. NEWMAN, and B. R. MOSS, Department of Animal and Range Sciences, Montana State University, Bozeman 59717

ABSTRACT

Barley cultivars Carlesberg II and its mutant Carlesberg/Risφ-56, and Bomi and its mutant Bomi/Risφ 1508 were extracted with 0.05*M* NaOH or 0.05*M* acetic acid to remove glutelin after sequential extraction of the albumin, globulin, and hordein fractions. Samples of these cultivars were also extracted with deionized water at pH 2, 4, 6, 8, 10, and 11. Albumin and globulin were isolated from a sample of Lenta barley, both before and after dialysis in deionized water, to remove natural salts. An isoelectric focusing technique more suitable for barley proteins was used to examine the extracted proteins. The glutelin extracted with acetic acid represented only 1.1% of the total protein and appeared, on the basis of isoelectric points, to be composed of albumin and globulin components. However, this may not be the true composition, because proteins with similar isoelectric points may differ in their other chemical and physical properties. Glutelin extracted with NaOH represented about 25% of the total protein and differed completely in electrofocusing composition from that extracted by acetic acid. The proteins extracted with deionized water at pH 2–8 resembled those extracted by acetic acid, whereas those extracted at pH 10 and 11 contained all protein fractions including glutelin. These results may indicate that the acetic acid extract consisted mainly of the remnants of albumin plus globulin left in the residue after albumin, globulin, and hordein were extracted. Removal of natural salts from a sample of Lenta flour by dialysis gave an albumin fraction much less contaminated with globulin and ensured more complete recovery of the globulin fraction.

Cereal Chem. 60(6):429-432

Isoelectric focusing (IEF), which includes isoelectrofocusing and electrofocusing, is one of the most efficient techniques for protein separation, and has been extensively reviewed (Righetti and Drysdale 1979). The technique has been applied to studies of maize proteins (Willson et al 1981), wheat proteins (Almgard and Clapham 1977), rice proteins (Padhye and Salunkhe 1979, Shadi and Djurtoft 1979), and barley proteins (Leback and Wrigley 1976; Shewry et al 1978, 1980).

In polyacrylamide gel electrophoresis, protein components move continuously through the gel and are separated according to size and charge. Spreading of the protein zones because of diffusion is a continuous process throughout electrophoresis. In IEF, however, protein components concentrate in a restricted zone on any part of the gel containing an ampholyte matching their isoelectric point (pI) and remain stationary as the pH is maintained. Thus, spreading because of diffusion is considerably reduced.

Glutelin contains most of the nitrogeneous constituents left in the grain meal after the saline and alcohol-soluble proteins are removed. This protein has been reported to be solubilized only by solutions of low pH value (mild acids) or high pH value (mild alkalis), and low ionic strength (Wall 1964). The extraction and solubilization of glutelin remains a problem, however; the solvents required tend to alter its native structure (Orth and Bushuk 1973).

Dilute acetic or lactic acids have been used most frequently for extracting wheat flour proteins. Mecham et al (1972) reported that acetic acid failed to solubilize part of the wheat protein. After repeated extraction of glutelin with 0.05*M* acetic acid (AcOH), a gel was formed that contained a high proportion of protein, which

[1]Published with the approval of the director of Montana Agriculture Experiment Station. Journal series no. 1271.

was extracted only when 0.04 mM $HgCl_2$ was added to the acetic acid extractant. Bietz and Wall (1975) found that more effective solvents, which tended to modify protein composition and lead to inconsistencies, were required to extract glutelin insoluble in dilute AcOH. These authors reported that the amounts of protein extractable by AcOH were relatively small, ranging from 6 to 12% of the total protein, depending on the wheat variety. After extensive extraction with dilute AcOH, much of the glutelin protein in wheat flour remained undissolved in the gel protein (Cluskey and Dimler 1967, Inamine et al 1967) or in the residue (Chen and Bushuk 1970). Orth and Bushuk (1972) found very little glutelin in the AcOH extract, particularly in wheats of good baking quality. Ali et al (1975), in working with Hiproly, Larker, Nordic, Dickson, and Conquest barley cultivars, reported the AcOH-soluble glutelin to be extremely low, ranging from 0.9 to 1.31% of the total protein.

In this paper, we describe conditions suitable for sharp separation of the components of some barley protein fractions through high-voltage IEF in thin-layer (1.5-mm) polyacrylamide gel plates (PAG plates). The main objective of our investigation, however, was to compare the composition of glutelins extracted from four barley cultivars both by AcOH and NaOH, through applications of thin-layer IEF.

MATERIALS AND METHODS

Barley Source and Treatment

The four barley cultivars Carlesberg II and its mutant Carlesberg II/Risø-56, and Bomi and its mutant Bomi/Risø 1508 were used in the present work. These cultivars were raised in 1979 at the Montana State University agronomy farm. Handling of the grains for analysis, separation of the combined albumin + globulin from a sample of whole meal, and sequential separation of albumin, globulin, and hordein from another sample of whole meal have been reported (El-Negoumy et al 1977, 1980).

In one set of experiments, the glutelins were extracted with 0.05M NaOH from the residue of the four barleys after removal of the albumin, globulin, and hordein. Forty milliliters of extractant was used in each of four extractions. The extracts were combined and then dialyzed against distilled water. In another set of experiments, the residues were extracted four times with 40 ml each of 0.05M AcOH. The extracts were combined for each sample and exhaustively dialyzed.

In another set of experiments, barley protein was extracted at various pHs from the whole meal. Six samples of 2 g each from each of the four barley cultivars in water suspension were extracted at pHs 2, 4, 6, 8, 10, and 11 as follows: The sample was suspended in 40 ml of deionized water, and the proper pH was obtained by dropwise addition of either 0.1M NaOH or 0.1M HCl. Each sample was extracted four times at each pH on a magnetic stirrer at room temperature for 30 min, before being centrifuged at 5,000 × g.

The supernatants from each pH were combined and dialyzed in a dialysis tank provided with a continuously flowing stream of distilled water at the rate of 500 ml/hr in the cold (5°C) room.

In the final experiment, 1 g of whole meal of the cultivar Lenta was extracted three times with 40 ml of deionized water each time to remove albumin. A second meal sample was suspended in 40 ml of water and exhaustively dialyzed to remove the naturally occurring salts, then albumin was extracted from it with deionized water. The residues from samples 1 and 2 were extracted three times each, with 40 ml of 0.5M NaCl plus 0.05M EDTA to remove the globulin. In a third sample, the meal was extracted three times, 40 ml each time with 0.5M NaCl plus 0.05M EDTA, to remove the combined albumin plus globulin.

Protein Content of Extracts

An aliquot 0.5–1.0 ml of each extract was analyzed for nitrogen by micro-Kjeldahl, and the protein content was calculated as N × 6.25.

Isoelectric Focusing

In preparation for electrofocusing, the extracts were exhaustively dialyzed against cold (5°C), distilled water, then perevaporated to about one fifth of the original volume and lyophilized. Complete solubilization of the freeze-dried proteins was impossible under the mild buffer conditions (0.05M glycine plus 3M urea) required for electrofocusing. The proteins were completely solubilized, however, by perevaporating the extracts (about 120 ml) to a volume of about 10 ml. The nitrogen content was determined by micro-Kjeldahl, and 0.05M glycine plus 3M urea was added to the extract in dry form. The nitrogen content was adjusted to 10 mg/ml with deionized water, then stirred with a magnetic stirrer at room temperature for 30 min.

The LKB 2117-301 Multiphor basic unit, LKB 2103 constant power supply unit, and the LKB thin-layer ampholine PAG plates, pH 3.5–9.5 were used in this investigation. A Haake model FT constant-temperature circulator connected to a PolyScience K-30 refrigeration unit was used to circulate the coolant in the Multiphor unit.

The PAG plates were either used whole or were cut to a size appropriate for the number of samples used. The gel plate was placed on the cooling platform over a very thin layer of kerosene, and any air bubbles were excluded. The coolant was kept at a constant temperature of 3° C by circulating a 35:65 (v/v) mixture of methanol and distilled water. The gel should be allowed to remain at that temperature for 15 min before sample application.

The electrode strips included in the PAG kit were wetted, with 1M NaOH as a catholyte and 1M phosphoric acid as an anolyte. Any free droplets were eliminated by gentle shaking. The strips were then placed at their proper positions on the gel. Samples (30 μl) containing about 300 μg of protein were pipetted on 5 × 10 mm slips of Gelman sepraphore III (cellulose acetate), then placed on the gel at a distance about one third of the gel width from the cathode. About 2 cm of the length of the gel was kept free of samples to be used for pH determination. The sample slips were removed from the gel after 20–30 min, depending on the gel size.

The best field-strength conditions for resolving barley protein components were 30 W, 1,800 V, and 20 mA. Focusing was completed when maximum voltage was reached (1.5–2 hr for whole gels and 30 min for half gels). These conditions are suitable only for cooling temperatures of 2–4° C. Higher temperatures require lower field strength to prevent overheating of the gel and distortion of protein zones.

The fixing and storing method recommended by LKB works well and is fairly rapid, but it leaves a dark background that is not easily removed in the final rinsings. We obtained excellent results when we used the method of Righetti and Drysdale (1975). Following electrofocusing, the gel was immediately immersed in 0.05% Coomassie blue R-250 plus 1% cupric sulfate dissolved in 10:25:65% (v/v) acetic acid–ethanol–distilled water. The gel was then transferred to the same solution containing 0.01% Coomassie blue R-250 but no cupric sulfate. The gel was finally washed two to three times in a 10:10:87% (v/v) solution of acetic acid–ethanol–distilled water. This technique leaves the gel background absolutely transparent.

Measurement of pH Gradient

The pH gradient was measured by LeBack and Wrigley (1976). Small pieces of the gel devoid of sample were sectioned linearly and the ampholyte eluted in 0.3 ml of 10 mM KCl in a test tube. The head space of the test tube was flushed with nitrogen to exclude CO_2, and the pH was measured at 25° C with a micro-pH electrode.

RESULTS AND DISCUSSION

Figure 1A shows electrofocusing patterns of some protein fractions of the four barley cultivars Carlesberg II, Carlesberg/Risø-56, Bomi, and Bomi/Risø 1508. Columns 1–4 represent albumin, 5–8 globulin, and 9–12 glutelin for these four cultivars, respectively. The major components in albumin are located within the isoelectric points range of pH 7.8–8.7. These components appear to be similar for the four barleys and included six major components located in the proximity of the cathode. Differences in the number and location of the minor components covering the isoelectric points range of pH 4.4–7.8 are indicated, however. The

major globulin components in columns 5–8 of Fig. 1A are also similar for the four barleys and are composed of two major components (α-, β-globulins) with isoelectric points of about pH 4.0 and 4.2, very close to the anode. The alkali-soluble proteins in patterns 9–12 of Fig. 1A showed two to three very faint protein components with isoelectric points of pH 7.3–8.4 (bands 2–4) in a dark, streaked background. This background is probably caused by hydrolysis of glutelin during its isolation in harsh alkaline conditions. This may also occur as a result of the breakdown of the cystinyl residues, which give rise to polypeptides of lower molecular weights. Column 1, appearing in all the glutelins in Fig. 1A at the cathode end of the sample strips, probably represents glutelin components that did not resolve during electrofocusing. These components may be in an aggregated form that cannot enter the gel. Mertz (1957), Orth and Bushuk (1973), and Singh and Sastry (1977) reported that glutelins are extractable only with solvents that tend to modify and break down their native chemical structure. The pH of the 0.05*M* NaOH extractant was 11.4. Working with soybean protein, Oshino and Okamoto (1975) found that the conformation of the protein changed above pH 11 as a result of decomposition of cystinyl residues. This observation suggested that naturally occurring disulfide groups may change to sulfhydryl and sulfinic acid groups, and thus the resulting sulfhydryl residues may exist as mercaptide groups (Wall 1971). Gawron and Odstrchel (1967) reported that alkali treatment of protein may lead to destruction of cysteine by B-elimination. The cleavage reaction of disulfide bands in protein under the influence of alkali was depicted by Donovan (1967) as follows:

$$2\ RS - SR + 2\ OH^- = 2\ RS^- + 2\ RSOH$$

$$2\ RSOH = RSH + RSO_2H$$

$$2\ H^+ + 2\ OH^- = 2\ H_2O$$

$$2\ RS - SR + 4\ OH^- = 2\ RS^- + RSO_2 + 2\ H_2O$$

Columns 1–4 of Fig. 1B are electrofocusing patterns that show the composition of glutelins extracted with 0.05*M* acetic acid from Carlesberg II, Carlesberg/Risø-56, Bomi, and Bomi/Risø 1508, respectively.

Examination of those patterns in comparison to the alkali-extracted glutelins in columns 9–12 of Fig. 1A indicates substantial differences in composition. It is evident that components with isoelectric points of pH 4.0–6.8 in Fig. 1B are totally absent from the glutelin patterns in Fig. 1A. Some of the components with isoelectric points between pH 7.8 and 8.7 in Fig. 1B may represent glutelin, although they appear to have more in common with the components of the same isoelectric point range in the albumin fractions of Fig. 1A (columns 1–4). Also, the two major components at the top of AcOH patterns in Fig. 1B, near the anode, appear to resemble those in the globulin fractions in Fig. 1A (patterns 5–8) both in location and isoelectric points. But although components may have similarities in isoelectric points, they may differ in other chemical and physical properties. The percentage of protein fractions extracted by 0.05*M* NaOH and 0.05*M* AcOH after extraction of albumin, globulin, and hordein are shown in Table I. The data indicate that very little protein was extracted by AcOH (0.9–1.3%) in comparison to that extracted with NaOH (23.8–26.1%). Ali et al (1975) also reported the amounts of AcOH-extractable protein from barley to be very low, ranging from 0.95 to 1.31% of the total protein. Reports in the literature (Cluskey and Dimler 1967, Inamine et al 1967) also indicate that much of the wheat glutelin remains in the gel protein or in the residue (Chen and Bushuk 1970) after repeated extraction with AcOH.

Figure 1C shows the electrofocusing composition of proteins extracted from water suspensions of Carlesberg II whole flour at various pH values. The other three barley cultivars had similar composition for the electrofocusing patterns. Columns 1–6 represent extracts at pH 11, 10, 8, 6, 4, and 2, respectively. The protein content of the extracts for four barley cultivars at these pH values, together with the protein content of one extraction with 0.5*M* NaCl plus 0.05*M* EDTA solution at pH 6.3 are presented in Table II. Examination of the patterns in Fig. 1C indicates that mainly globulin was extracted at pHs 2, 4, 6, and 8 together with small amounts of albumin. The percentages of protein extracted at these pHs (Table II) ranged from .7% at pH 2 to 17.3% at pH 8. At the higher pH values of 10 and 11 (Fig. 1C, columns 5, 6) a mixture of albumin, globulin, glutelin, and probably hordein was extracted in amounts ranging from about 50% at pH 10 to 86% at pH 11. These results support the conclusion that both the amounts and

TABLE I
Percent of Glutelin of Total Protein Extracted by 0.05*M* NaOH and 0.05*M* Acetic Acid

Barley Cultivar	0.05*M* Acetic Acid (pH 2.3)	0.05*M* NaOH (pH 11.4)
Carlesberg II	1.1	23.8
Carlesberg/Risø-56	1.3	24.9
Bomi	0.9	26.1
Bomi/Risø 1508	1.2	24.3

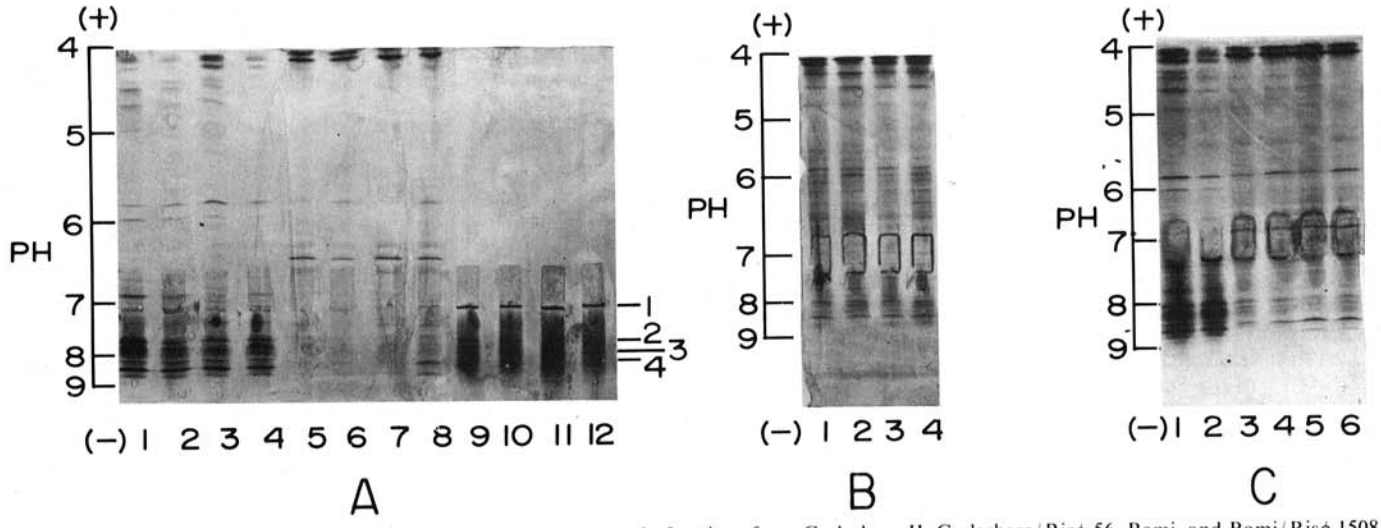

Fig. 1. Electrofocusing of various protein fractions. **A,** Control: protein fractions from Carlesberg II, Carlesberg/Risø-56, Bomi, and Bomi/Risø 1508. Columns 1–4 = albumin, 5–8 = globulin, 9–12 = alkali-extracted glutelin. **B,** Acetic acid-extracted glutelin. Columns 1–4 represent protein extracted from Carlesberg II, Carlesberg/Risø-56, Bomi, and Bomi/Risø 1508, respectively. **C,** Water extracts of Carlesberg II whole meal at various pHs. Columns 1–6 represent pH 11, 10, 8, 6, 4, and 2, respectively. Conditions: thin-layer ampholine polyacrylamide gel plates, pH 3.5–9.5. Coolant temperature 3°C. Field strength 30 W, 1,800 V, and 20 mA.

TABLE II
Effect of pH on Amounts of Protein Extracted from Whole Grain of Four Barley Cultivars

Barley Cultivar	Percent of Protein						
	pH 2	pH 4	pH 6	pH 8	pH 10	pH 11	One Extraction with NaCl-EDTA Solution (pH 6.3)
Carlesberg II	0.7	2.8	8.2	13.1	61.4	82.5	23.9
Carlesberg/Risø-56	0.9	4.6	10.3	17.3	58.7	79.4	20.8
Bomi	0.8	6.7	8.5	15.0	52.3	86.2	27.5
Bomi/Risø 1508	0.9	3.9	7.8	12.6	49.8	82.4	24.1

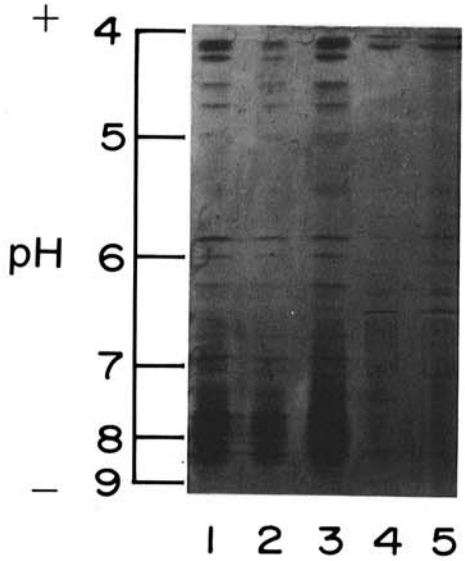

Fig. 2. Electrofocusing of albumin and globulin from Lenta barley. Column 1, Albumin extracted with deionized water; 2, albumin extracted from sample previously dialyzed against distilled water to remove naturally occurring salts; 3, albumin + globulin extracted with 0.5*M* NaCl + 0.05*M* EDTA; 4, globulin extracted with 0.5*M* NaCl + 0.05*M* EDTA from residue obtained after removal of albumin in column 1; 5, globulin extracted with 0.5*M* NaCl + 0.05*M* EDTA from residue obtained after removal of albumin in column 2. Electrofocusing conditions: thin-layer ampholine polyacrylamide gel plate, pH 3.5–9.5. Cooling temperature 3°C. Field strength 30 W, 1,800 V, and 20 mA.

composition of the proteins extracted may be dependent on the pH of the solvent. Table II also shows that upon extraction of these barley cultivars with a solution of 0.5*M* NaCl + 0.05*M* EDTA at pH 6.3, about 2–3.5 times the amount of protein extracted at pH 6.0 from a water solution was obtained. This indicates that the amount of protein extracted was more dependent on the kind of solvent used rather than on its pH. The pH of the AcOH extractant in Table I is between 2 and 3. Similarities in composition between the AcOH extract from Carlesberg II (Fig. 1B, column 1) and its water extract of pH 2 (Fig. 1C, column 6) are evident, especially with regard to the globulin components. This, together with the low yield of protein obtained in both cases (0.7–0.9 and 0.9–1.3%, respectively) led to the conclusion that AcOH may extract the remnants of albumin and globulin left in the residue after these proteins are removed from the meal.

The effect of solvent composition on the composition of protein extracted from barley is further illustrated by the results in Fig. 2. Column 3 of Fig. 2 represents the salt-soluble protein (albumin + globulin) extracted from Lenta barley. In column 1, the albumin from Lenta barley was extracted with deionized water, whereas in column 2 the albumin was recovered after the flour sample was dialyzed and the natural salts removed. Columns 4 and 5 represent globulin extracted with 0.5*M* NaCl plus 0.05*M* EDTA from the residues in columns 1 and 2, respectively. Removal of natural salts by dialysis (column 2) yielded an albumin that was much less contaminated with globulin in comparison to the nondialyzed sample (column 1). Also, the globulin from the dialyzed residue (column 5) was much more completely recovered than that obtained from the residue from the undialyzed flour sample (column 4).

LITERATURE CITED

ALI, M. R., McDONALD, C. C., and BANASIK, O. J. 1975. Protein properties of a Hiproly barley cross. Proc. Am. Soc. Brew. Chem. 33:28.

ALMGARD, G., and CLAPHAM, D. 1977. Swedish wheat cultivars distinguished by content of gliadins and isozymes. Swed. J. Agric. Res. 7:137.

BIETZ, J. A., and WALL, J. S. 1975. The effect of various extractants on the subunit composition and associations of wheat glutenin. Cereal Chem. 52:145.

CHEN, C. H., and BUSHUK, W. 1970. Nature of protein in Triticale and its parental species. I. Solubility, characteristics and amino acid composition of endosperm proteins. Can. J. Plant Sci. 50:9.

CLUSKEY, J. E., and DIMLER, R. J. 1967. Characterization of acetic acid-insoluble fraction of wheat gluten protein. Cereal Chem. 44:611.

DONOVAN, J. W. 1967. Spectrophotometric observation of the alkaline hydrolysis of protein disulfide bonds. Biochem. Biophys. Res. Commun. 29:273.

EL-NEGOUMY, A. M., NEWMAN, C. W., and MOSS, B. R. 1977. Chromatographic fractionation and composition of salt-soluble protein from Hiproly (CI 3947) and Hiproly Normal (CI 4362) barleys. Cereal Chem. 54:332.

EL-NEGOUMY, A. M., NEWMAN, C. W., and MOSS, B. R. 1980. Solubility of barley proteins in various phosphate buffer extractants. Nutr. Rep. Int. 21:99.

GAWRON, O., and ODSTRCHEL, G. 1967. Kinetic studies on the alkaline decomposition of cysteine derivatives and peptides. J. Am. Chem. Soc. 89:2263.

INAMINE, E. S., NOBLE, E. G., and MECHAM, D. K. 1967. Solubilization and fractionation of wheat flour protein insoluble in dilute acetic acid. Cereal Chem. 44:143.

LEBACK, D. H., and WRIGLEY, C. W. 1976. Isoelectric focusing. Page 306 in: Chromatographic and Electrophoretic Techniques. N. N. Smith and S. W. T. Seakins, eds. Heinmann, London.

MECHAM, D. D., COLE, E. W., and NG, H. 1972. Solubilizing effect of "gel" protein of flour. Cereal Chem. 49:21.

MERTZ, E. T., and BRESSANI, R. 1957. Studies on corn proteins. I. New methods of isolation. Cereal Chem. 34:63.

ORTH, R. A., and BUSHUK, W. 1972. A comparative study of the proteins of diverse baking qualities. Cereal Chem. 49:263.

ORTH, R. A., and BUSHUK, W. 1973. Studies of glutelin. I. Comparison of preparative methods. Cereal Chem. 50:106.

OSHINO, K., and OKAMOTO, S. 1975. Molecular interaction of denatured soybean proteins. Cereal Chem. 52:9.

PADHYE, V., and SALUNKHE, D. K. 1979. Extraction and characterization of rice proteins. Cereal Chem. 56:389.

RIGHETTI, P. G., and DRYSDALE, J. W. 1979. Isoelectric focusing. Page 341 in: Techniques in Biochemistry and Molecular Biology. North Holland Publ. Co., Amsterdam.

SHADI, A. I., and DJURTOFT, R. 1979. Studies of rice proteins by crossed immunoelectrophoresis, gel electrophoresis, and isoelectric focusing. Cereal Chem. 56:402.

SHEWRY, P. R., ELLIS, J. R. S., PRATT, H. M., and MIFLIN, B. J. 1978. A comparison of methods for the extraction and separation of hordein from 29 barley varieties. J. Sci. Food Agric. 29:433.

SHEWRY, P. R., FAULKS, A. J., and MIFLIN, B. J. 1980. Effect of high lysine mutations on the protein fractions of barley grains. Biochem. Genet. 18:133.

SINGH, U., and SASTRY, L. V. S. 1977. Studies on the proteins of the mutants of barley grains. I. Extraction and electrophoretic characterization. Cereal Chem. 54:1.

WALL, J. S. 1964. Cereal proteins. Page 315 in: Symposium of Food Proteins and Their Reactions. H. W. Schultz and A. F. Anglemeyer, eds. Avi Publ., Westport, CT.

WALL, J. S. 1971. Disulfide bonds. Determination, location, and influence on molecular properties of proteins. J. Agric. Food Chem. 19:619.

WILLSON, C. M., SHEWRY, P. R., and MIFLIN, B. J. 1981. Maize endosperm protein compared by sodium dodecyl sulfate gel electrophoresis and isoelectric focusing. Cereal Chem. 58:275.

[Received April 2, 1982. Accepted May 27, 1983]