Cereal Chemistry

Vol. 54 January-February 1977 No. 1

STUDIES ON THE PROTEINS OF THE MUTANTS OF BARLEY GRAIN. I. EXTRACTION AND ELECTROPHORETIC CHARACTERIZATION

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ABSTRACT Cereal Chem. 54(1): 1-12

Solubility fractionation and polyacrylamide gel electrophoresis of the proteins of a barley variety, two mutants produced therefrom, and Hiproly barley were carried out after studying the favorable conditions for their quantitative extraction. Comparison of the proportions of the various extracted protein fractions (including the glutelin fraction extracted by employing the acetic acid, urea, and cetyltrimethyl ammonium bromide (AUC) solvent system) and the qualitative and quantitative changes in the electrophoretic patterns in the proteins from different barleys indicated interesting differences in the protein composition of these barleys. The electrophoretic changes were found most striking in the glutelin and albumin fractions and less striking in the globulin and prolamin fractions. The changes in the proportion of the protein fractions were most conspicuous in the prolamin fraction, less in the glutelin and albumin fractions, and least in the globulin fraction of the mutants. These changes appear to be nutritionally more favorable in the Notch-2 mutant.

The discovery of *opaque*-2 and *floury*-2 mutant genes (1,2) in corn developed renewed interest among plant breeders to evolve varieties with better nutritional quality in different cereals. In order to upgrade the nutritional quality of the protein of grains by genetic manipulation, an understanding of the nature and genetic differences of proteins from different varieties of grain is an important prerequisite.

The recent identification of the 'Hiproly' barley of Ethiopian origin from the World Barley Collection (3) with a high-protein and high-lysine content and the subsequent development of Notch mutants (4) with high protein and high lysine at this Institute have generated interest in the nature of genetic changes induced in these mutants. Some earlier work on the proteins of barley indicated interesting differences in water-soluble, salt-soluble (5), and alcohol-soluble protein fractions (6) of some genetically different varieties, including 'Hiproly'. However, more comprehensive study is required, especially on the storage protein fractions, *viz.*, the prolamins and glutelins.

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The study of cereal proteins in general poses problems in extraction and solubility and, as a consequence, different workers (7–9) have studied the proteins of wheat, corn, and sorghum by electrophoresis. The poor solubility was circumvented by using urea in the system. Nevertheless, the extraction and study of the glutelin fraction remain a problem, as they require the use of solvents which tend to modify the native chemical character of the proteins, making these solvents unsuitable for study.

In the present study, the solubility fractionation of the proteins of the parent variety NP-113, and two mutants Notch-1 and Notch-2 produced therefrom, together with the Hiproly barley for comparison, and the polyacrylamide gel electrophoresis of the different protein fractions of these barleys have been carried out. This includes extraction and electrophoresis of the glutelin.

MATERIALS AND METHODS

Grain Samples

Barley (*Hordeum vulgare*) samples of four different varieties, NP-113, Notch-1, Notch-2, and Hiproly, grown under similar cultural conditions have been used in the present study. Notch-1 and Notch-2 are induced mutants of NP-113. These were isolated from the progeny of ethylmethanesulfonate-treated seeds (4).

Separation of Protein Fractions from the Grain

Hundred-mesh flour samples defatted with acetone at room temperature (25°–27° C) were used for the extraction of the protein fractions. The defatted flour sample was successively extracted with the solvents, water, 0.5*M* sodium chloride solution, 70% ethanol, and 0.1*N* alkali, respectively, to separate the classical protein fractions described by Osborne (10): albumins, globulins, prolamins, and glutelins.

The extraction was carried out with each solvent by gentle shaking for 2 hr with a flour-to-solvent ratio of 1:10 (w/v). The extract was clarified by centrifugation and the residue was washed similarly for 1 hr with two 5-ml portions of the respective solvents before extraction of the next fraction. The extracts of each fraction were combined and diluted to 25 ml. All the extractions were carried out at room temperature (25°–27° C).

The water-soluble fraction was lyophilized without dialysis. The salt-soluble and alkali-soluble fractions (after neutralization with acetic acid) were lyophilized after dialysis. The alcohol-soluble fraction was obtained in dry form by rotary evaporation under vacuum at 37° C. All the protein fractions were stored in the frozen state.

Alternative Method for Glutelin Preparation

After the albumin, globulin, and prolamin were separated, the residue was extracted with a 0.1*M* acetic acid solution containing 3*M* urea and 0.01*M* cetyltrimethyl ammonium bromide (AUC solvent) (11) in the usual ratio of 1:10 (w/v). The protein was precipitated from the extracts by saturating the solution with ammonium sulfate in the cold. The precipitate was allowed to settle overnight, washed with distilled water to remove ammonium sulfate, and dried

under vacuum over P_2O_5 in a desiccator at room temperature. This fraction was labeled AUC-soluble glutelin.

Direct Extraction with AUC Solvent

This was carried out by gently shaking a flour-to-solvent mixture of 1:10 (w/v) for 2 hr. The extract was clarified by centrifugation and the residue was extracted similarly with two 10-ml portions of solvent for 1-hr periods. The extracts were pooled and made up to a final volume of 50 ml. The protein was precipitated as described earlier for AUC-soluble protein.

Determination of the Protein Content

The protein content of each fraction and the total protein content of the flour samples were determined by micro-Kjeldahl nitrogen analysis and by multiplying the percentage nitrogen by the conventional factor of 6.25. Moisture correction was applied for the flour samples. Corrections were made for the nonprotein nitrogen (NPN) in the grain samples and the albumin fractions. The NPN of the flour samples was determined by extracting the NPN with a 10% solution of trichloroacetic acid. The albumin fraction was precipitated from 10% trichloroacetic acid solution and the NPN in the supernatant was determined.

Electrophoresis

For electrophoresis, 300 μg and 400 μg, respectively, of freeze-dried water-soluble and salt-soluble protein samples in 0.1 ml of 4*M* urea were applied to each gel tube and electrophorized with operating pH 8.3 by the procedure of Davis (12). For clear identification of the minor protein bands, the amount of 400 μg of the salt-soluble protein was found suitable.

Alcohol-Soluble Proteins. Three hundred micrograms of flash-evaporated alcohol-soluble protein in 0.1 ml of 0.1*M* acetic acid containing 4*M* dimethylformamide and 4*M* urea was applied to each gel tube and examined using the procedure of Chen and Bushuk (13).

AUC-Soluble Proteins. Before electrophoresis, glutelin disulfide bonds were reduced by treating 1–2% protein solutions in 8*M* urea-phosphate buffer, pH 7.5, with 2-mercaptoethanol at about 100*M* excess over total disulfides at room temperature for 3 hr. The reduced protein in solution was reacted for 1 hr with 1:1 molar ratio of acrylonitrile to the initial 2-mercaptoethanol, as described by Huebner and Wall (14). The solution was dialyzed against distilled water and lyophilized. The electrophoresis was carried out under conditions similar to those for alcohol-soluble proteins, except the operating pH was 2.9, which was suitable for electrophoresis of this fraction.

Protein bands were stained for 1 hr with 0.5% solution of Amido black in 7% acetic acid. Unabsorbed dye was removed by repeated washings of the gels with 7% acetic acid. Protein bands were scanned in a Chromoscan (Joyce Loebl; England) which bears a linear scale.

RESULTS AND DISCUSSION

Table I shows the solubility fractionation, the protein content of grain samples, the results of direct extractions with the AUC solvent system, and

TABLE I
Distribution of Protein Fractions in Different Barley Varieties[a]

S. No.	Variety	100-Grain Weight g		Total Protein	Albumin	Globulin	Prolamin	Glutelin Alkali Extn.	Glutelin AUC Extn.	Extraction Efficiency		Prolamin %	
										Alkali extn.	AUC extn.	AUC glutelin % Ratio	AUC Direct Extn.
1	NP-113	2.96	a	13.8	1.9	1.6	4.1	3.4	4.6	...	...	...	...
			b	4.08	0.56	0.45	1.21	1.00	1.36	...	...	...	...
			c	...	13.9	11.0	29.7	24.6	33.3	79.2	87.9	0.89	83.3
2	Notch-1	2.31	a	17.2	3.2	1.7	4.0	5.2	6.0	...	...	...	...
			b	3.97	0.73	0.39	0.92	1.20	1.38	...	...	...	...
			c	...	18.5	9.9	23.2	30.2	34.9	81.8	86.5	0.66	86.5
3	Notch-2	2.45	a	19.0	3.4	2.1	4.0	6.1	7.6	...	...	...	...
			b	4.65	0.84	0.51	0.98	1.49	1.86	...	...	...	...
			c	...	18.0	11.0	21.1	32.1	40.0	82.2	90.1	0.53	81.9
4	Hiproly	3.22	a	21.4	5.2	1.7	4.7	7.2	8.6	...	...	...	...
			b	6.89	1.67	0.54	1.51	2.31	2.76	...	...	...	...
			c	...	24.3	7.9	22.0	33.6	40.2	87.8	94.4	0.55	85.8

[a]Values in line a represent g protein/100 g flour; those in line b represent mg protein/grain; and those in line c represent percentage of the total protein.

results of extraction with this solvent system after successive removal of the classical protein fractions albumin, globulin, and prolamin. The table also presents information on the grain weight for each variety, the protein content, and the content of the protein fractions on a single-grain basis.

Extraction of Protein Fractions

The application of the AUC solvent system for the extraction of proteins in the present study has been warranted in view of the following: a) the likely damage to the covalent bonds of the alkali-extracted proteins; b) the rather low efficiency of extraction when dilute alkali is employed (this is about 80%, Table I); and c) the difficult quantitative separation of the alkali extract from the residue due to gelatinization.

A comparative picture of the effect of the two solvent systems presented in Table I shows that the AUC solvent system is invariably superior to the alkali system since the efficiency of extraction has improved. It may be noted that this solvent system singly can extract as much as the total of the percentages extracted by water, dilute salt solution, aqueous alcohol, and dilute alkali, successively. The mechanism whereby it extracts so efficiently may be its action on hydrogen bonds and the lyophilic activity of the cetyltrimethyl ammonium bromide—a detergent. The effect of bromide ion on the proteins cannot, of course, be ruled out (15). Further studies are needed to establish the suitability of this solvent system in protein extraction. If the solvent is suitable, direct extraction could be employed. However, the extraction efficiency is lower when direct extraction is employed (Table I).

Protein Content of the Varieties

Considerable variation in the protein content of the total flour of these barleys has been observed (13.8 to 19.0%, excluding Hiproly, which shows 21.4%, Table I). Comparison of the protein content expressed as percentage of flour shows that Notch-1 has a 24% and Notch-2 a 38% increase over that of the parent variety NP-113. The high-protein content and higher lysine content of the Notch mutants over the parent variety NP-113 have been established earlier (16). Hiproly has 56% more protein than NP-113 variety. When the values are computed for the protein content on the per grain basis, the protein content in Notch-2 is about 14% greater, and in Hiproly 70% greater, than that of NP-113. Notch-1, however, shows a lower value than the parent.

Comparison of the Protein Fractions

When the protein fractions of the different varieties are compared (see Table I), a 1.5–1.7-fold increase of albumin in the Notch mutants over that of NP-113 was noted on the flour basis. Hiproly has 2.7 times more protein than NP-113. On the per grain basis, there is an increase of about 30% in Notch-1 and 50% in Notch-2, and nearly a threefold increase in Hiproly. The proportion of this fraction (percentage of protein) is about 30% higher in Notch mutants and about 75% higher in Hiproly barley.

The variations in the globulin fractions are not so prominent, except in Notch-2, where about a 30% increase is found on the flour basis. However, on the per grain basis there is a slight reduction in Notch-1.

A reduction of 24% in prolamin fraction in Notch-1 and 19% in Notch-2, and a

25% increase in Hiproly on the per grain basis, have been observed. On the flour basis, however, no significant reduction is noted except for Hiproly, which shows about a 15% increase. The proportion of this fraction (percentage of protein) is about 20–30% lower in Notch mutants and Hiproly.

The AUC-soluble glutelin fraction shows some interesting changes among the varieties. On the per grain basis, an increase of about 37% is found for Notch-2 mutant and a twofold increase for Hiproly, whereas Notch-1 shows a negligible increase. When expressed on the flour basis, there is about a 30, 65, and 87% increase in Notch-1, Notch-2, and Hiproly, respectively. The proportion of glutelin has also increased by about 20% (percentage of protein basis) in Notch-2 and Hiproly.

It is known that, for increased nutritive value of the proteins of the cereal grains, an increase in albumins, globulins, and glutelins, and particularly a decrease in prolamins, are important (17). The present results are largely in agreement with this trend. While the prolamin fraction, which is known to be deficient in lysine, shows a reduction (percentage of protein basis) in the mutants and Hiproly, there is an increase in the albumin and glutelin fractions. Although the content of prolamin in Hiproly is greater than that in NP-113 on the flour basis, it is lower when expressed as percentage of total protein. The changes in the proportion of these fractions appear to be most prominent in the prolamin, less in the glutelin and albumin, and least in the globulin fractions of the mutants.

Although from a nutritional point of view composition based on percentage of total constituents is satisfactory, from the genetic viewpoint considerations on the per grain basis gain importance. In order to assert that a variety has good protein content with good nutritional quality, consideration of its 'per grain' values becomes dominant. It is also desirable to obtain a larger quantity of better quality protein from the same number of grains with the same grain weight as the parent, while grain yield per unit area is maintained. In the present study, it has been observed that, compared to NP-113, while there is an increase of 24% of protein in Notch-1 and 38% in Notch-2 on the flour basis, there is about a 15% increase in Notch-2 on the per grain basis but no increase for Notch-1 on this basis (Table I). There is also a reduction in the prolamin fraction accompanied by an increase in the albumin and glutelin fractions. These changes on the per grain basis also favor the nutritional quality of the protein. The grain weight, however, is lower in these mutants, although the yield per plant is maintained (18).

Electrophoretic Comparison of the Fractions

The higher nutritive value of Hiproly (19) may be due to good nutritive proteins that are either absent from other barleys or present in low levels. The changes in protein composition may be preliminarily visualized by electrophoresis. Some major changes in the proteins of the Notch mutants and Hiproly have been observed in this study.

The albumin fractions show seven distinct and three faint protein bands with altered proportions in each variety (Fig. 1, A–D). Thus, the proportion of the band 4 appears in a decreasing order in NP-113, Notch-1, and Notch-2 (Fig. 1, A–C). The other bands appear generally in lower proportions in Notch-1 and Notch-2 than in NP-113, except bands 1 and 7, which are in higher proportions in Notch-1 and Notch-2, respectively. In addition, the faint bands designated as a, b, and c are in considerably higher proportions in Hiproly (Fig. 1, D). The bands

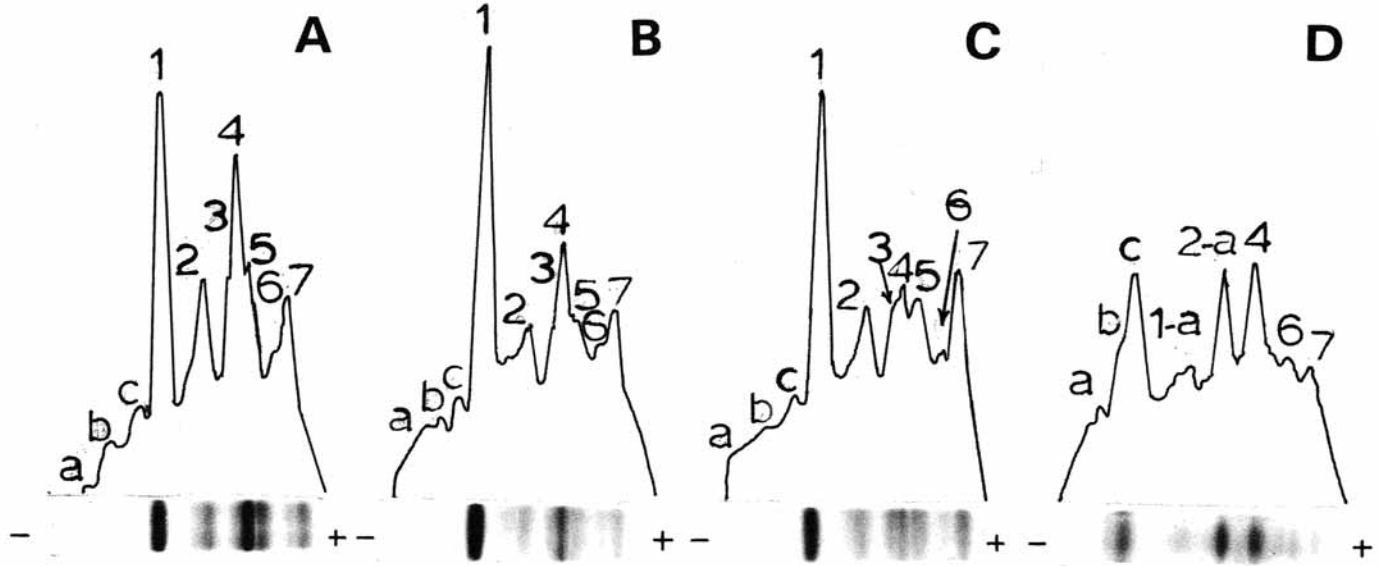

Fig. 1. Electrophoretic patterns and densitographs of albumins of: A) NP-113, B) Notch-1, C) Notch-2, and D) Hiproly barley.

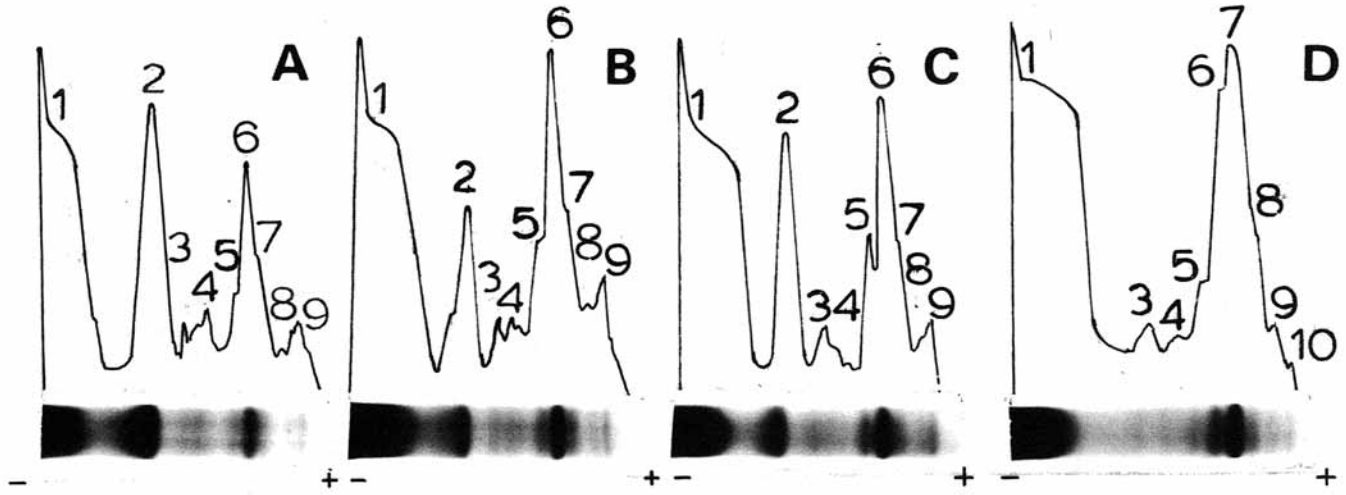

Fig. 2. Electrophoretic patterns and densitographs of globulins of: A) NP-113, B) Notch-1, C) Notch-2, and D) Hiproly barley.

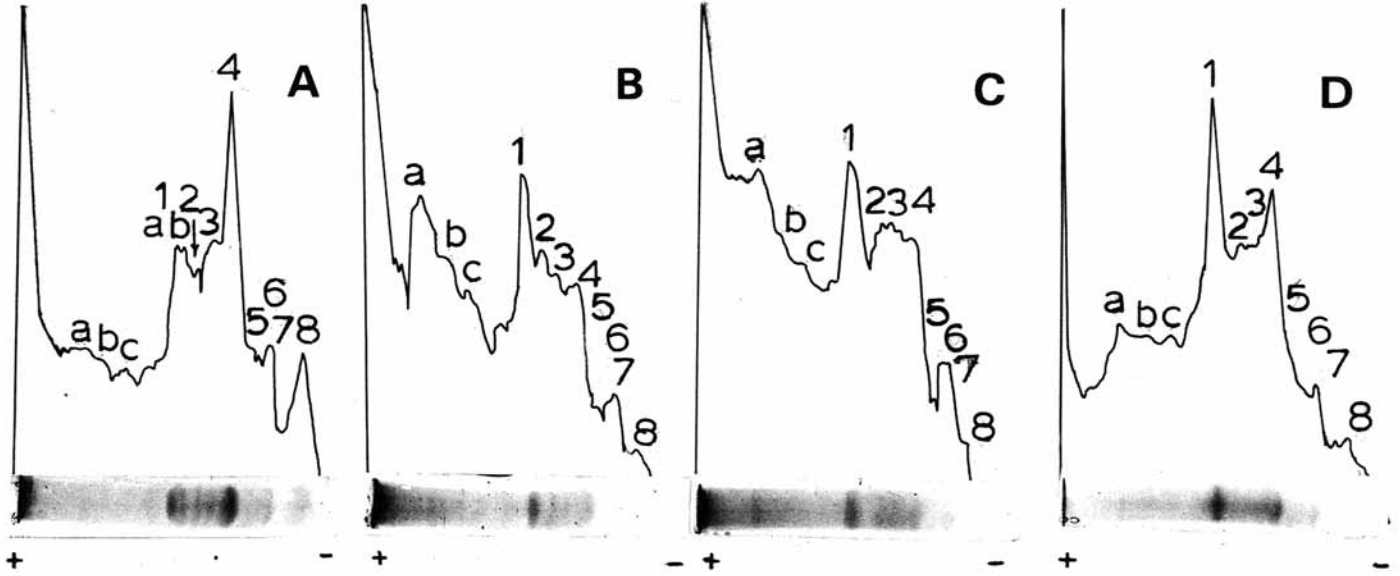

Fig. 3. Electrophoretic patterns and densitographs of prolamins of: A) NP-113, B) Notch-1, C) Notch-2, and D) Hiproly barley.

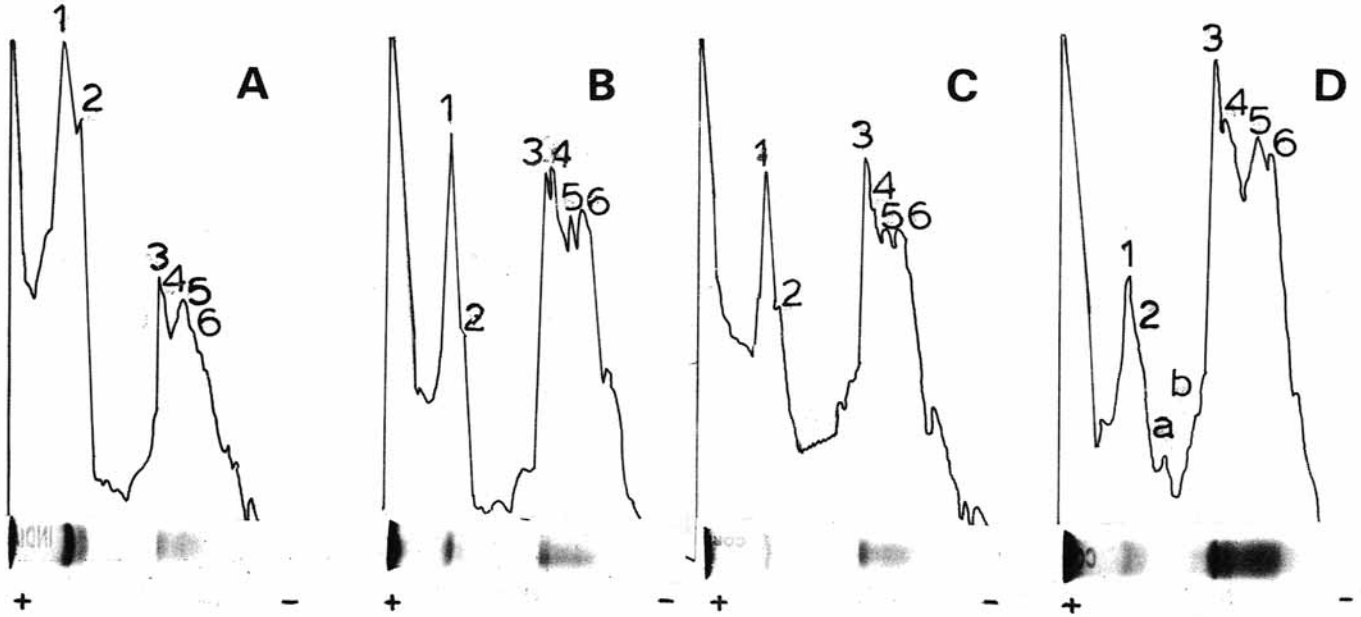

Fig. 4. Electrophoretic patterns and densitographs of glutelins of: A) NP-113, B) Notch-1, C) Notch-2, and D) Hiproly barley.

1, 2, 3, and 5 seem to be totally absent in Hiproly. But the bands 1 and 2 make their appearance as 1-a and 2-a, with altered mobility.

The globulin fractions show about nine protein bands (Fig. 2, A–D). All the varieties show an intense and diffuse protein band at the origin. The main differences in the patterns are found in the bands 2, 5, 6, and 7. Band 2 decreases and bands 5 and 6 increase in NP-113, Notch-2, and Notch-1 (Fig. 2, A, C, and B). Band 2 is totally absent and 7 is of considerable intensity in Hiproly (Fig. 2, D). The bands moving faster than band 6 are faint in NP-113.

The prolamin fractions show about 8 components in addition to a few slow-moving minor bands (Fig. 3, A–D). Some protein remains at the origin of the running gel in all varieties except Hiproly, where it is negligible. Band 4 is the most intense in NP-113 (Fig. 3, A) followed by Hiproly (Fig. 3, D), Notch-2 (Fig. 3, C), and Notch-1 (Fig. 3, B), in that order. Band 1 resolves into 1-a and 1-b in NP-113 while, in the other varieties, it remains as a single band. The minor bands designated as a, b, and c make their appearance in Notch-1, Notch-2, and Hiproly, but are absent in NP-113. Also, the fast-moving band 8 found in NP-113 has very low intensity in the other varieties.

The glutelin fraction failed to separate satisfactorily in spite of several attempts using different buffer systems. This is perhaps the reason for neglect of this fraction by other workers. Since glutelin is highly cross-linked by disulfide bonds and thus forms polymers of high molecular weight, the protein was studied after reduction. The reduced protein was alkylated with acrylonitrile to maintain the electric charge on the molecules and to facilitate a meaningful comparison.

Thus, this fraction after reduction shows about 6 bands (Fig. 4, A–D). The major differences are in bands 1 and 2, which tend to decrease in proportion in NP-113, Notch-1, Notch-2, and Hiproly, in order. Similarly, bands 3, 4, 5, and 6 increase in these varieties in the same order. All the varieties show protein at the origin, and this may be attributed to unfolding of the molecules on reduction and to steric hinderance. Further, the bands a and b are observed only in Hiproly.

The differences in the proportions of the protein fractions as shown by the electrophoretic patterns, particularly those patterns of the glutelin and albumin fractions of the Notch mutants, may be related to the nutritive quality of the grain. This appears to be the case for Hiproly also, although it is a different genotype. The relation of the altered protein composition to the nutritional quality of the grain requires further studies on isolation of protein components and their amino acid composition.

Acknowledgments

The authors wish to thank M. S. Naik, Head of the Division of Biochemistry, for his help and H. C. Bansal for the supply of seed. U. Singh is grateful to the Indian Council of Agricultural Research, New Delhi, for financial assistance.

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[Received June 10, 1975. Accepted October 7, 1975]