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Studies on the Proteins of the Mutants of Barley Grain. 2. Fractionation and Characterization of the Alcohol-Soluble Proteins

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Fractionation and characterization of the alcohol-soluble proteins of a barley variety, its mutants, and Hiproly, a high-protein and high-nutritive barley isolated from the world barley collection, indicate quantitative and qualitative changes in the mutants. The 35% ethanol-soluble subfraction is higher in proportion in the mutants and Hiproly, and this could have resulted from the higher proportions of the polar amino acids in these proteins among which are also some limiting amino acids like lysine and threonine. The digestibility, as observed by the in vitro procedure, of this subfraction in all the varieties also is higher and, presumably as a result, the hordein fraction of these mutants also shows better digestibility. It appears that, in view of the presence of more polar amino acids which include lysine in this subfraction and better digestibility, its enrichment could be a nutritionally favorable index of better grain quality.

Earlier studies on the proteins of a barley variety, NP-113, its mutants (Notch-1 and Notch-2), and Hiproly indicated considerable changes in the classical protein fractions of the mutants, and these changes appeared to be nutritionally favorable (Singh and Sastry, 1977). Such changes might be attributed to either the enrichment of the protein species already existing as indicated in the case of wheat gluten or due to the synthesis of the new species of the proteins as in triticale or both (Yong and Unrau, 1964). In the present investigation, attempts have been made to identify these changes in the mutants and their relationship to nutritional quality. Consequently, the alcohol-soluble proteins have been fractionated, amino acid composition of the subfraction determined, and nutritional quality evaluated by preliminary in vitro evaluation procedures.

MATERIALS AND METHODS

The grain samples, namely, NP-113, Notch-1, Notch-2, and Hiproly, used in this investigation, and the protein fractions therefrom, were obtained as described earlier (Singh and Sastry, 1977). The determination of the protein content and electrophoresis of the proteins were also described earlier. The gel system (Singh and Sastry, 1977) with an operating pH 4.0 was employed for characterization of the protein subfractions.

Fractionation of Hordein. After extraction of hordein with 70% ethanol, distilled water was added to the extract until a final concentration of 35% ethanol was obtained. After allowing the protein to precipitate for 30 min, the suspension was centrifuged. The residue was washed with 35% ethanol and ethanol and dried under vacuum. This formed the 35% ethanol-insoluble fraction. The supernatant was "flash-evaporated" at 37 °C and the residue dried under vacuum. This fraction was designated as 35% ethanol-soluble fraction.

Preparation of α- and β-Hordeins. The procedure of Turner et al. (1965) was followed. Two hundred milligrams of the hordein was suspended in 5 mL of 95% ethanol. The suspension was shaken continuously for 2 h and centrifuged. The residue was washed twice, and the washings were pooled with the first supernatant and flash-evaporated at 37 °C. This fraction is referred to as the α-hordein. The pellet which was dried in vacuum was designated as β-hordein.

Gel Filtration of Hordein. The protein sample was dissolved in a sample containing 4 M dimethylformamide (DMF) and 0.1 M acetic acid. The solution was dialyzed against the same solvent for 24 h to remove contaminating pigments. After adjusting the protein concentration to 20 mg/4 mL of the solution, the sample was applied at the top of the Sephadex G-100 column (2 × 50 cm) previously equilibrated with the same solvent after measuring the total absorption at 280 nm. Elution was effected with the same solvent. Three-milliliter fractions were collected with the aid of a LKB-Radi Rac Siphon control automatic fraction collector. Fifty fractions were collected at the rate of 12 mL/h. The optical density of each fraction was also monitored at 280 nm. The tubes containing the protein under each peak of the elution profile were pooled, dialyzed against distilled water, and lyophilized to dryness.

Determination of Amino Acid Composition of Protein. About 10 mg of protein (N × 6.25) was hydrolyzed with 10 mL of distilled hydrochloric acid (6 N) for 24 h at 110 ± 1 °C in evacuated sealed tubes. Excess acid was removed by repeated washing with distilled water and it was subsequently evaporated under reduced pressure at 50 °C in a rotary flash-evaporator. The residue was taken up in citrate buffer (pH 2.0), and an aliquot was analyzed on a Technicon Sequential Multisample amino

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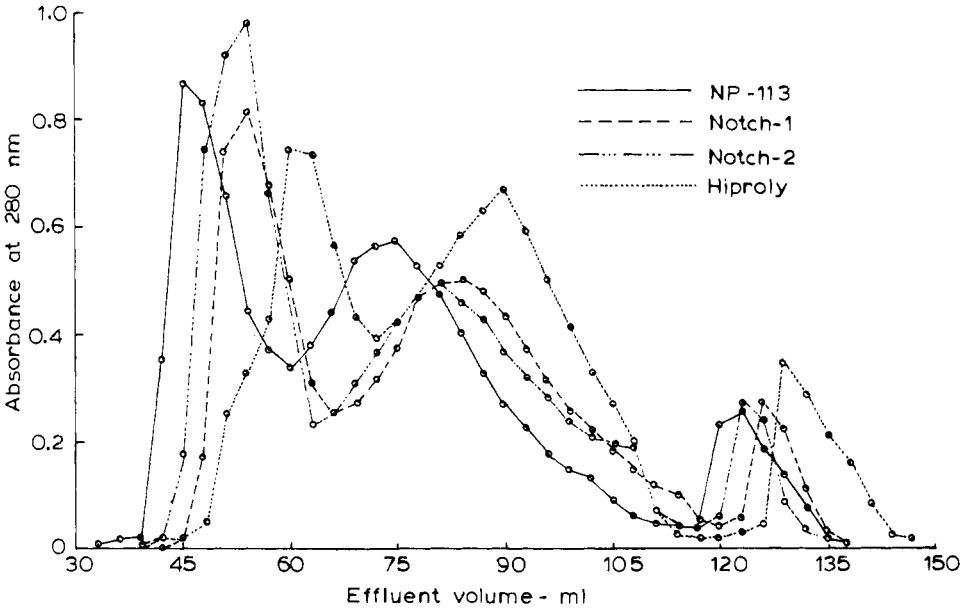

Figure 1. Elution profile of hordeins of different barleys from the Sephadex G-100 column; the proteins eluted in the first, second, and third peaks for each variety in the order of emergence from the column are referred to as fraction I, fraction II, and fraction III, respectively.

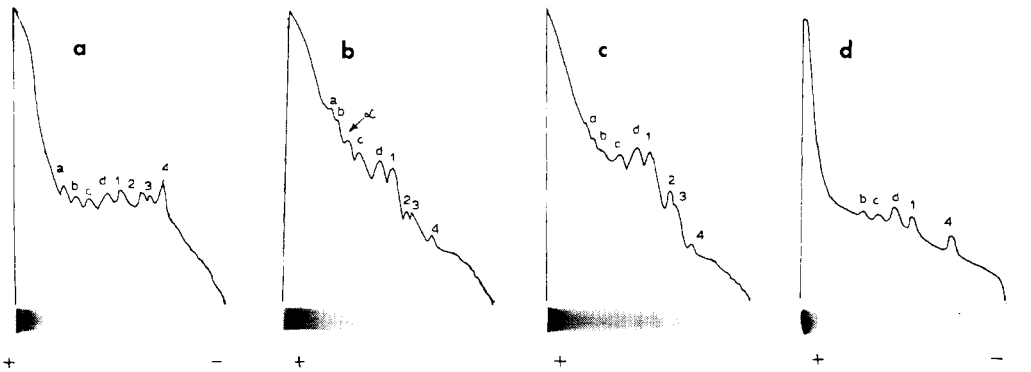

Figure 2. Disc electrophoretic patterns of fraction I of the hordeins from (a) NP-113, (b) Notch-1, (c) Notch-2, and (d) Hiproly, together with their densitographs.

acid analyzer. The total amino nitrogen of the hydrolysate was also assayed by the method of Rosen (1957) for measuring the recovery of the amino acids.

The chemical score value was calculated as described by Block and Mitchell (1946):

$$\text{chemical score} = \frac{\text{g/(16 g of N of the amino acid in sample)} \times 100}{\text{g/(16 g of N of same amino acid in egg)}}$$

Amino acid composition of egg protein was taken from literature (National Academy of Sciences, 1963).

In Vitro Digestibility of Protein in Flour Samples and the Isolated Fractions. Fifty milligrams of protein was incubated with 20 mg of pepsin in 10 mL of 0.1 N sulfuric acid for 24 h at 37 °C. The pepsin digest was buffered with 1 g of potassium monohydrogen phosphate, adjusted to pH 8.4, and incubation was continued with 10 mg of trypsin at 37 °C for another 24 h. The samples were covered with toluene during incubation. Enzyme blank was prepared without protein. At the end of the incubation period, 10 mL of 10% trichloroacetic acid was added. After allowing 30 min, the residue was centrifuged off and nitrogen determined in an aliquot of the supernatant by the procedure used by Rosen (1957). Digestibility was calculated as follows:

$$\%\ \text{protein digestibility} = \frac{(\text{mg of N in supernatant}) \times 100}{\text{mg of N in sample}}$$

RESULTS

Fractionation on Sephadex Columns. The elution profiles of the hordein proteins of the four barleys from the Sephadex column are presented in Figure 1 and the percentage distribution of the fractions in Table I.

The figure shows that hordein can be separated into three fractions—two major and one minor—namely, fractions I, II, and III in the order of their emergence from the column. Fraction I represents about 38–40% of the protein applied, fraction II about 50–64%, and fraction III about 7–9%, and the total recovery varies between 98 and 102%.

Fractions I and II were studied by polyacrylamide gel electrophoresis (fraction III was omitted as it represents only a minor fraction) in gel system C with an operating pH 4.0, which gave the best resolution of the protein species of hordein. The electrophoretic patterns of fractions I and II are presented in Figures 2 and 3, respectively. It can be noted that fraction I consisted mainly of components of low and medium electrophoretic mo-

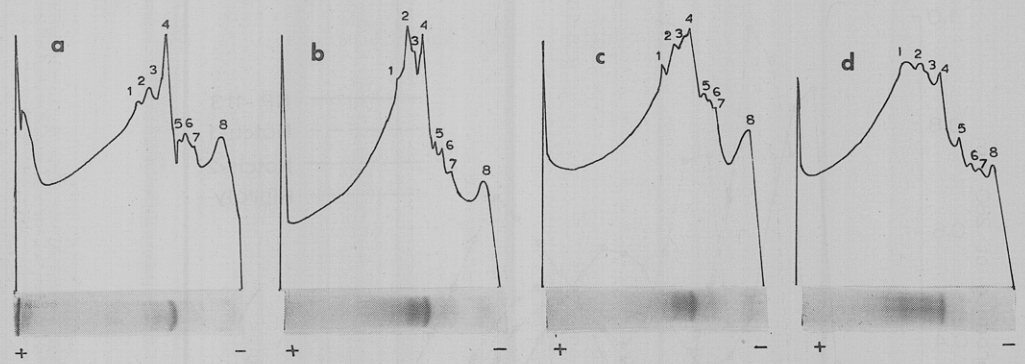

Figure 3. Disc electrophoretic patterns of fraction II of hordeins from (a) NP-113, (b) Notch-1, (c) Notch-2, and (d) Hiproly, together with their densitographs.

Table I. Distribution of the Hordein Proteins of Different Varieties in Sephadex Fractions

	Protein recovery, %			
Variety	Fraction I (peak I)	Fraction II (peak II)	Fraction III (peak III)	Total
NP-113	38.6	52.8	8.8	100.2
Notch-1	38.9	50.6	8.7	98.2
Notch-2	40.3	51.3	7.5	99.1
Hiproly	39.3	54.1	9.1	102.5

Table II. Solvent Fractionation of Hordein from Different Barleys

	Protein, %			
	35 % ethanol		95% ethanol	
Variety	Soluble	Insoluble	Soluble	Insoluble
NP-113	24.6	75.4	16.2	83.8
Notch-1	44.8	55.2	16.3	83.7
Notch-2	41.7	58.3	17.3	82.7
Hiproly	41.3	58.7	15.2	84.8

bilities, while fraction II contained components of medium and high electrophoretic mobilities. Thus, the electrophoretic bands 5–8 of the unfractionated hordein did not appear in fraction I but appeared in fraction II. In addition, some bands of low mobility, a–d, appeared more clearly in fraction I than they did in original hordein. This could be due to enrichment of these components on fractionation.

Qualitative and quantitative differences were also observed between fraction I of the different barleys. Thus, Hiproly failed to show bands a, 2, and 3. Band 4 appeared in decreasing order of intensity in NP-113, Notch-1, and Notch-2, and the bands d and 1 in increasing order. In addition, the band marked α appeared in Notch-1 in fraction I. In fraction II, bands 1, 2, and 3 appeared in higher intensities in the mutants and Hiproly.

Solvent Fractionation of Hordein. Results of solvent fractionation of the prolamins of the four barleys in Table II show that the 95% ethanol-soluble (α-hordein) proteins represent 15.2 to 17.3% of the total hordein.

Electrophoretic patterns of these α- and β-hordeins presented in Figure 4 show that the α-hordein had predominantly the fast-moving components, whereas the β-hordein had both fast- and slow-moving components. No difference was found in the electrophoretic patterns of the α-hordeins of NP-113 and mutants which showed three bands each. Hiproly, however, showed only two bands. Marked differences were also not observed in the electrophoretic patterns of β-hordeins of these four barleys.

Table II also shows the results of fractionation of hordein using ethanol into 35% ethanol-soluble and ethanol-insoluble hordeins. It may be noted that, while about 25%

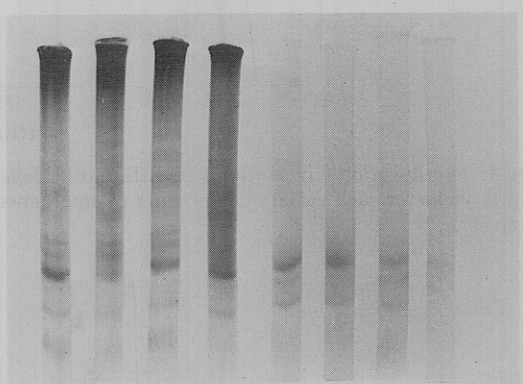

Figure 4. Disc electrophoretic patterns of β- and α-hordeins from different barleys: 1 and 5, β- and α-hordeins of NP-113; 2 and 5, β- and α-hordeins of Notch-1; 3 and 7, β- and α-hordeins of Notch-2; 4 and 8, β- and α-hordeins of Hiproly, respectively.

was the soluble fraction in NP-113, about 40–44% formed the soluble fraction in the mutants and Hiproly. This subfraction was invariably in lower proportions than the insoluble fraction. The ratio of soluble to insoluble proteins (in 35% ethanol) was only 0.3 in NP-113, while it was 0.8 in Notch-1 and 0.7 in Notch-2 and Hiproly. The altered protein make-up in the mutants as compared with that of NP-113 was consistent with that in the Hiproly, which is known to be nutritionally superior.

Electrophoretic patterns of these two fractions presented in Figures 5a–d and 6a–d, respectively, show that the fast-moving components were predominant in the 35% ethanol-soluble fraction and more protein was mobile in this fraction than that in the insoluble fraction. The insoluble fraction contained the bands with low or medium mobility.

Some differences in the electrophoretic patterns of the 35% ethanol-soluble fraction of the four barleys were noted. Band 4 was most intense in NP-113, followed by Notch-1 and Notch-2, while it was not distinct in Hiproly. Bands α_1 to α_4 were identified only in the Notch-2 mutant (Figure 5a–d). In the case of 35% ethanol-insoluble fractions, Band 1 was most conspicuous in Notch-2. The α_2 band was found only in Notch-2 and the α_1 band was in higher proportion in Notch-1 (Figure 6a–d).

Amino Acid Analyses of the Hordeins and Their Fractions. The results of amino acid analysis of hordein and 35% ethanol-soluble fraction (Table III), and fraction I and fraction II of hordein (Table IV) of the four barleys obtained by gel filtration on Sephadex column together with the chemical score values showed that hordein was low in lysine, methionine, threonine, and arginine and high in glutamic acid and proline. The chemical score values

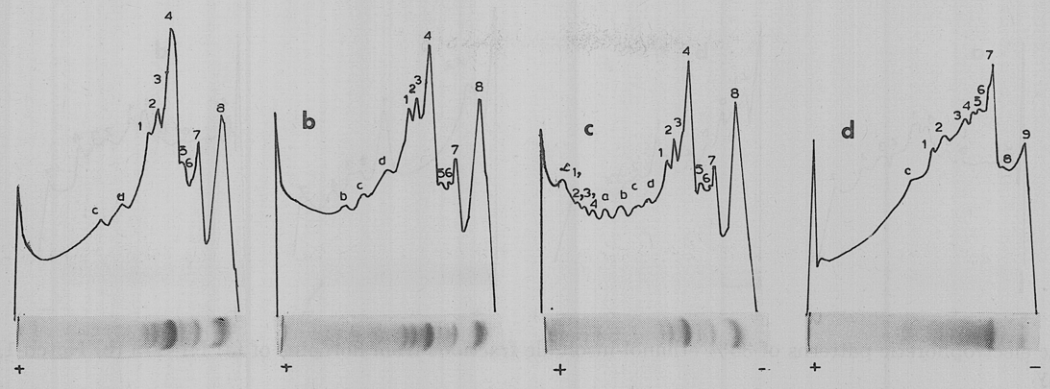

Figure 5. Disc electrophoretic patterns of 35% ethanol-soluble fractions from hordeins of (a) NP-113, (b) Notch-1, (c) Notch-2, and (d) Hiproly.

Table III. Amino Acid Composition and Chemical Score of Hordein and 35% Ethanol-Soluble Fraction of Different Varieties

	Amino acid content, g/16 g of N							
	Hordein				35% ethanol-soluble			
Amino acid	NP-113	Notch-1	Notch-2	Hiproly	NP-113	Notch-1	Notch-2	Hiproly
Lysine	0.79	0.81	0.81	0.83	1.26	1.34	1.32	1.60
Histidine	1.90	1.82	1.84	1.76	1.86	1.90	1.80	1.81
Arginine	2.76	3.06	2.78	2.82	4.24	4.84	4.43	3.48
Aspartic acid	1.61	2.30	2.37	2.35	3.20	3.48	3.51	4.30
Threonine	1.30	1.96	1.96	1.74	1.83	2.43	2.08	2.10
Serine	2.60	2.80	2.81	2.40	3.23	4.29	3.86	3.65
Glutamic acid	36.44	35.86	35.80	34.82	30.23	29.98	30.46	31.04
Proline	18.76	17.98	16.40	16.70	15.14	14.23	13.48	14.10
Glycine	2.30	2.23	2.32	2.13	3.07	4.21	4.03	3.59
Alanine	2.54	2.63	2.45	2.60	3.68	4.26	4.26	4.52
Cystine	2.72	2.66	2.18	1.95	3.03	2.86	2.11	2.70
Valine	3.70	3.54	3.71	3.46	4.66	4.40	4.57	4.67
Methionine	0.79	0.94	0.80	1.07	1.13	1.62	1.87	1.59
Isoleucine	3.98	3.60	3.82	2.40	4.28	4.03	4.71	4.05
Leucine	7.36	7.68	7.90	7.80	8.49	7.69	8.89	8.03
Tyrosine	4.74	4.16	4.26	4.30	3.89	3.52	3.40	3.21
Phenylalanine	8.31	8.12	8.89	9.32	6.37	6.19	6.22	5.76
Chemical score, %	12	12	12	13	19	20	20	24

Table IV. Amino Acid Composition and Chemical Score of Fractions I and II of Hordein of Different Varieties

	Amino acid content, g/16 g of N							
	Fraction I				Fraction II			
Amino acid	NP-113	Notch-1	Notch-2	Hiproly	NP-113	Notch-1	Notch-2	Hiproly
Lysine	0.84	0.81	0.78	0.86	0.67	0.68	0.66	0.75
Histidine	1.74	1.88	1.78	1.48	1.95	1.75	1.65	1.35
Arginine	3.87	3.81	2.94	3.65	1.49	1.84	2.05	1.76
Aspartic acid	1.68	1.66	1.71	1.57	1.14	1.20	1.38	1.03
Threonine	1.58	1.44	1.59	1.29	0.82	0.91	0.89	0.73
Serine	3.31	3.04	3.07	3.54	1.92	2.15	2.20	1.69
Glutamic acid	34.96	34.23	33.89	32.90	37.86	38.45	37.34	37.80
Proline	17.26	16.83	17.39	18.48	19.75	19.68	20.72	22.07
Glycine	2.65	2.51	2.49	2.43	1.67	1.83	1.98	1.93
Alanine	2.17	1.90	1.99	1.88	1.88	2.14	2.21	1.94
Cystine	3.49	3.62	3.36	2.83	1.80	1.61	1.39	1.02
Valine	4.87	5.04	5.02	4.43	2.36	2.44	2.52	2.12
Methionine	0.85	0.97	0.67	1.07	0.51	0.40	0.41	0.59
Isoleucine	5.01	4.97	5.26	4.59	3.60	4.30	4.38	4.37
Leucine	7.85	8.35	7.85	7.14	6.65	6.61	6.81	6.60
Tyrosine	3.63	3.02	3.39	4.19	5.89	4.11	4.56	4.41
Phenylalanine	7.08	7.38	7.61	9.31	11.12	12.92	10.57	11.97
Chemical score, %	13	12	11	13	10	10	10	11

were also low for hordein and were 12 for the hordein of NP-113, Notch-1, Notch-2, and 13 for Hiproly. Only minor differences were observed in the amino acid composition of the different barleys as observed in the case of the electrophoretic patterns of hordein (Singh and Sastry, 1977, Figures 3a–d). Threonine was higher in mutants and Hiproly, and methionine was higher in Hiproly. Cystine was lower in Notch-2 and Hiproly.

An examination of the amino acid composition of the subfraction of hordein, namely, the 35% ethanol-soluble fraction (Table III), indicated that lysine, arginine, aspartic acid, threonine, serine, methionine, and isoleucine were

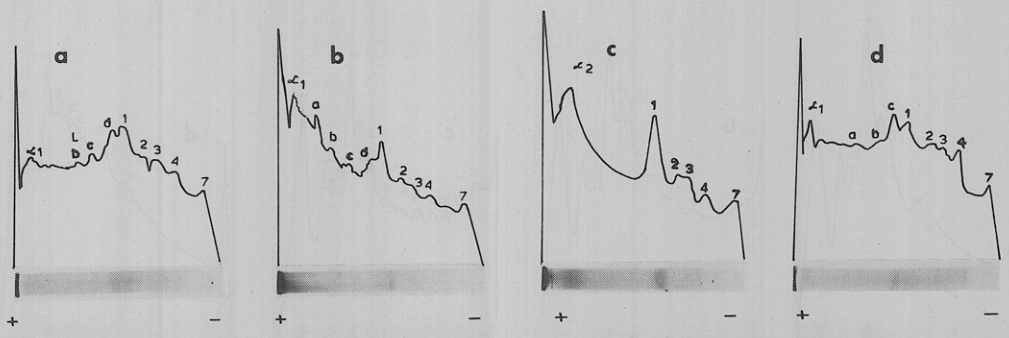

Figure 6. Disc electrophoretic patterns of 35% ethanol-insoluble fractions from hordeins of (a) NP-113 (b) Notch-1, (c) Notch-2, and (d) Hiproly.

Table V. In Vitro Digestibility of the Proteins of the Four Barleys

Variety	Total flour protein, %	Hordein, %	35% ethanol-soluble subfraction, %
NP-113	75.2	54.3	65.4
Notch-1	73.8	61.8	68.9
Notch-2	81.4	63.9	67.8
Hiproly	80.5	65.6	69.6

higher, whereas glutamic acid, proline, tyrosine, and phenylalanine were lower in 35% ethanol-soluble fraction as compared with the native hordein. This trend was noted in all varieties. The chemical score values also were higher compared with the respective original hordeins, viz., 19 for NP-113, 20 for the mutants, and 24 for Hiproly as against 12 for NP-113 and mutants and 13 for Hiproly.

The amino acid composition of Sephadex-separated fractions I and II of hordein (Table IV) shows that it is not greatly different from native hordein (Table III). When compared with fraction II, fraction I shows higher lysine, arginine, glycine, serine, threonine, cystine, methionine, valine, and isoleucine and lower glutamic acid, proline, tyrosine, and phenylalanine. The chemical score values are only slightly better than those of fraction II.

Digestibility of the Hordeins and Their Fractions. The native hordein and 35% ethanol-soluble fractions of these different barleys were also subjected to in vitro digestibility tests. The results are summarized in Table V. When the digestibilities of the total flour protein of different barleys were compared, Notch-2 had the highest digestibility followed by Hiproly, NP-113, and Notch-1, although the differences between the first two and the later two were not large. Hordein of all the varieties had the lowest digestibility compared with the digestibility of the total flour protein and the 35% ethanol-soluble fractions. Among the hordeins of the four barleys, that of NP-113 showed the lowest digestibility, followed by Notch-1, Notch-2, and Hiproly, the values for the later three being considerably larger compared with that of NP-113.

When the digestibilities of the different barleys were compared with their respective ethanol-soluble subfractions, the subfraction invariably showed higher digestibility. These differences are more marked for NP-113 and Notch-1.

DISCUSSION

Fractionation of the isolated classical protein fractions into subfractions could help in identification of the changes brought about by mutation in the strategy of improving the grain protein quality. In the present investigation, the hordein proteins were fractionated further on Sephadex columns and by using different concentrations of ethanol. The qualitative and quantitative changes in the separated fractions were looked for in the different barleys by electrophoresis and determination of the proportions of the subfractions. The isolated subfractions were further characterized by determining the amino acid composition and in vitro digestibility to examine the contribution of these changes to the nutritional qualities of the total flours.

Sephadex column fractionation and fractionation into α- and β-hordeins at 95% ethanol concentration did not reveal any change in the proportions between these barleys. However, in the case of Sephadex column fractionation, differences in elution volumes of fractions I, II, and III were indicative of the possibly lowered molecular size of the proteins in the mutants. The Hiproly barley also showed proteins of possibly even much lower molecular size compared with those of NP-113 and the mutants. The reduction in molecular size of the proteins in the mutants requires confirmation by other methods such as ultracentrifugal analysis. The increase in concentration of electrophoretic bands "d" and "1" and decrease of band "4", also seen originally in the unfractionated hordein (Singh and Sastry, 1977; Figure 3), were seen in fraction I, though not as clearly as in fraction II. This indicates an alteration in the proportion of the existing species of proteins.

Similar fractionation of the alcohol-soluble proteins of wheat and triticale on Sephadex columns has been reported earlier (Beckwith, 1972; Chen and Bushuk, 1970). Fraction II was suggested to contain predominantly the alcohol-soluble proteins whereas fraction I was shown to contain a contaminant from the glutelin fraction. Wasik and Bushuk (1974) also confirmed this observation while studying wheat glutelin. Support for such a suggestion can perhaps also be adduced from the data on amino acid composition. When the amino acid compositions of fractions I and II (Table IV) are compared, fraction I shows relatively more favorable composition. Fraction I contains a higher level of amino acids like lysine, arginine, threonine, methionine, and valine and a lower level of glutamic acid and proline as compared with fraction II. These higher levels probably resulted from the suggested contaminant from glutelin present in this fraction, as for wheat and triticale proteins (Beckwith, 1972; Chen and Bushuk, 1970; Wasik and Bushuk, 1974). The higher levels of glutamic acid and proline, more characteristic of prolamins, and the lower levels of the basic amino acids and other essential amino acids might suggest that fraction II represents more truly a prolamin-type protein.

The lack of any major difference in the proportion or electrophoretic pattern of the α-hordein fraction, soluble in the less polar 95% ethanol representing more truly a prolamin type of protein, indicates that in this prolamin type of protein no major changes have taken place. Earlier, Nelson (1969) observed no change in the amino acid composition of alcohol-soluble proteins of maize mutants. Among the α- and β-hordeins, the α fraction appears to

be relatively pure, since it showed only two–three components electrophoretically.

The presence of higher quantities of 35% ethanol-soluble proteins in the mutants and Hiproly than in NP-113 might be a reflection of the lower hydrophobic interactions due to a higher proportion of amino acids with polar side chains in the proteins, causing the proteins to remain in solution in a more polar solvent like 35% ethanol compared with 70% ethanol. The prolamin fraction was soluble in nonpolar solvents like ethanol, chloroform, and methanol, owing primarily to the presence of large proportions of amino acids with nonpolar side chains and consequent hydrophobic interactions of these proteins with the solvent (Tracey, 1966).

Although no major differences were noted in overall amino acid composition (Table III) and electrophoretic patterns of the hordeins (Singh and Sastry, 1977, Figure 3) of the different barleys, the data on the amino acid composition (Table IV) of the ethanol-soluble subfraction favor the view that higher quantities of the 35% ethanol-soluble fraction would result from lower hydrophobic interactions due to higher proportions of the amino acids with polar side chains. It may be noted from the results obtained that, among the polar amino acids, the content of the amino acids like lysine, arginine, aspartic acid, and threonine was higher while the content of proline and phenylalanine groups as nonpolar was lower in the 35% ethanol-soluble fractions. Though the content of valine, methionine, and isoleucine grouped as nonpolar also was higher and that of glutamic acid grouped under polar amino acids was lower in this subfraction, it appeared that in the 35% ethanol-soluble subfraction the polar to nonpolar amino acid balance favored the polar amino acids. Electrophoretically, this fraction also showed the components of higher mobilities, indicating a more basic nature of the proteins, which could result only from the predominance of the polar residues.

The presence of a higher proportion of some of the limiting amino acids like lysine, threonine, and methionine, and the higher digestibility of this 35% ethanol-soluble subfraction (Table V) endow this fraction with better nutritive quality. The higher digestibility of the hordeins from the mutants and Hiproly compared with that of NP-113 also presumably owes to the higher proportion of the 35% ethanol-soluble subfraction.

The electrophoretic patterns of the 35% ethanol-soluble subfractions and also the insoluble subfractions did show qualitative (such as the bands α_1 to α_4, 1, and 4) and quantitative differences in addition to the changes already indicated in the total proportion of the subfraction.

It thus becomes clear that there was considerable alteration in the protein make-up of the alcohol-soluble proteins of these mutants, and there occurred both enrichment of the existing species and formation of new species of proteins. Furthermore, in view of the presence of proteins containing more of the polar amino acids which include lysine among others in the 35% ethanol-soluble subfraction, its enrichment could be a nutritionally favorable index of better grain quality. The improved digestibility of the flours of Notch-2 and Hiproly themselves could have resulted from the overall alteration in the classical protein types, viz., the albumins, the globulins, the prolamins, and the glutelins (Singh and Sastry, 1977), in addition to the 35% ethanol-soluble subfraction.

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