CHROMATOGRAPHIC FRACTIONATION AND COMPOSITION OF THE COMPONENTS OF THE SALT-SOLUBLE PROTEINS FROM HIPROLY (CI 3947) AND HIPROLY NORMAL (CI 4362) BARLEYS[1]

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ABSTRACT

Fractionation of the protein complex in Hiproly (CI 3947) and Hiproly Normal (CI 4362) barleys showed that the salt-soluble protein fraction accounted for 41.8 and 43.5% of the total lysine, respectively. Salt-soluble and glutelin fractions together accounted for 78.7% of the total lysine in Hiproly and 80.6% in Hiproly Normal. Salt-soluble protein fractions from these two barleys contained identical protein components, as indicated by comparison of the electrophoretic mobilities of components of chromatographic fractions. One chromatographic fraction accounted for 50.3% of the lysine in the salt-soluble proteins of Hiproly and 43.5% in Hiproly Normal. The first three chromatographic fractions from the salt-soluble protein of Hiproly contained 52% more lysine than did comparable fractions in Hiproly Normal. These fractions contained higher percentages of aspartic acid, lysine, and valine and lower percentages of tyrosine, glutamic acid, and cystine/2 in Hiproly than in Hiproly Normal.

The Ethiopian barleys, Hiproly (CI 3947) and its lower lysine isogene (CI 4362), were isolated from the world's barley collection (1). The isogene CI 4362 has been unofficially designated Hiproly Normal, as it is phenotypically and agronomically similar to Hiproly. Both barleys are high in protein, but the protein of Hiproly barley contains substantially more lysine and has higher nutritional value than Hiproly Normal protein (2,3). Fractionation of the protein complex of high-lysine Emir barley by Ingversen and Køple (4) indicated that the salt-soluble fraction representing 24% of the protein accounted for 44% of the total lysine. Fractionation of that protein by them on Sephadex G-100 yielded four major protein peaks varying in lysine content between 5.3 and 7.3%. They suggested the existence of three high-lysine protein groups in the salt-soluble fraction of Emir barley. In a later publication (5), these investigators reported the high-lysine barleys Hiproly, CI 7115, and its mutants 29 and 86 to contain major proteins identical to those in the normal lysine barley Carlesberg II, as indicated by comparison of their molecular weights and electrophoretic mobilities.

The object of the present investigation was to locate the lysine-rich component or components in the salt-soluble proteins of Hiproly high-lysine barley (CI 3947). The lysine distribution and the protein composition of the salt-soluble proteins of both Hiproly and Hiproly Normal barleys as depicted by chromatography and electrophoresis were determined. The salt-soluble protein includes both the albumins and globulins. The distinction between these two classes of proteins in the literature was based on a solubility criteria which is difficult to reproduce in the laboratory. Chromatography and electrophoresis of

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these protein classes by others (6) showed extensive heterogeneity and carry-over of the proteins in these solubility classes.

MATERIALS AND METHODS

Barley Source and Treatment

The barley grains used in this work were raised at Montana State University Agronomy farm in adjoining plots of irrigated land in 1973. The grains were first ground on a hammer mill through a 3.2-mm screen, then ground to a fine powder through a Burr mill. The flour was defatted with cold acetone (10 ml/g) by stirring for 18 hr at −20°C, then air drying after washing with ethyl ether.

Analytical Methods

All chemicals used were reagent grade. An 8*M* solution of urea was treated with charcoal, then passed through a mixed-bed resin (AG 501-X8, 25–50 mesh, from Bio-Rad Labs, Richmond, Calif.). The final pH of the urea solution was 7. Sodium dodecyl sulfate (SDS) was recrystallized from pure ethanol.

Recovery of the Major Protein Classes

Twenty-five grams of whole barley flour was extracted with 250 ml of solvent in a 300-ml centrifuge bottle by vigorous stirring with a magnetic stirrer for 1 hr at 5°C. Three different solvents were used: albumin, globulin + nonprotein nitrogen: 0.5*M* NaCl, 0.05*M* EDTA-Na; hordein: 55% isopropyl alcohol; glutelin: 0.0125*M* $Na_2B_4O_7$, 0.043*M* NaOH, 0.5% sodium dodecyl sulfate. The number of extractions made for each class of proteins was determined according to the recommendation of Ingversen and Kϕle (4). The 55% (v/v) isopropyl alcohol and water extract of hordein was freed from alcohol using a rotary evaporator. The salt-soluble, hordein, and glutelin extracts were exhaustively dialyzed against cold, running, distilled water for 24 hr, then reduced to an eighth of their volume and freeze-dried.

Nitrogen Determination

Nitrogen was determined by the conventional Kjeldahl technique.

Chromatographic Fractionation of the Salt-Soluble Proteins of Barley

1. Sample Preparation. The distinction between albumins (water-soluble) and globulins (salt-soluble) included in the Osborne fractionation of cereal proteins was considered unimportant in the present investigation and, hence, the salt-soluble proteins are defined as the protein extracted with 0.5*M* NaCl solution. The freeze-dried salt-soluble proteins (1.2 g) were dispersed into 200 ml of 0.03*M*, pH 8, glycine-NaOH buffer containing 0.04 g ethylenediaminetetraacetic acid (EDTA) and 1.2 ml 2-mercaptoethanol. After adjusting the pH of the sample solution to 8, it was dialyzed against 4 liters of glycine buffer containing 0.6% 2-mercaptoethanol.

2. Column Preparation. The diethylaminoethyl cellulose ion-exchanger (DEAE-cellulose), 0.95 meq/g, was obtained from Sigma Chemical Company. The coarse powder (21 g) was prepared in the normal manner, equilibriated with the chromatographic buffer, and packed into a 2.5 × 40-cm column (K 25/40 from Pharmacia Fine Chemicals, Upsala, Sweden) to a height of 40 cm using 10

mm nitrogen gauge pressure. Four liters of pH 8, 0.03*M* glycine buffer containing 3.3*M* urea and 0.05% 2-mercaptoethanol was pumped through the column at a rate of 13.5 ml/min, using a Master-Flex pump (Cole-Parmer Instrument Co.) fitted with a model 7013 pump head and a Master-Flex controller.

3. Chromatography. The sample was pumped through the column at a rate of 13.5 ml/min, followed by stepwise elution with pH 8, 0.03*M* glycine buffer containing 3.3*M* urea, 0.05% 2-mercaptoethanol, and a NaCl gradient from 0.0 to 1.5%.

4. Recovery of Proteins from Chromatographic Elution Fractions. The elution fractions from each chromatographic peak were pooled, then dialyzed against cold (5°C), running, distilled water for 24 hr. The fractions were then reduced to 1/8 their original volume and freeze-dried.

Electrophoresis and Molecular-Weight Determination of Protein Components

The molecular-weight markers used for the estimation of the molecular weights of the salt-soluble protein components in the chromatographic elution fractions were: Cytochrome C, 12,400; chymotrypsinogen, 25,000; ovalbumin, 45,000; albumin (bovine), 67,000; and γ-globulin (human), 167,000 obtained from Schwartz-Mann and catalase, 240,000 obtained from Boehringer Mannheim.

The SDS subunits for the molecular-weight markers were prepared in the presence of 0.002*M* N-ethylmaleimide, while the subunits for the barley protein components were prepared in the presence of 2-mercaptoethanol, as recommended by Orth and Bushuk (7).

Gel Preparation. The gel buffer contained 7.8 g $NaH_2PO_4 \cdot H_2O$, 38.6 g Na_2HPO_4, and 2 g SDS/liter. A 22% Cyanogum-41 solution was prepared in deionized water and the insoluble materials were removed by filtration through Whatman No. 1 filter paper. For a typical gel slab, 40 ml Cyanogum-41 solution was degassed and mixed with 70 ml gel buffer, 0.35 ml N,N,N,N-tetramethylethylenediamine (T.E.M.E.D.), and 0.2 ml 2-mercaptoethanol. After adding 5 ml of a freshly prepared 10% solution of ammonium persulfate and mixing, the gel slab was polymerized in an E.C. vertical gel electrophoresis apparatus, using 6-mm spacers. The gel slab was 6 mm thick, 12.8 cm wide, and about 13 cm in length.

Electrophoresis. After addition of the electrode buffer (gel buffer diluted 1:1 with deionized water) and removal of the slot-former, samples of the protein solutions (100 μg) were introduced and allowed to settle for 10 min. Electrophoresis was conducted at 80 mA at room temperature for 14 hr. Following electrophoresis, the gels were removed and stained with Coomassie Blue dye according to Koenig *et al.* (8).

Molecular-Weight Determination of the Components of the Salt-Soluble Proteins. The mobilities of the molecular-weight markers were determined and plotted against their known molecular weights on a semilogarithmic scale. The molecular weights of the salt-soluble protein components in the chromatographic fractions were estimated from their electrophoretic mobilities according to Weber and Osborn (9).

TABLE I
Nitrogen and Lysine Contents[a] of the Osborne Fractions of Whole Hiproly (CI 3947)[b] and Hiproly Normal (CI 4362)[c] Barleys

Parameter	Hiproly (CI 3947)					Hiproly Normal (CI 4362)				
	Total	Salt-soluble proteins	Hordein	Glutelin	Insoluble proteins	Total	Salt-soluble proteins	Hordein	Glutelin	Insoluble proteins
% Nitrogen (g/100 g whole grain)	2.99	0.78	0.96	1.02	0.15	2.91	0.74	1.02	0.93	0.14
% Total nitrogen	...	26.8	32.9	35.1	5.20	...	26.0	36.2	32.8	5.1
Lysine (mg/100 g whole grain)	752.0	274.0	98.0	242.0	42.0	510.0	184.0	40.0	157.0	42.0
% Total recovered lysine	...	41.8	14.8	36.9	6.4	...	43.5	9.5	37.1	9.9
% Lysine in whole barley or fractions	4.0	5.6	1.6	3.8	4.4	2.8	4.0	0.6	2.7	4.7

[a]Averages of triplicate determinations.
[b]Hiproly (CI 3947): recovery of nitrogen = 2.92 g (97.6%); recovery of lysine = 656 mg (87.2%).
[c]Hiproly Normal (CI 4362): recovery of nitrogen = 2.83 g (97.3%); recovery of lysine = 465 mg (82.9%).

Amino Acid Analysis

The lysine content of the Osborne protein fractions and the amino acid composition of the chromatographic fractions of the salt-soluble proteins of the two barley isogenes were determined on 24 hr, 6*N* HCl hydrolysate samples according to Spackmann *et al.* (10). The analyses were performed on a Durrum single-column liquid chromatograph. A PDP-8 computer continuously monitored the analyzer and computed the data. A 24-hr 6*N*-HCl hydrolysis in vacuum results in a serine value low by approximately 10% and threonine low by about 5% due to destruction by acid; thus, the values for these amino acids were

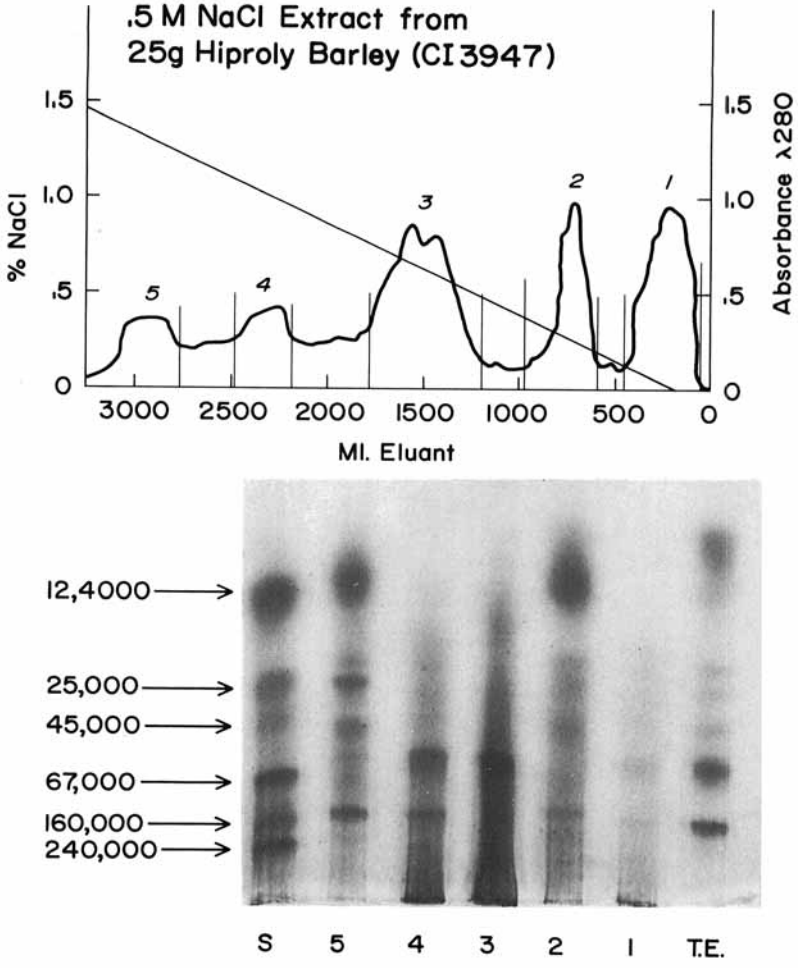

Fig. 1. Chromatographic elution absorbance pattern of the salt-soluble protein from Hiproly barley (CI 3947) and electrophoretic patterns of the proteins from elution peaks. T. E. = Total salt-soluble protein extract; S = mixture of molecular-weight markers; and 1, 2, 3, 4, 5 = proteins from peaks of the chromatogram.

adjusted to that level. Cysteine and cystine were oxidized to cysteic acid using performic acid, and are reported as cystine/2 according to the method of Hirs (11). Tryptophan was determined by a 48-hr alkaline hydrolysis at 135°C by the method of Hugli and Moore (12).

RESULTS AND DISCUSSION

Composition of the Osborne Fractions of Hiproly and Hiproly Normal Proteins

Table I presents the nitrogen and lysine distribution in the various Osborne protein fractions from Hiproly (CI 3947), containing 2.99% nitrogen, and from Hiproly Normal (CI 4362), containing 2.91% nitrogen. The nitrogen and lysine recoveries from these fractions were 97.6 and 87.2%, respectively, for Hiproly and 97.3 and 82.9%, respectively, for Hiproly Normal. These recoveries were obtained following five successive extractions for each class of proteins (4). Although the salt-soluble protein contained 26.8% of the total nitrogen in Hiproly and 26% in Hiproly Normal, it accounted for 41.8 and 43.5% of the total lysine, respectively. The data in Table I also indicate that lysine in these barleys occurred in the salt-soluble and glutelin protein fractions (78.7% of the total lysine in Hiproly and 80.6% in Hiproly Normal). The salt-soluble and the glutelin proteins contained 90 and 85 mg more lysine per 100 g of grains, respectively, in Hiproly than in Hiproly Normal proteins. Thus, it is concluded that the higher lysine content of Hiproly barley protein (4.0%) in comparison to Hiproly Normal protein (2.8%) is due largely to the higher lysine content of its salt-soluble and glutelin proteins. Although the hordein protein fraction represented 35.1% of the total protein in Hiproly and 36.2% in Hiproly Normal, it only accounted for 14.8 and 9.5% of the total lysine, respectively. Thus, the hordein fraction of barley is of minor importance as a contributor of lysine. The results presented in Table I confirm those reported by Ingversen and Kφle (4) on high-lysine Emir barley.

Chromatography of the Salt-Soluble Proteins and Electrophoresis of Their Chromatographic Fractions

The chromatographic elution absorbance patterns of the salt-soluble proteins,

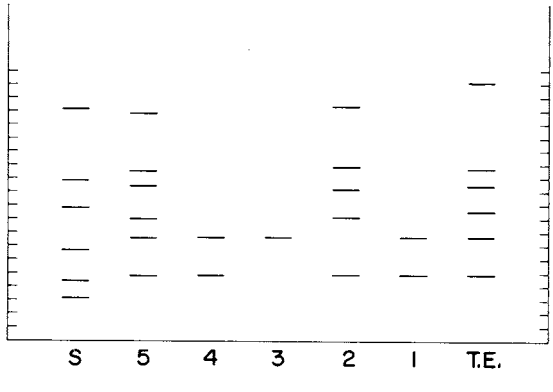

Fig. 2. Diagrammatic illustration showing band positions in the electrophoretic patterns in Fig. 1.

together with the electrophoretic patterns representing the proteins from each elution fraction, are illustrated in Figs. 1 and 2 for Hiproly and Figs. 3 and 4 for Hiproly Normal. The concentration of protein in the samples applied to the slots in the electrophoretic gels in Figs. 1 and 3 was 100 μg/10 μl. Similar chromatographic patterns were given by both Hiproly and Hiproly Normal barleys, with each containing five major fractions. The lack of sharpness in the peaks indicated that each contained more than one protein component. This is confirmed by the composition of the electrophoretic patterns representing each fraction. The similarity in electrophoretic composition of comparable fractions

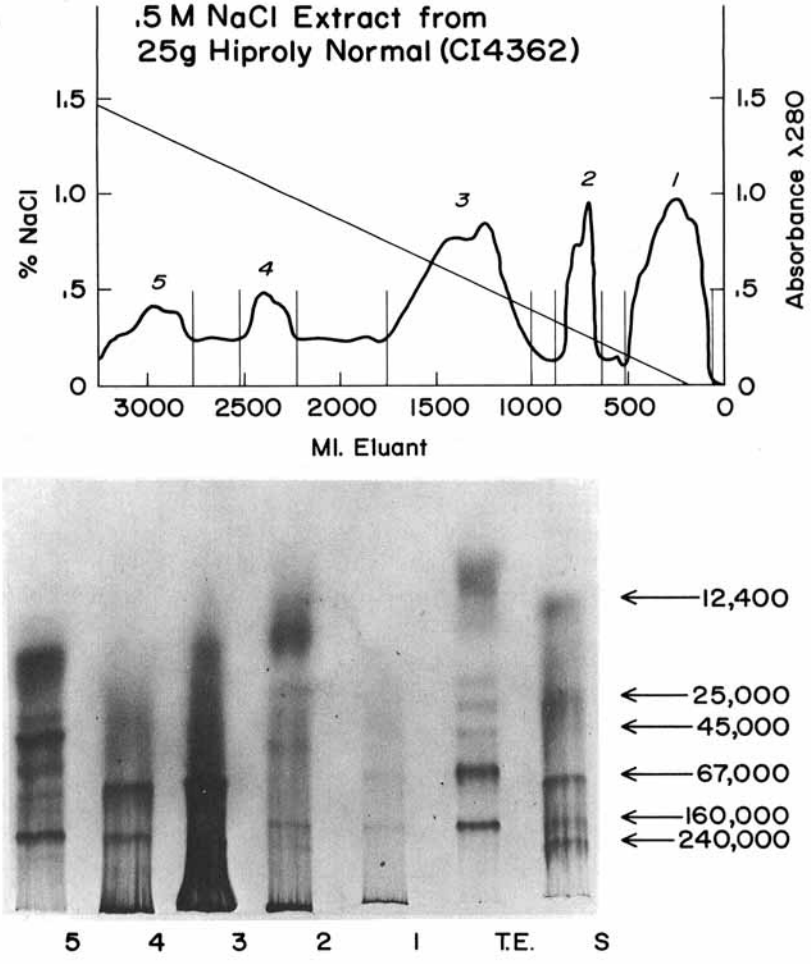

Fig. 3. Chromatographic elution absorbance pattern of the salt-soluble protein from Hiproly Normal barley (CI 4362) and electrophoretic patterns from elution peaks. T. E. = Total salt-soluble protein extract; S = mixture of molecular-weight markers; and 1, 2, 3, 4, 5 = proteins from peaks 1, 2, 3, 4, 5 of the chromatogram.

from Hiproly and Hiproly Normal is also evident from comparing the patterns in Figs. 1 and 3.

The electrophoretic pattern for fraction 1 showed traces of two components of low mobilities, while that for peak 2 showed a major component of high mobility and several minor components of lower mobilities. The proteins in fraction 3 had high affinity for staining with Coomassie Blue. Fraction 4 represented by pattern 4 in Figs. 1 and 3 contained two major components, while fraction 5 represented by pattern 5 contained a mixture of components and resembled the total salt-soluble extract (T.E.) in composition.

It is evident from these results that further research should be conducted on chromatography of these proteins to establish more suitable buffers and conditions for separation. Nimmo *et al.* (13) fractionated the water-soluble protein extracts of wheat flour chromatographically using glycine-phosphate gradient buffer as eluant. Each of the fractions obtained contained groups of proteins as demonstrated by polyacrylamide gel electrophoresis.

The molecular weights of the protein components in the chromatographic fractions in Figs. 1 and 3 were estimated from SDS polyacrylamide gel electrophoretic patterns (see **Materials and Methods**). Weber and Osborn (9) have shown that SDS electrophoresis can be used to determine the molecular weights of polypeptides with an accuracy of at least ±10%. Examination of these data indicated that the two barley isogenes contained identical components when the molecular weights of similar fractions were compared. The lack of a clean-cut separation of these compounds in different fractions was demonstrated by the fact that components with a mol wt of 60,000 occurred in peaks 1, 3, and 4, while those with a mol wt of 150,000 occurred in peaks 1, 2, and 4. It is possible that these components represent different proteins with the same molecular weights.

The nitrogen and lysine distributions in the chromatographic fractions of the salt-soluble proteins from Hiproly and Hiproly Normal barleys are presented in Table II. It should be observed that the percentage nitrogen in these fractions varied from 21.5% in fraction 5 from Hiproly Normal to 94.9% in fraction 3 from Hiproly. Fractions 1 and 5 in both barley types were characterized by their low protein contents as compared to fractions 2, 3, and 4. The lower protein content

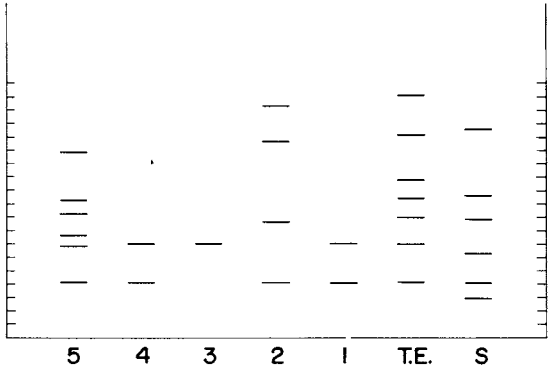

Fig. 4. Diagrammatic illustration showing band positions in the electrophoretic patterns in Fig. 3.

TABLE II
Nitrogen and Lysine Contents of the Chromatographic Fractions of Salt-Soluble Proteins from Hiproly (CI 3947) and Hiproly Normal (CI 4362) Barleys

	Hiproly (CI 3947)							Hiproly Normal (CI 4362)						
		Nitrogen content[a]			Lysine content[b]				Nitrogen content[a]			Lysine content[b]		
Fraction No. (Peaks)	Fraction weight g	% Nitrogen in fractions	Nitrogen g	% Total nitrogen recovered	mg	% Total lysine recovered	% Lysine in fractions	Fraction weight g	% Nitrogen in fractions	Nitrogen g	% Total nitrogen recovered	mg	% Total lysine recovered	% Lysine in fractions
1	0.602	32.0	0.031	26.2	15.0	24.4	7.84	0.640	31.1	0.032	28.8	11.0	25.2	5.5
2	0.139	88.9	0.019	16.4	9.0	14.6	7.28	0.138	84.3	0.019	17.4	6.0	13.3	5.17
3	0.366	94.9	0.056	47.6	31.0	50.3	8.90	0.294	86.8	0.042	37.8	19.0	43.5	7.3
4	0.064	83.8	0.009	7.3	5.0	8.1	5.10	0.118	89.6	0.016	14.5	7.0	16.1	7.8
5	0.070	26.1	0.003	2.5	1.6	2.6	4.93	0.052	21.5	0.002	1.5	0.6	1.4	6.5
Total	1.241	...	0.118	100.0	61.6	100.0	...	1.242	...	0.101	100.0	43.6	99.5	...

[a]Nitrogen: determined by micro-Kjeldahl.
[b]Lysine: from amino acid analysis.

TABLE III
Amino Acid Composition[a,b] of the Chromatographic Fractions from the Salt-Soluble Proteins of Hiproly (CI 3947) and Hiproly Normal (CI 4362) Barleys

	Fraction 1		Fraction 2		Fraction 3		Fraction 4		Fraction 5	
Parameter	**CI 3947**	**CI 4362**	**CI 3947**	**CI 4362**	**CI 3947**	**CI 4362**	**CI 3947**	**CI 4362**	**CI 3947**	**CI 4362**
Alanine	4.6	4.6	6.1	5.4	5.4	5.4	5.4	4.4	5.5	5.0
Arginine	5.0	5.2	6.6	7.1	8.9	9.1	8.4	9.4	7.3	7.6
Aspartic acid	7.3	5.9	8.0	7.1	8.4	6.9	8.7	7.8	8.5	8.1
Cystine/2[c]	2.9	3.2	1.2	2.7	1.9	3.4	3.1	3.9	3.2	3.3
Glutamic acid	20.0	22.5	15.4	18.4	14.1	14.9	14.6	13.0	13.8	13.9
Glycine	7.2	6.1	6.1	6.2	6.6	6.5	5.4	5.2	10.1	11.2
Histidine	1.9	2.1	1.8	2.7	2.4	2.9	2.7	2.6	2.4	2.2
Isoleucine	3.9	3.8	4.0	3.4	3.4	3.4	3.4	3.2	3.5	3.2
Leucine	5.3	6.0	5.6	6.3	7.1	7.2	8.2	7.8	7.7	7.2
Lysine	7.8	5.4	7.3	5.2	8.9	7.3	5.1	7.8	4.9	6.5
Methionine	1.9	1.8	3.1	2.3	2.1	2.1	2.2	2.3	2.1	2.2
Phenylalanine	4.2	4.3	4.5	4.8	4.6	4.6	5.3	4.8	4.9	4.6
Proline	9.2	9.9	7.4	7.9	5.3	5.4	5.6	6.5	5.4	5.8
Serine	4.4	4.9	5.8	5.1	5.2	5.2	4.9	4.6	4.8	4.1
Threonine	4.6	4.4	4.5	4.4	4.3	4.2	4.4	4.2	4.5	4.0
Tryptophan[d]	1.7	1.1	1.2	1.8	1.6	1.3	2.1	1.8	1.7	1.6
Tyrosine	3.0	4.0	2.8	3.5	3.1	4.1	3.8	4.3	3.4	3.8
Valine	5.3	4.7	8.8	5.9	6.8	6.2	6.7	6.4	6.3	5.8

[a]g Amino acids/100 g amino acids recovered.
[b]Twenty-four-hour hydrolysis. The serine values have been increased by 10% and threonine values by 5% to compensate for destruction by acid.
[c]Performic acid—oxidized prior to acid hydrolysis. Calculated from cysteic/alanine ratio according to Hirs (11).
[d]Forty-eight-hours' alkaline hydrolysis. Calculated from tryptophan-histidine ratio according to Hugli and Moore (12).

may be due to the higher concentration of linked carbohydrates in those fractions. The existence of covalent association of carbohydrates with certain chromatographic albumin fractions of water-soluble proteins from wheat has been reported by Nimmo *et al.* (13) and Waldschmidt-Leitz (14). Reports in the literature also indicate that pentosans associated and chromatographed with wheat albumin fractions (15,16). Fractions 1, 2, and 3 from Hiproly contained 26.2, 16.4, and 47.6% of the recovered nitrogen, respectively, which corresponded to 24.4, 14.6, and 50.3% lysine, or 90.2% of the total nitrogen and 89.3% of the total lysine. Similar fractions from Hiproly Normal contained 28.8, 17.4, and 37.8% of the recovered nitrogen corresponding to 25.2, 13.8, and 43.5% lysine, respectively, or 84% of the total nitrogen and 82.5% of the total lysine. It should be emphasized that the third fraction in both barleys containing one major electrophoretic component was the largest contributor to the lysine content of the salt-soluble proteins (50.3% in Hiproly and 43.5% in Hiproly Normal). Fractions 4 and 5 in both barleys were minor contributors of nitrogen and lysine in the salt-soluble proteins. The lysine content of the first three fractions from Hiproly exceeded that in the same fractions from Hiproly Normal by 19 mg, or about 52%.

The amino acid compositions of the various chromatographic fractions from the salt-soluble protein of Hiproly and Hiproly Normal are presented in Table III. Fractions 1, 2, and 3 from Hiproly appear to contain higher percentages of aspartic acid, lysine, and valine, and lower percentages of tyrosine, glutamic acid, and cystine/2 than comparable fractions from Hiproly Normal. Fractions 1, 2, and 3 contained 2.4, 2.1, and 1.6% more lysine, respectively, in comparison to the same fractions from Hiproly Normal. These three fractions should be considered major contributors to the high-lysine character of Hiproly.

In conclusion, the salt-soluble proteins in Hiproly and Hiproly Normal barleys contained similar protein components, as indicated by comparison of the electrophoretic mobilities of components of chromatographic fractions. However, the three major chromatographic fractions 1, 2, and 3 were substantially richer in lysine in Hiproly than in Hiproly Normal. In order to raise the lysine content of barley proteins, the genetic control mechanism responsible for regulation of these proteins must be modified. Therefore, besides the obvious interest in increasing the salt-soluble proteins as a whole, the genotypic control of the components in chromatographic fractions 1, 2, and 3 should be of particular interest in breeding work for improving barley protein quality.

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