Journal of Experimental Botany, Vol. 29, No. 110, pp. 677–692, June 1978

An Evaluation of Techniques for the Extraction of Hordein and Glutelin from Barley Seed and a Comparison of the Protein Composition of Bomi and Risø 1508

P. R. SHEWRY, J. M. HILL, H. M. PRATT, M. M. LEGGATT, AND B. J. MIFLIN

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, U.K.

Received 23 August 1977

ABSTRACT

Whole seed of barley (cv. Julia) was ground, and the meal extracted to remove lipids, non-protein nitrogen compounds, albumins, and globulins.

Four procedures for extracting hordein and glutelin from this meal were then compared. The composition of the isolated fractions was monitored by amino acid analysis and SDS polyacrylamide gel electrophoresis. More hordein was extracted by 55% (v/v) propan-2-ol containing 2% (v/v) 2-mercaptoethanol at 60 °C than at 20 °C or by sequential extraction with 55% propan-2-ol alone followed by 55% propan-2-ol plus 0·6% 2-mercaptoethanol.

After hordein extraction glutelins were successfully extracted from the residual meal by reduction and alkylation in buffer containing 8 M urea, and were precipitated by dialysis against water. Small amounts of hordein were recovered from the alkylated glutelin by washing with hot 70% (v/v) ethanol plus 0·7% (v/v) acetic acid. The acid alcohol-insoluble glutelin was free from hordein polypeptides.

Glutelins were also extracted sequentially using borate buffer at pH 10 with 0·6% mercaptoethanol followed by the same buffer with 1% SDS.

Two procedures were used to compare the hordein and glutelin composition of endosperms of high lysine (Risø 1508) and normal (Bomi, Julia) barley varieties. The hordein extracted at 60 °C by 55% propan-2-ol plus 2% 2-mercaptoethanol represented almost 50% of the total N of the endosperm of Bomi and Julia, and 16% of Risø 1508. The high lysine mutant (Risø 1508) had more glutelin and salt-soluble nitrogen than Bomi.

Electrophoretic analysis of the component polypeptides of the hordein of Bomi and Risø 1508 showed several differences in the bands present, and in their relative proportions. In contrast the hordein-free glutelins of all three varieties appeared to have similar polypeptide compositions.

Investigation of the salt-soluble fraction confirmed that the high lysine gene in Risø 1508 results in increases in both protein and non-protein nitrogen components.

The results obtained on the distribution of nitrogen between the various fractions in the seeds of Bomi and Risø 1508 and on the amino acid analysis and polypeptide composition differ considerably from those published by other workers, in which a classical Osborne type extraction was used, and we conclude that such methods should not be used for barley.

INTRODUCTION

Osborne (1895, 1924) extracted the protein from cereal seeds and separated it into various fractions according to its solubility in different solvents. In wheat, barley, and maize one of the largest fractions was soluble in aqueous ethanol and this he named prolamin. The fraction remaining after sequential extraction of the seed meal by water, salt, and alcohol was largely soluble in alkali and was termed glutelin. These terms prolamin and glutelin are still widely used, although often not clearly defined. Bishop (1928, 1929) showed that hordein, the prolamin of barley, was not particularly soluble in aqueous ethanol at room temperature and that the addition of reducing agents improved extraction (Bishop, 1939), as was shown independently by Lontie, Rondelet, and Dulcino (1953) and Lontie and Voets (1959). More recently Moureaux and Landry (1968), Landry and Moureaux (1970), and Sodek and Wilson (1971) used a similar technique for the sequential extraction of maize seed protein. Moureaux and Landry (1968) and Landry and Moureaux (1970) termed the fraction extracted in propan-2-ol with mercaptoethanol a glutelin but Sodek and Wilson (1971) called it prolamin since it had a similar amino acid composition and some of the same polypeptides as the prolamin fraction extracted in propan-2-ol alone.

Despite the marked advantages associated with the use of a reducing agent and higher temperatures many workers still use classical Osborne procedures to extract hordein (Brandt, 1975; 1976; Ingversen, Køie, and Doll, 1973; Køie, Ingversen, Andersen, Doll and Eggum, 1976; Andersen and Køie, 1975; Lauriere, Charbonnier, and Mossé, 1976; Bansal, Srivastava, Eggum, and Mehta, 1977; Balaravi, Bansal, Eggum, and Bhaskaran, 1976; Singh and Sastry, 1977; Munck, 1972). Besides giving low recoveries of hordein, these procedures also lead to contamination of the glutelin and residue fractions with hordein, as was demonstrated for wheat and maize by Paulis, James, and Wall (1969) and Bietz and Wall (1973).

The problem in the evaluation of extraction techniques is partly one of definition of the prolamin fraction, especially when, like hordein, it is not readily soluble in aqueous ethanol at room temperatures. Characterization of the hordein fraction solely by solubility properties and amino acid analysis is not satisfactory since it averages the properties of many different polypeptides. Several techniques have been used to separate hordein polypeptides including different types of electrophoresis and molecular sieve chromatography. Having thus identified such polypeptides by their electrophoretic properties, as well as by their amino acid composition, it is possible to define them as hordein components and to recognize their presence as contaminants in other protein fractions of the grain.

In this paper we report a comparison of different techniques for hordein extraction from seed meal from which the salt-soluble nitrogen has been removed. We define hordein as not only those polypeptides extracted in aqueous alcoholic solvents at room temperature, but also polypeptides with the same electrophoretic mobility which remain in the tissue after such extraction. Further support for the identification of these polypeptides as hordein is provided by amino acid analysis of the fractions containing them. We define glutelin as the protein fraction remaining after the salt-soluble nitrogen fraction and all the hordein polypeptides have been

extracted. The procedures used are based on the observations of Bishop (1928, 1929, 1939), Lontie, Rondelet, and Dulcino (1953), Lontie and Voets (1959), Paulis *et al.* (1969), and Bietz and Wall (1973). The techniques of hordein polypeptide separation used are similar to those we have published previously (Shewry *et al.*, 1977).

A high lysine mutant of barley termed Risø 1508 has been identified and preliminary analyses of its protein composition made (Ingversen *et al.*, 1973; Køie *et al.*, 1976; Andersen and Køie, 1975; Brandt, 1975, 1976) which have shown that the mutant has much less hordein than the parent variety, Bomi. When the hordein fractions were compared by single and two-dimensional polyacrylamide electrophoresis it was found that the polypeptide composition of the hordein of the mutant differed from the parent variety (Shewry, Pratt, Charlton, and Miflin, 1977). Since the original characterization used techniques which have been found to incompletely extract hordein we have used improved techniques to make a detailed comparison of the nitrogen fractions of these two lines. We have also investigated the effect of mutation on the polypeptide composition of the hordein-free glutelin. After most of the work was completed an EEC workshop was held in our laboratory in which we and workers from other laboratories compared current techniques, including some of those reprinted here. The results of this workshop have been published elsewhere (Miflin and Shewry, 1977) and the conclusions reached, although necessarily tentative because of the limited nature of the results, are essentially in agreement with the same detailed comparisons presented in this paper.

MATERIALS AND METHODS

Extraction procedures

Whole seed of Julia (field-grown at Rothamsted, 1975 crop) and freeze-dried, hand-dissected endosperms of Bomi, Risø 1508 (field-grown at Risø, 1975 crop), and Julia were ground in a Glen Creston hammer mill to pass through a 0·5 mm sieve. Defatting and extraction of salt-soluble nitrogen were carried out on an orbital shaker at 4 °C. The meal (50 g Julia whole grain, 5 g endosperms) was extracted with butan-1-ol (2 × 10 ml g^{-1} for 30 min) and petroleum ether (40–60° b.r.) (1 × 10 ml g^{-1} for 30 min) to remove lipids (Bietz and Wall, 1972) followed by 0·5 M NaCl (3 × 10 ml g^{-1} for 1 h) to remove salt-soluble nitrogen. Aliquots of the salt-soluble fraction were mixed with equal volumes of 10% (w/v) TCA and stood overnight at 4 °C to precipitate albumins and globulins which were removed by centrifugation. After salt extraction, the meal was lyophilized, finely ground, and 1 g subsamples removed for extraction of hordein and glutelin.

Four extraction procedures were compared. All 60 °C extractions were carried out in 250 ml screw-capped centrifuge bottles in a shaking water bath and all 20 °C extractions in 50 ml screw-capped centrifuge bottles in a Baird and Tatlock Multivortex shaker.

Procedure 1. This is based on the work of Lontie *et al.* (1953) and Landry and Moureaux (1970). The 1 g samples of meal were shaken at 20 °C for 1 h with 10 ml of the following solvents:

(i) 55% (v/v) propan-2-ol (hordein-1);
(ii) 55% (v/v) propan-2-ol + 0·6% (v/v) 2-mercaptoethanol (hordein-2);
(iii) 0·05 M borate buffer, pH 10. + 0·6% (v/v) 2-mercaptoethanol (glutelin-1);
(iv) 0·05 M borate buffer, pH 10, + 0·6% (v/v) 2-mercaptoethanol + 1% SDS (glutelin-2).

Each extraction was repeated twice. The nomenclature of the hordein fractions follows that used by Sodek and Wilson (1971) for the zein fractions of maize.

Procedure 2. Hordein was extracted by shaking 1 g meal at 60 °C for 3 times 1 h with 10 ml 55% (v/v) propan-2-ol + 2% (v/v) 2-mercaptoethanol. The residual solvent was removed under

vacuum and the meal was then alkylated using a modification of the method of Friedman, Krull, and Cavins (1970). After shaking overnight at 4 °C with 100 ml 0·133 M Tris/nitrate buffer, pH 7·5, containing 8 M urea, 3 μM disodium EDTA, 0·075% KCl, and 1% (v/v) 2-mercaptoethanol, the reduced protein was alkylated by shaking for 4·5 h at 20 °C with 4·5 ml 4-vinylpyridine.

The reaction was terminated by the addition of glacial acetic acid to pH 3 and the residue removed by centrifugation at 23 000 *g* for 30 min. The residue and supernatant (containing the alkylated glutelin) were dialysed for several days against tap water followed by distilled water. The alkylated glutelin, which precipitated during dialysis, was removed by centrifugation. Acid alcohol-soluble glutelin was extracted by shaking the alkylated glutelin pellet 3 times for 30 min at 60 °C with 10 ml 70% (v/v) ethanol containing 0·7% (v/v) acetic acid (Bietz and Wall, 1973).

Procedure 3. This was the same as procedure 2 except that the extraction of hordein was at 20 °C.

Procedure 4. The extraction of hordein was omitted and a combined alkylated hordein and glutelin fraction was extracted after direct alkylation of the meal (as in procedure 2). A mixture of hordein and acid alcohol-soluble glutelin was prepared by shaking the alkylated protein pellet 3 times for 30 min at 60 °C with 70% ethanol + 0·7% acetic acid (1 × 20 ml followed by 2 × 10 ml).

After the removal of aliquots for nitrogen determination, the supernatants were dialysed against distilled water to remove solvents and buffers and finally lyophilized.

Samples of the extracted hordein and glutelin-1 fractions were pyridylethylated using a modification of the procedure of Friedman *et al.* (1970) (Shewry *et al.*, 1977). Nitrogen was determined by Kjeldahl analysis.

Fractionation of the salt-soluble nitrogen

The salt-soluble fractions, prepared from milled whole seed of Bomi and Risø 1508, were separated into protein and non-protein components by either (a) adding TCA to 5% (w/v), (b) adding ethanol to 70% (v/v), or (c) dialysing against 0·5 M NaCl. The nitrogen content of all the subfractions obtained was determined. Aliquots of the total fraction, the TCA supernatant, and dialysed fraction were separated in 0·5 M NaCl on a 32 cm × 1·5 cm column of Sephadex-G75 which was calibrated with bovine serum albumin and L-lysine. Lysine in the eluate was determined using the ninhydrin-cadmium reagent of Blackburn (1965).

Amino acid determination

Protein hydrolysis. Samples (100 mg seedmeals, 10 mg extracted proteins) were hydrolysed with 6 N HCl under nitrogen in sealed tubes at 110 °C for 21 h (Kirkman, 1974). *S*-2-Pyridylethylcysteine (PEC) for standards was prepared as described by Cavins and Friedman (1970).

Amino Acid Analysis

The amino acids were analysed using a Technicon TSM-1 Autoanalyser. A single column analytical system was devised using a glass column filled with a strongly acidic ion-exchange resin, Chromobeads Type C3, obtained from Technicon, giving a column 230 mm × 5 mm. The amino acids were eluted by sequential application of four separated sodium citrate buffers and the system was designed to resolve all the expected amino acids plus PEC and to allow accurate determination of lysine (c. 1%) in the presence of a large ammonia peak derived from glutamine and asparagine. The first buffer was pH 3·05 (0·2 M Na^+) for 28 min followed by pH 4·10 (0·2 M Na^+) for 30 min, pH 7·70 (0·6 M Na^+) for 30 min and pH 9·00 (1·0 M Na^+) for 40 min. All buffers were 0·05 M with respect to citrate and the flow rate was 0·32 ml min^{-1} at 64 °C. The 2-methoxyethanol concentration in the first buffer (7·5% (v/v)) was critical in achieving separation of serine and threonine. Cystine was eluted as a frontal band with the pH 4 buffer. The pH 7·7 buffer eluted histidine, PEC, lysine, and ammonia, and would elute arginine. Changes in the ionic strength of this buffer markedly affected the elution of PEC and can cause it to merge with lysine. The pH 9·0 buffer was used to decrease the time needed to elute arginine. The total elution time was 128 min and the total running time including regeneration was 165 min.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS slab-gel system was as described previously (Shewry *et al.*, 1977).

Samples of hordein, acid alcohol-soluble glutelin, and glutelin-1 and -2 fractions were dissolved in 1% SDS (with 0·5% 2-mercaptoethanol when unalkylated) and heated to 80 °C for 2 min. They were then dialysed against 0·1% SDS (with 0·05% 2-mercaptoethanol) prior to electrophoresis.

Alkylated glutelin was extracted for electrophoresis by shaking the glutelin pellet for 16 h at 4 °C with a small volume of 0·058 M Tris/HCl buffer, pH 6·7, containing 8 M urea and 1% SDS. After centrifugation the supernatant was electrophoresed. The protein loadings were adjusted to give the best separation of each fraction. Apparent molecular weights were determined in a previous study (Shewry *et al.*, 1977). The nomenclature of the hordein bands follow Køie *et al.* (1976) and a comparison of this nomenclature with that of other workers has been made previously (Shewry *et al.*, 1977).

RESULTS

Comparison of extraction procedures

Milled whole grain of Julia was extracted to remove lipids and salt-soluble nitrogen and then used for a comparison of the four methods of extracting hordein and glutelin. Eight replicate 1 g samples were used for each extraction procedure and the mean amounts of nitrogen recovered in the fractions, together with the standard deviations, are given in Table 1. Procedures 1, 2, and 3 all gave good reproducibility between replicates but procedure 4 was less satisfactory in this respect.

The relatively large amounts of glutamic acid + glutamine and proline residues and extremely small amounts of lysine found in hordein means that the levels of these amino acids can serve as indicators of the purity of the hordein and glutelin fractions. The amounts of these amino acids in the various fractions are, therefore, given in Table 1. The composition of the fractions was also monitored by determining the polypeptide patterns by SDS polyacrylamide gel electrophoresis (Figs 1, 2).

The amount of protein extracted by aqueous propan-2-ol at 20 °C (designated hordein-1) in procedure 1 was only 24% of the total seed N and a further 9% was removed by a subsequent extraction with 0·6% 2-mercaptoethanol also present (designated hordein-2). Increasing the 2-mercaptoethanol concentration to 2% (procedure 3) resulted in more protein being extracted by a single solvent than by the two sequential extractions of procedure 1. Further increases in protein extraction were obtained by extracting at 60 °C when almost 40% of the total seed N was extracted (procedure 2). There was no evidence from the amino acid composition (Table 1), or from the electrophoresis pattern (Fig. 1b) to suggest that the greater amounts of N extracted in procedure 2 were due to contamination of the hordein fraction by other proteins. The beneficial effect of alkylation on the hordein separation pattern is shown by comparing Fig. 1b with Fig. 1a, thus confirming our previous findings (Shewry *et al.*, 1977). Comparison of Fig. 1d with Fig. 1e shows the differences in the relative distribution of polypeptides extracted sequentially in the absence and presence of a reducing agent. Although the group of bands of approximate molecular weight 67 000 (designated C bands) are almost completely extracted by propan-2-ol, alone the addition of 2-mercaptoethanol results in a further extraction of the bands between molecular weight 30 000 and 51 000 (B bands).

The total amounts of N in the hordein and acid alcohol-soluble glutelin fractions (43–44%) were similar in procedures 2 and 3 and only slightly greater than when the two fractions were extracted in a single step by direct alkylation (procedure 4). The protein extracted by this method contained mostly hordein, but also some

TABLE 1. *The nitrogen content and amino acid composition of protein fractions extracted from barley var. Julia*

Four procedures for the extraction of hordein and glutelin are compared.

	mg N g^{-1} dry wt.		% Total N	Amino acid composition[a]		
	mean	s.d.		Glutamate + glutamine	Proline	Lysine
Original milled seed	12·56	—	100	20·6	11·9	3·6
Salt-soluble fraction	3·56	—	26·9	14·2[b]	8·7[b]	5·3[b]
Procedure 1						
Hordein-1	3·22	0·07	24·3	32·3	23·8	0·9
Hordein-2	1·17	0·09	8·8	32·0	20·9	1·1
Glutelin-1	0·29	0·02	2·2	18·3	10·2	3·6
Glutelin-2	3·34	0·05	25·2	13·8	4·7	5·3
Residue	1·33	0·04	10·1	13·8	7·9	4·7
Total N recovered[c]	12·91	—	97·5	—	—	—
Procedure 2 (60 °C hordein extraction)						
Hordein	5·27	0·11	39·8	29·3	19·6	1·0
Acid alcohol-soluble glutelin	0·54	0·06	4·1	19·0	11·2	3·9
Acid alcohol-insoluble glutelin	1·21	0·13	9·1	13·2	7·3	4·8
Residue	2·05	0·13	15·5	10·7	6·6	5·0
Total N recovered[c]	12·62	—	95·4	—	—	—
Procedure 3 (20 °C hordein extraction)						
Hordein	4·88	0·05	36·9	30·9	20·4	0·8
Acid alcohol-soluble glutelin	0·85	0·08	6·4	19·2	11·3	3·6
Acid alcohol-insoluble glutelin	1·42	0·12	10·7	12·6	6·3	5·1
Residue	1·66	0·15	12·6	10·9	6·6	6·2
Total N recovered[c]	12·37	—	93·5	—	—	—
Procedure 4 (direct alkylation)						
Hordein + acid alcohol-soluble glutelin	5·49	0·22	41·4	28·4	19·5	1·5
Acid alcohol-insoluble glutelin	2·21	0·19	16·7	14·1	8·1	4·4
Residue	1·00	0·23	7·6	10·7	6·7	4·6
Total N recovered[c]	12·26	—	92·6	—	—	—

[a] Expressed as mole %.
[b] Salt-soluble protein only.
[c] Includes salt-soluble nitrogen.

other polypeptides, and had slightly higher lysine (1·5%) than the hordein fractions extracted by other procedures.

Polyacrylamide gel analysis of the glutelin fractions was difficult due to the presence of stain-absorbing material which was only partially removed by prolonged dialysis and high speed centrifugation of the protein samples. The resolution of the glutelin fractions from procedure 1 was not affected by alkylation (results not shown). The acid alcohol-insoluble glutelin prepared by procedures 2, 3, and 4 was difficult to dissolve for electrophoresis; the best method was shaking it

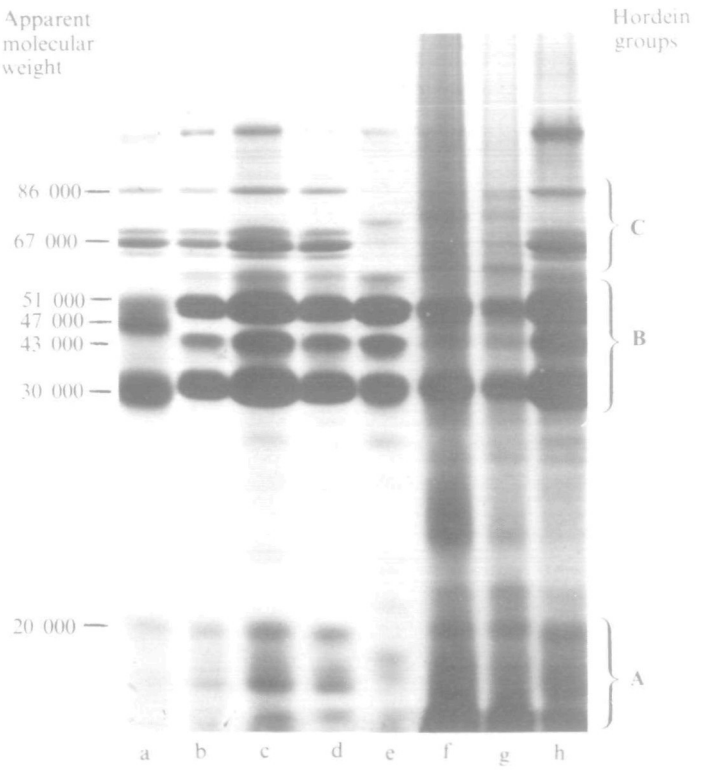

FIG. 1. SDS polyacrylamide gel electrophoresis of hordein and acid alcohol-soluble glutelin fractions extracted from milled whole seed of barley var. Julia. (a) Total hordein (procedure 2); (b) PE total hordein (procedure 2); (c) PE total hordein (procedure 3); (d) PE hordein 1 (procedure 1); (e) PE hordein 2 (procedure 1); (f) PE acid alcohol-soluble glutelin (procedure 2); (g) PE acid alcohol-soluble glutelin (procedure 3); (h) PE hordein + acid alcohol-soluble glutelin (procedure 4).

with a small volume of 8 M urea and 1% SDS (an anionic detergent) at pH 7. Extremes of pH and other detergents such as Triton X-100 (non-polar) and cetyltrimethylammonium bromide (cationic) were less effective.

The acid alcohol-insoluble glutelin fractions prepared by the three alkylation procedures had similar amino acid and polypeptide compositions and were free from residual hordein bands. There was some evidence for residual hordein-like polypeptides in the glutelin-1 and glutelin-2 fractions of procedure 1 (Fig. 2f and g, see arrows). However, this contamination was not sufficient to markedly affect the overall amino acid composition of the glutelin-2 fraction in that its glutamic acid + glutamine and lysine content was similar to the acid alcohol-insoluble glutelin of procedures 2 and 3. In this case the presence of low lysine hordein polypeptides may have been balanced by more complete extraction of lysine-rich proteins which remained in the residues with procedures 2 and 3. There was little difference in the amino acid composition of the acid alcohol-insoluble glutelins of procedures 2, 3, and 4 or in their polypeptide patterns, which were similar to the glutelin fractions of procedure 1 (Fig. 2b, c, d, f, and g). Some of the polypeptides in the glutelin fraction had similar mobilities on the gels to some of the hordein bands, notably the mol. wt. 47 000 and 51 000 polypeptides in unalkylated hordein preparations.

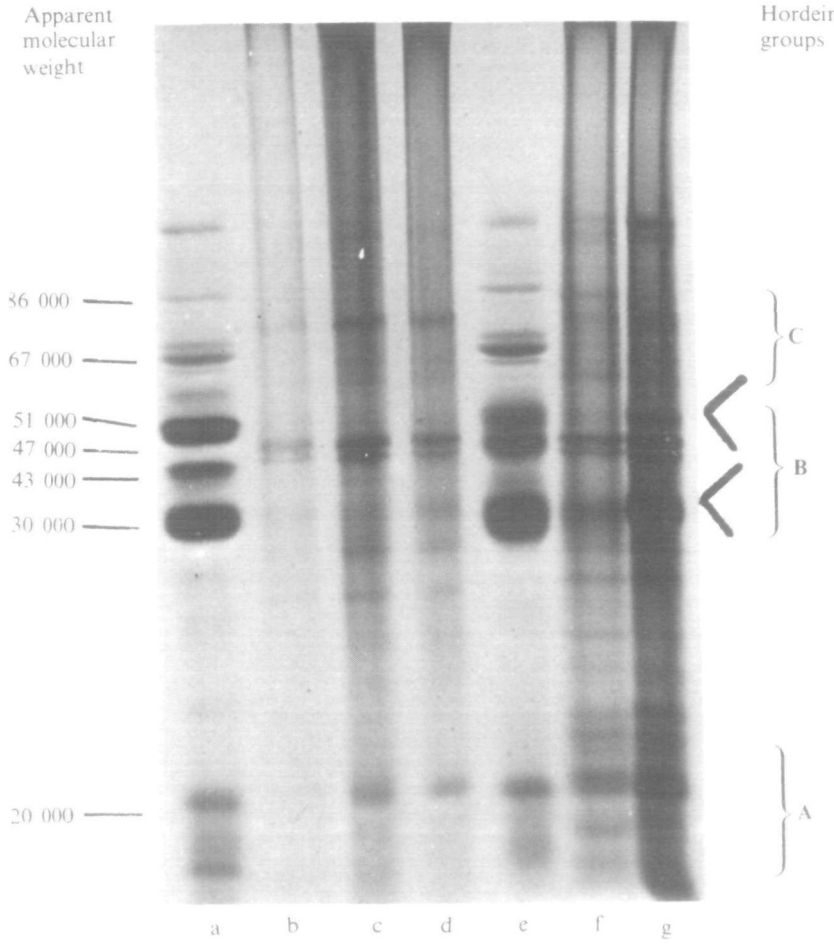

FIG. 2. SDS polyacrylamide gel electrophoresis of hordein and glutelin fractions extracted from milled whole seed of barley var. Julia. (a) PE total hordein (procedure 2); (b) PE acid alcohol-insoluble glutelin (procedure 2); (c) PE acid alcohol-insoluble glutelin (procedure 3); (d) PE acid alcohol-insoluble glutelin (procedure 4); (e) total hordein (procedure 2); (f) glutelin 1 (procedure 1); (g) glutelin 2 (procedure 1). Residual hordein bands in the glutelin-1 and glutelin-2 fractions are indicated by arrows.

However, alkylation changed the mobility of the mol. wt. 47 000 hordein band (cf. Fig. 2a and e) but had no effect on the glutelin bands (cf. Fig. 2d and f).

The residue of procedure 1 contained more glutamic acid + glutamine and proline than that from the other procedures, suggesting possible contamination with residual hordein.

Comparison of hordein and glutelin from high-lysine and normal barleys

Procedures 1 and 2 were used to compare the hordein and glutelin composition of endosperms of high lysine (Risø 1508) and normal (Bomi, Julia) barleys. The meal was extracted to remove lipids and salt-soluble N prior to subsampling. Duplicate 1 g subsamples were used for hordein and glutelin extraction and the means of the N recovered are present in Table 2. Again the amount of hordein N

TABLE 2. *The nitrogen content and amino acid composition of protein fractions extracted from endosperms of normal (Julia, Bomi) and high lysine (Risø 1508) varieties of barley*

	Julia[a]				Bomi[a]				Risø 1508[a]			
	% Total N	Amino acid composition[c]			% Total N	Amino acid composition[c]			% Total N	Amino acid composition[c]		
		Glx[d]	Pro	Lys		Glx[d]	Pro	Lys		Glx[d]	Pro	Lys
Original meal[b]	100	23·9	14·5	3·1	100	23·1	15·1	2·9	100	15·4	9·1	4·7
Salt-soluble fraction	22·1	—	—	—	20·1	—	—	—	35·4	—	—	—
Albumin + globulin	13·5	15·6	9·2	4·6	11·4	13·8	9·0	4·2	19·6	11·7	8·1	4·8
Non-protein nitrogen	8·6	NA	NA	NA	8·7	NA	NA	NA	15·8	NA	NA	NA
Procedure 1												
Hordein 1	32·1	32·1	23·1	0·8	26·2	32·4	24·2	0·9	6·7	23·9	16·6	2·7
Hordein 2	8·3	31·9	22·4	0·7	13·3	33·6	23·2	0·4	1·1	29·9	19·2	1·3
Glutelin 1	2·6	16·0	8·1	4·0	2·8	14·2	9·3	4·8	4·8	19·8	7·9	3·5
Glutelin 2	22·3	15·4	8·7	5·1	24·5	15·5	9·5	5·4	36·5	12·8	7·0	5·4
Residue	9·2	20·7	11·6	3·2	9·3	17·9	10·2	4·0	13·1	15·9	8·8	3·9
Total N recovered[e]	96·6	—	—	—	96·2	—	—	—	97·6	—	—	—
Procedure 2												
Hordein	49·9	32·6	22·3	0·9	47·4	32·6	21·8	0·9	15·6	25·4	15·0	2·5
Acid alcohol-soluble glutelin	5·0	21·1	12·7	3·2	4·1	18·6	11·6	3·4	13·2	15·8	7·2	5·0
Acid alcohol-insoluble glutelin	8·5	10·8	7·0	5·2	9·8	12·7	6·1	5·3	14·6	12·1	5·8	5·1
Residue	10·7	12·2	6·5	5·0	11·9	11·8	6·5	5·3	14·8	11·3	5·9	4·9
Total N recovered[e]	96·2	—	—	—	93·3	—	—	—	93·6	—	—	—

NA = not analysed.

[a] The 100-endosperm weights for Julia, Bomi, and Risø 1508 were 4·02, 3·84, and 3·39 g respectively.

[b] The meals of Julia, Bomi, and Risø 1508 contained 13·6, 15·0, and 16·6 mg N g^{-1} dry wt. respectively.

[c] Expressed as mole %.

[d] Glutamic acid + glutamine.

[e] Includes salt-soluble fraction.

extracted from Bomi and Julia was greater using procedure 2 (almost 50% of the total endosperm N) than with procedure 1 (approximately 40%). The hordein-1 fraction accounted for only 25–30% of the total endosperm N.

The hordein fractions from Bomi and Julia had a typical hordein amino acid composition with a predominance of glutamic acid + glutamine (30–34%) and proline (22–24%) residues and with little lysine (<1%) present. In Risø 1508, however, the hordein extracted by procedure 2 and the hordein-1 fraction were both lower in glutamic acid + glutamine (24–25%) and proline (15–17%), and higher in lysine (>2%) than in Bomi. The hordein-2 fraction of 1508 was more typical with only 1·3% lysine.

The high lysine content of the Risø 1508 hordein-1 fraction can probably be explained by consideration of the polypeptide banding pattern (Fig. 3) which shows that it contained a relatively large proportion of low molecular weight (<20 000) bands. This group of bands, designated the A bands (Køie *et al.*, 1976) are relatively high in lysine (Ivanov, Mesrob, and Prusik, 1968; Shewry *et al.*, 1977).

The differences between the hordein banding patterns of Bomi and Risø 1508 are most noticeable in the hordein-2 fraction (Fig. 3c, f); Risø 1508 consists mainly of the mol. wt. 51 000 band with no mol. wt. 43 000 band and very little of the mol. wt. 30 000 band. Analysis of hordein polypeptides extracted by procedure 2 from Bomi and Risø 1508 (Fig. 3a, d) shows the same features, notably the larger amount of mol. wt. 51 000 band, the absence of the mol. wt. 43 000 band, and the

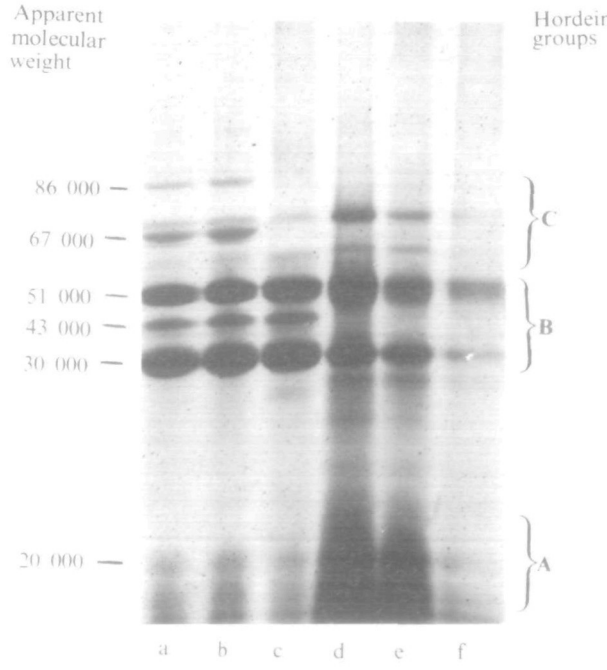

FIG. 3. SDS polyacrylamide gel electrophoresis of hordein fractions extracted from endosperms of barley vars Bomi and Risø 1508. (a) Bomi PE total hordein (procedure 2); (b) Bomi PE hordein 1 (procedure 1); (c) Bomi PE hordein 2 (procedure 1); (d) Risø 1508 PE total hordein (procedure 2); (e) Risø 1508 PE hordein 1 (procedure 1); (f) Risø 1508 PE hordein 2 (procedure 1). The hordein fractions of Julia are essentially similar to those of Bomi and are not shown.

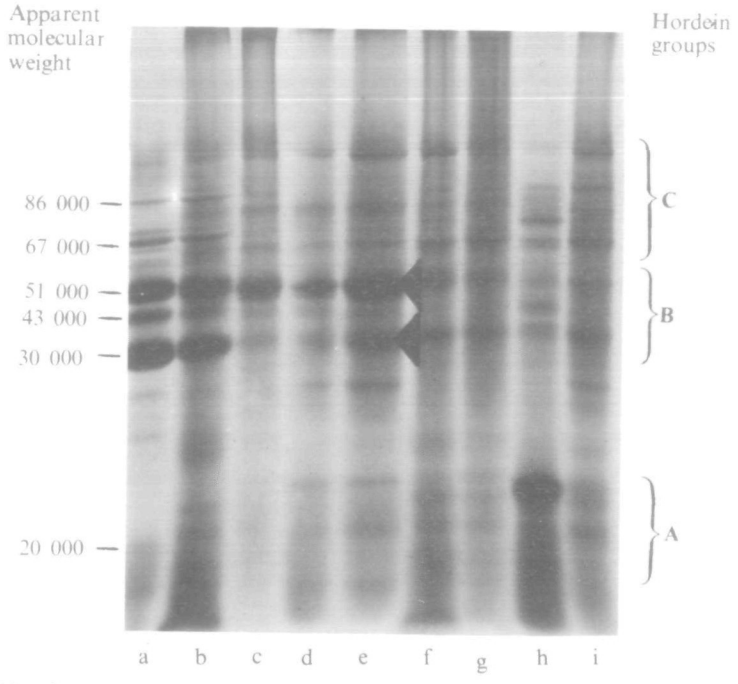

Fig. 4. SDS polyacrylamide gel electrophoresis of glutelin fractions extracted from endosperms of barley vars Bomi and Risø 1508. (a) Bomi PE total hordein (procedure 2); (b) Bomi PE acid alcohol-soluble glutelin (procedure 2); (c) Bomi PE acid alcohol-insoluble glutelin (procedure 2); (d) Bomi glutelin 1 (procedure 1); (e) Bomi glutelin 2 (procedure 1); (f) Risø 1508 PE acid alcohol-soluble glutelin (procedure 2); (g) Risø 1508 PE acid alcohol-insoluble glutelin (procedure 2); (h) Risø 1508 glutelin 1 (procedure 1); (i) Risø 1508 glutelin 2 (procedure 1). Residual hordein bands in the glutelin-1 and glutelin-2 fractions of Bomi are shown by arrows.

much larger amount of low molecular weight material in Risø 1508. Differences are also apparent in the group of polypeptides around mol. wt. 67 000 (Fig. 3a, d), in which the triple banding pattern in Bomi is replaced by a single band in Risø 1508. There is some evidence in Fig. 3 to suggest that the hordein-1 of Risø 1508 contains some minor bands not present in Bomi; these are probably either minor hordein components or low level contaminants which are only seen because large amounts of material were extracted to obtain the protein separated on the gel. When hordein from an equivalent weight of Bomi seed is separated these bands also appear on the gel. The banding pattern of Bomi is similar to that previously found in Julia: the hordein-1 is rich in mol. wt. 67 000 and 86 000 polypeptides while the hordein-2 consists predominantly of the mol. wt. 30 000, 43 000, and 51 000 bands.

The acid alcohol-soluble glutelin fractions of Bomi and Julia accounted for 4–5% of the total seed N and again contained both hordein and glutelin polypeptides (Fig. 4b) with an intermediate amino acid composition (Table 2). The glutelin-1 fraction was only a minor component (less than 3% of the total seed N) and the amino acid analysis suggested the presence of residual hordein. SDS polyacrylamide gel electrophoresis of the glutelin-1 and glutelin-2 fractions likewise suggested the presence of some hordein contamination particularly of the mol. wt.

30 000 band (arrows). As in the previous experiment several polypeptides were common to the acid alcohol-insoluble glutelins and the glutelin-1 and -2 fractions.

In Risø 1508 the acid alcohol-soluble glutelin fraction accounted for about 13% of the total seed N; its amino acid composition was more glutelin-like than in Bomi and Julia indicating little hordein contamination. In contrast, the glutelin-1 fraction of Risø 1508 contained relatively more glutamic acid + glutamine and less lysine than in Bomi, probably reflecting the low hordein extraction of Risø 1508 by procedure 1. However, there is no clear evidence for hordein contamination in the polypeptide pattern (Fig. 4) except perhaps in the low molecular weight region. In total the glutelin fractions formed a larger proportion of the total seed N in Risø 1508 than in Bomi or Julia.

Comparison of the glutelin fractions of Bomi and Risø 1508 demonstrated that many of the polypeptides occurred in both, particularly when the acid alcohol-insoluble glutelin fractions (Fig. 4c, g) are compared. The major difference is in the major band of mol. wt. approx. 50 000 which is greater in Bomi than Risø 1508.

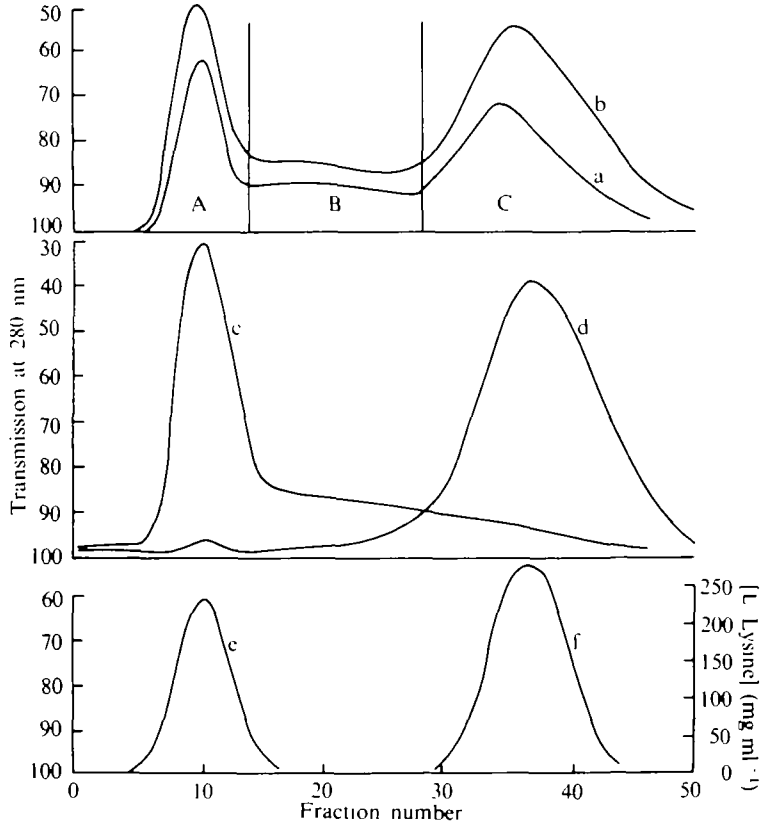

FIG. 5. The separation of salt-soluble fractions of Bomi and Risø 1508 on Sephadex G-75. Aliquots (5 ml) of the total salt-soluble fraction of Bomi (a) and of the total (b), dialysed (c). and 5% TCA-soluble (d) salt-soluble fractions of Risø 1508 were applied to a 32 cm × 1·5 cm column of Sephadex G-75 and eluted with 0·5 M NaCl. The transmission of the eluate at 280 nm was measured with an LKB uvicord. The column was calibrated with bovine serum albumin (e) and L-lysine (f). The areas marked A, B, and C are discussed in the text.

The residues from procedure 1 for all three varieties contained relatively more glutamic acid + glutamine and proline and less lysine than the glutelin fractions, suggesting the presence of residual hordein. In contrast the residues from procedure 2 had an amino acid composition similar to the glutelin fractions indicating minimal contamination with hordein and suggesting that all the hordein had been extracted.

Investigation of the salt-soluble fraction

To confirm that the high lysine gene in Risø 1508 caused increases in both the protein and non-protein components of the salt-soluble fraction, a further study was made of salt-soluble fractions prepared from whole grain of Bomi and Risø 1508. These fractions contained 23% of the total seed N in Bomi and 39% in Risø 1508. The N retained during dialysis was 16% in Bomi and 25% in Risø 1508 compared to 14% and 24% respectively precipitated by 5% TCA. Addition of ethanol to 70% precipitated less N: 9% in Bomi and 17% in Risø 1508. Examination of the supernatants by SDS polyacrylamide gel electrophoresis showed only traces of low molecular weight polypeptides in the TCA supernatants while the ethanol supernatants contained more proteins with a wider range of molecular weights. It was therefore concluded that the addition of TCA to a final concentration of 5% gave an accurate determination of the amount of protein in the salt-soluble fractions.

This was confirmed by separation of the fractions on a column of Sephadex-G75 (Fig. 5). Separation of the total salt-soluble fractions showed the presence of two peaks which co-eluted with bovine serum albumin and L-lysine (areas A and C in Fig. 5) separated by a low peak of material of intermediate molecular weight (area B). When equal amounts of salt-soluble fractions of Risø 1508 and Bomi (equivalent to 0·15 g tissue) were applied to the column, the u.v. absorbance of all three peaks was higher in Risø 1508. When the 5% TCA supernatant was applied to the column only traces of the high and medium molecular weight peaks were present and an aliquot which had been dialysed against 0·5 M NaCl contained only traces of the low molecular weight peak.

DISCUSSION

The comparison of methods demonstrated that almost complete removal of hordein can be achieved by repeated extraction of finely milled barley flour with 55% propan-2-ol plus 2% 2-mercaptoethanol at 60 °C (procedure 2). Evidence that the protein extracted is hordein was provided by amino acid analysis, and the polypeptide patterns given by SDS polyacrylamide gel electrophoresis. There was some evidence for residual hordein in the glutelin fraction, particularly in that part of the alkylated glutelin fraction soluble in acid-alcohol. However, this was less than 10% of the hordein already extracted and, on the basis of amino acid analysis and electrophoretic pattern, contained glutelin-like polypeptides as well as hordein. Consequently, the amount of hordein unextracted by procedure 2 is not likely to exceed more than about 5% of the total. In contrast, procedure 1, which is similar to the Landry and Moureaux (1970) and Sodek and Wilson (1971) methods for

maize, extracts less alcohol-soluble protein from barley than either procedure 2 or 3, and electrophoretic analysis of the glutelin fractions shows the presence of residual hordein. There is also some evidence, from amino acid analysis, for hordein contamination of the residue.

Procedure 1 also allows an evaluation of the classical Osborne procedure (using aqueous alcohol at room temperature in the absence of reductant) to be made. In the original work ethanol was used, but propan-2-ol is now more common, because it appears to be more effective. However, the amounts of hordein extracted are still small. For example, only 43–64% of the total hordein (as measured in procedure 2) was extracted. Similar results have been calculated for zein (Miflin, 1978). Also, in a recent collaborative comparison of various extraction methods (Miflin and Shewry, 1977) it was found that a classical Osborne procedure extracted only 20% of the total seed nitrogen compared to about 40% by three other more exhaustive methods using reducing agents. Consequently, although the Osborne method is still widely used, there is little to recommend it particularly because it can lead to erroneous conclusions.

The results of the comparison of the extraction methods re-emphasize and confirm points made by other workers. These are: (1) the importance of repeated extractions (Bishop, 1928), (2) the need to use greater than ambient temperatures (Bishop, 1928; Preaux and Lontie, 1975), and (3) the need to include a reducing agent (Bishop, 1939; Lontie *et al.*, 1953; Lontie and Voets, 1959).[1]

Not only does the extraction method affect the total amount of the hordein fraction, it also affects its polypeptide and amino acid composition. We have recently demonstrated differences in the polypeptide composition of hordein-1 and hordein-2 fractions extracted from Julia, Bomi, and Risø 1508 (Shewry *et al.*, 1977). The present study confirms these observations and also demonstrates that the hordein-1 and hordein-2 fractions of Bomi and Julia differ from the total hordein in their polypeptide composition, although all are similar in their amino acid composition. In Risø 1508 the fractions also differ in their amino acid composition, the unusual composition of the hordein-1 and total hordein fractions probably being due to the relatively large amount of the low molecular weight A or α-hordein. This has been shown to contain less glutamic acid + glutamine and proline and more lysine than other hordein fractions (Ivanov *et al.*, 1968).

Incomplete hordein extraction may also lead to misleading values for the total amount and the amino acid composition of the glutelin fraction. Thus Ingversen *et al.* (1973) originally found that the amount of glutelin N was similar in Bomi and Risø 1508 (39% of the total seed N). This has subsequently been disproved during recent re-investigations by Køie and Nielson and by Shewry, Pratt, and Miflin (Miflin and Shewry, 1977) which demonstrated increased glutelin in Risø 1508 (37% compared to 25%), a conclusion confirmed by the present investigation.

The presence of hordein in the glutelin fraction affects its amino acid composition, and if different varieties or treatments have different levels of

[1] The use of higher temperatures can probably be replaced by a highly effective mechanical stirring apparatus as used by Køie and Nielsen, 1977 (see Miflin and Shewry, 1977).

contamination incorrect conclusions may be drawn. Thus Ingversen *et al.* (1973) found that the amino acid composition of glutelins from Bomi and Risø 1508 differed, and they suggested that this might indicate a different polypeptide composition in the two lines. However, in the present study there were few differences in either the amino acid composition or the electrophoretic banding pattern. Andersen and Køie (1975) reported that the amino acid composition of the glutelin fraction changes with increasing nitrogen content of the grain. However, as the hordein was extracted at room temperature without a reducing agent the changes are probably due to increasing amounts of hordein contamination.

Previous studies (Brandt, 1976; Lauriere *et al.*, 1976; Singh and Sastry, 1977) of the polypeptide composition of barley glutelins used techniques that result in incomplete extraction of hordein and thus it is not surprising that patterns obtained show a high degree of contamination with hordein polypeptides. When this contamination is removed it can be seen (Figs 2, 4) that the glutelin fraction has a distinctive and complex electrophoretic banding pattern. Although the alkylation procedures are of value in providing hordein-free glutelin fractions, the small amounts of hordein remaining after exhaustive extraction can conveniently be ignored in many studies and the glutelins extracted in a single fraction using 1% SDS and 0·6% 2-mercaptoethanol at pH 10.

The exact nature of the glutelin-1 fraction is uncertain. As in maize it has a similar amino acid composition to the acid alcohol-soluble glutelin fraction (Misra and Mertz, 1976), but it does not resemble this in its polypeptide pattern. Also, its contribution to the total seed nitrogen of normal varieties is small (less than 3% in Bomi and Julia) compared to approximately 8% in maize (Moureaux and Landry, 1968; Landry and Moureaux, 1970).

The results on the salt-soluble fraction confirm the suggestion of Brandt (1976) that increases in both non-protein and protein components of the salt-soluble nitrogen fraction occur in Risø 1508. This is in contrast to the work of Ingversen, Køie, and Doll (1973) and Andersen and Køie (1975) which did not consider the contribution of non-protein components. Increased levels of free amino acids also occur in high lysine mutants of maize and sorghum (Mertz, Misra, and Jambunathan, 1974).

The results presented here show that the mutation in Risø 1508 causes a decrease in the proportion of seed nitrogen in hordein to a third of that in Bomi, a doubling of the proportion of the seed nitrogen in glutelin, and increases of 70% and 80% in that in the albumin plus globulin and TCA-soluble nitrogen fractions respectively. Since the amount of nitrogen per endosperm is nearly the same in Bomi and Risø 1508 (576 μg compared to 563 μg), the changes represent real changes in amounts per endosperm.

Besides affecting the amount of hordein the mutation also affects its overall amino acid composition and the relative distribution of polypeptides within the fraction. In contrast there is no evidence for any change in the nature of the glutelin polypeptides.

ACKNOWLEDGEMENTS

We wish to thank Dr. Hans Doll and his colleagues at Risø for seed of Bomi and Risø 1508 and for useful discussions, and Dr. J. S. Wall and colleagues for advice on alkylation methods. The research was supported by EEC contract No. 473.

LITERATURE CITED

ANDERSEN, A. J., and KØIE, B., 1975. *Agron. J.* **67,** 695–8.

BALARAVI, S. P., BANSAL, H. C., EGGUM, B. O., and BHASKARAN, S., 1976. *J. Sci. Fd Agric.* **27,** 545–52.

BANSAL, H. C., SRIVASTAVA, K. N., EGGUM, B. O., and MEHTA, S. L., 1977. Ibid. **28,** 157–60.

BIETZ, J. A., and WALL, J. S., 1972. *Cereal Chem.* **49,** 416–30.

—— —— 1973. Ibid. **50,** 537–47.

BISHOP, L. R., 1928. *J. Inst. Brew.* **34,** 101–18.

—— 1929. Ibid. **35,** 316–22.

—— 1939. *The proteins of barley grain with special reference to hordein.* MSc. thesis. University of Birmingham.

BLACKBURN, S., 1965. In *Methods of biochemical analysis.* Ed. D. Glick. Interscience, New York. Vol. 8, 1–45.

BRANDT, A., 1975. *FEBS Lett.* **52,** 288–91.

—— 1976. *Cereal Chem.* **53,** 890–901.

CAVINS, J. F., and FRIEDMAN, M., 1970. *Analyt. Biochem.* **35,** 489–93.

FRIEDMAN, M., KRULL, L. H., and CAVINS, J. F., 1970. *J. biol. Chem.* **245,** 3868–71.

INGVERSEN, J., KØIE, B., and DOLL, H., 1973. *Experientia,* **29,** 1151–2.

IVANOV, CH., MESROB, B., and PRUSIK, Z., 1968. *Can. J. Biochem.* **46,** 1301–7.

KIRKMAN, M., 1974. *J. Chromat.* **97,** 175–91.

KØIE, B., INGVERSEN, J., ANDERSEN, A. J., DOLL, H., and EGGUM, B. O., 1976. In *Evaluation of seed protein alterations by mutation breeding.* Proc. Research Co-ordination Meeting, Hahnenklee, 1975, IAEA, Vienna. Pp. 55–61.

LANDRY, J., and MOUREAUX, T., 1970. *Bull. Soc. Chim. biol.* **52,** 1021–37.

LAURIERE, M., CHARBONNIER, L., and MOSSÉ, J., 1976. *Biochimie,* **58,** 1235–45.

LONTIE, R., RONDELET, J., and DULCINO, J., 1953. *Eur. Brew. Conv.,* Nice. Pp. 33–8.

—— and VOETS, T., 1959. *Eur. Brew. Conv.,* Rome. Pp. 27–36.

MERTZ, E. T., MISRA, P. S., and JAMBUNATHAN, R., 1974. *Cereal Chem.* **51,** 304–7.

MIFLIN, B. J., 1978. In *Nitrogen in the environment.* Ed. D. R. Nielsen and J. G. MacDonald. Academic Press. New York (in press).

—— and SHEWRY, P. R., 1977. (Eds.). *Techniques for the separation of barley and maize seed protein.* Publ. by the Commission for European Communities, Luxembourg.

MISRA, P. S., and MERTZ, E. T., 1976. *Cereal Chem.* **53,** 699–704.

MOUREAUX, T., and LANDRY, J., 1968. *C. r. hebd. Séanc. Acad. Sci., Paris,* **266,** 2302–5.

MUNCK, L., 1972. *Hereditas,* **72,** 1–128.

OSBORNE, T. B., 1895. *J. Am. chem. Soc.* **17,** 539–67.

—— 1924. *The vegetable proteins.* Longmans, London. Pp. 1–154.

PAULIS, J. W., JAMES, C., and WALL, J. S., 1969. *J. agric. Fd Chem.* **17,** 1301–5.

PREAUX, G., and LONTIE, R., 1975. In *The chemistry and biochemistry of plant proteins.* Ed. J. B. Harborne and C. F. Van Sumere. Academic Press, London. Pp. 89–111.

SHEWRY, P. R., PRATT, H. M., CHARLTON, M. J., and MIFLIN, B. J., 1977. *J. exp. Bot.* **28,** 597–606.

SINGH, U., and SASTRY, L. V. S., 1977. *Cereal Chem.* **54,** 1–12.

SODEK, L., and WILSON, C. M., 1971. *J. agric. Fd Chem.* **19,** 1144–50.