Effects of Genotype and Nitrogen Nutrition on Protein Aggregates in Barley[1]

FLORENCE BÉNÉTRIX,[2] AHMAD SARRAFI,[3] and JEAN-CLAUDE AUTRAN[2]

ABSTRACT

Cereal Chem. 71(1):75–82

Eight barley cultivars were grown and treated by the application of nitrogen at six different times and rates. Phosphate-sodium dodecyl sulfate extraction and size-exclusion high-performance liquid chromatography were used to separate different sizes of protein aggregates and monomers. Their compositions were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was found that the major protein fractions do not vary in the same way. The insoluble residue and the total protein content were significantly influenced by nitrogen fertilization. The size-exclusion high-performance liquid chromatography fraction F4, which is rich in C-hordein subunits, was also significantly influenced by nitrogen fertilization, while some others (F3 and F5) were essentially cultivar-dependent. Fraction F4 emerged as the best criterion for distinguishing between winter and spring barleys, and its quantitation could be recommended as a screening test of barley samples. This study provides a relevant approach for the understanding of the functional properties of hordeins in malting quality. It further supports the hypothesis that increased amounts of C hordeins impair malting quality by limiting water diffusion during the steeping phase.

Protein content and composition of barley are of considerable importance in malt quality (Shewry and Miflin 1983, Smith and Lister 1983). High-protein barleys generally impair processing quality by altering the malt modification, extract yield, filtration during brewing, clarity of beer, and foam stability (Van den Berg et al 1981). Accordingly, it is largely accepted that relatively low-protein barleys (11.0–11.5% protein content) are best suited for malting and brewing.

The most abundant proteins in barley endosperm are the hordeins. These primary storage proteins can be separated into three groups: sulfur-rich B-hordein subunits, sulfur-poor C-hordein subunits, and high molecular weight D-hordein subunits (Shewry and Miflin 1983). On the assumption that aggregated hordeins (especially B-hordein subunits) that are associated by disulfide bonds might form a persisting matrix around the starch granules and might therefore restrict the hydrolysis of starch by amylases during mashing, early studies suggested that malting quality was related to the amount of B-hordein subunits (Baxter and Wainwright 1979, Slack et al 1979, Baxter 1981, Shewry et al 1981). Hence, the tendency of B- and D-hordein subunits to form complexes (Smith and Simpson 1983) or greater amounts of gel protein (Smith and Lister 1983) was thought to be an indicator of poor malting quality. Skerritt and Janes (1992) also observed a specific elevation of B1- and B2-hordein subunits in poor malting samples. The same authors also demonstrated that hordein subunits within a cultivar differed in their extractability from the aggregate at any given reductant concentration as sulfhydryl reducing-agent concentrations were increased. D-hordein subunits were the slowest to be extracted, which suggests that they form the backbone of the gel protein (Skerritt and Janes 1992). However, so far, microscopic studies and physico-chemical attempts to support these suggestions have been inconclusive.

Recently, several authors suggested that C-hordein subunits might also be responsible for quality differences between barley samples. Skerritt and Henry (1988) and Bénétrix (1993) found that the C hordeins were more poorly modified in malting than were the other storage proteins. Janes and Skerritt (1993) observed that the relative amount of C-hordein subunits decreased markedly with increased malt extract values. Moreover, according to Millet (1991), the subaleurone layer of the barley kernel had a large proportion of C-hordein subunits that presumably limited water diffusion during the steeping phase.

The protein content of grain endosperm and its composition are influenced by a number of extrinsic factors, such as cultivar and environmental conditions. For instance, high levels of nitrogen fertilizers tend to increase the protein content in endosperm tissue (Zoschke 1970), specifically the hordein subunits (Palmer and Bathgate 1976). The accumulation of hordeins also responds dynamically to nitrogen nutrition (Lásztity 1984, Corke and Atsmon 1988).

Because of the high degree of homology between hordein polypeptides, it has been difficult to ascertain whether variations in nitrogen fertilization influence the various hordein subunits in the same way and whether the aggregative structures found in barley endosperm are the same. Shewry et al (1983) and Miflin

(1987) have clearly demonstrated that the proportion of C-hordein subunits observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns increased during sulfur-starvation conditions. However, electrophoretic studies of hordeins have led to controversial results about polypeptide composition-quality relationships (Shewry et al 1980, Riggs et al 1983). These relationships do not appear to adequately follow qualitative or quantitative changes in the polypeptide composition in response to increasing nitrogen supply (El-Negoumy et al 1979, Smith et al 1986). Alternatively, Marchylo and Kruger (1984) separated hordeins on the basis of surface hydrophobicity by using the reverse-phase high-performance liquid chromatography (RP-HPLC) procedure developed by Bietz (1983). That study indicated that the ratio of B-hordein subunits to C-hordein subunits was influenced by nitrogen fertilization, although no qualitative change in the hordein elution profiles could be attributed to environmental factors or to grain protein content. Griffiths (1987) found that the B-C ratio was very sensitive to nitrogen availability and, more recently, Gille and Montembault (*personal communication*) reported that the nitrogen supply more specifically influenced the proportion of C-hordein subunits.

Because SDS-PAGE, like RP-HPLC, is restricted to the study of monomeric proteins or reduced subunits, and because most storage proteins occur as large aggregates in the mature endosperm, using size-exclusion chromatographic techniques to investigate protein complexes might provide a better insight into barley quality. For example, Millet (1991) used size-exclusion high-performance liquid chromatography (SE-HPLC) to show that the size distribution of barley protein aggregates was influenced by genotype and could be related to malting quality. In the case of bread wheat, Scheromm et al (1992) showed that, according to the cultivar, the total amount of the highest molecular weight fractions varied in response to changes in the level of nitrogen supply. To our knowledge, however, no detailed study of barley protein aggregates in relation to cultivars and nitrogen fertilization has so far been reported.

This study was undertaken to: 1) characterize the size distribution of unreduced barley proteins by SE-HPLC, 2) determine the respective contribution of cultivar and nitrogen fertilization (time and rate of nitrogen application) to the distribution of protein aggregates, and 3) evaluate the stability of cultivars in response to changes in nitrogen nutrition.

MATERIALS AND METHODS

Barley Samples

Eight barley cultivars with four replicates were grown using a randomized block design in a controlled greenhouse at Ecole Nationale Supérieure Agronomique de Toulouse. Each replicate consisted of one pot with three plants per treatment. Samples included the French cultivars Flamenco and Clarine (two-rowed winter cultivars), Plaisant and Pirate (six-rowed winter cultivars), Triumph and Bérénice (two-rowed spring cultivars), and two American cultivars, Karl and Traill. The malting qualities of the cultivars are as follows: Flamenco, totally unsuitable; Clarine, the best of the French malting two-rowed winter barleys; Plaisant, the only six-rowed winter barley used by French maltsters; Pirate, reduces malting yields; Triumph, still a quality reference cultivar; and Bérénice, medium quality. The two American malting six-rowed spring cultivars, Karl and Traill, have the same genetic origin (Wesenberg et al 1976). However, the former contains a low percentage of total protein at various nitrogen levels.

Treatment

Nitrogen was applied in different amounts and at different stages of barley plant development. The pots were watered daily with a basic Hoagland-modified nutrient solution containing 110 kg of nitrogen (or nitrogen units [U]) per hectare, supplied as NH_4NO_3. The six nitrogen treatments, N1–N6, differed by 40-U applications (per hectare) of nitrogen at several growth stages: N1, no additional nitrogen added (110 kg); N2, 40 U at early tillering (150 kg); N3, 40 U at stem extension (150 kg); N4, 40 U

at heading (150 kg); N5, 40 U at stem extension + 40 U at heading (190 kg); N6, 40 U at early tillering + 40 U at stem extension + 40 U at heading (230 kg).

The results were also interpreted on the basis of a nitrogen nutrition index, calculated according to Peterson et al (1992). The seeds were harvested at maturity and milled in a Cyclotec laboratory mill with a 0.5-mm screen. Protein content (N × 6.25) was determined for whole grains by the Kjeldahl method using a Cu-Se catalyst.

Protein Extraction

Ground seeds (40 mg) were stirred for 2 hr at 60°C in $0.1 M$ sodium phosphate buffer (pH 6.9) containing 2% SDS. Extractions were followed by centrifugation at $37,500 \times g$ for 30 min at 20°C. The amount of protein extracted by the phosphate-SDS buffer was defined as the soluble fraction (F_s), expressed on a total protein basis (% F_s).

SE-HPLC

Instrumentation for SE-HPLC was described previously (Dachkevitch and Autran 1989). A TSK4000-SW size-exclusion analytical column (7.5 × 300 mm, 450Å, Beckman Instruments, Gagny, France) protected by a guard column (7.5 × 75 mm, 250Å) was used. A $0.1 M$ sodium phosphate buffer (pH 6.9) containing 0.1% SDS was used as the eluent with a flow rate of 0.7 ml/min. Supernatant (20 μl) was applied to the column using an automated sample injector. The column effluent was monitored at 214 nm and 0.1 absorbance units full scale, and the chromatograms were analyzed using Spectra-Physics (San Jose, CA) analytical software. The total area under the elution curve corresponds to the soluble fraction (F_s). By calibrating the data of total area with the Kjeldahl analysis of the soluble extract, the insoluble residue fraction (F_i) was calculated (% F_s + % F_i = 100).

A fast-protein liquid chromatography system (Pharmacia, Uppsala, Sweden) was used with a preparative Superose 6 gel-filtration column to further characterize the fractions corresponding to the different sizes of aggregates or monomers. Analysis was performed using the same buffer for protein extraction and elution as for SE-HPLC, with a 0.4 ml/min flow rate and 200-μl sample loading (Lundh and MacRitchie 1989).

Apparent molecular weights of major peaks were estimated by calibrating the column with three unreduced protein standards: thyroglobulin (669,000), alcohol dehydrogenase (150,000), and bovine serum albumin (66,000).

Electrophoresis

The subunit composition of SE-HPLC peaks was determined by 13% SDS-PAGE. To recover concentrated proteins from col-
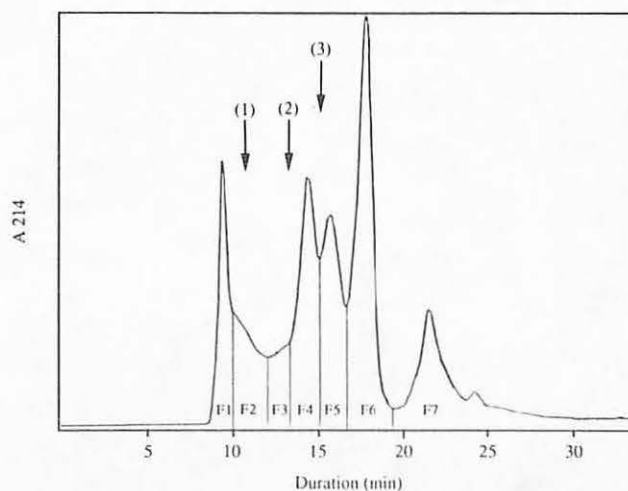

Fig. 1. Typical elution pattern of unreduced barley storage proteins extracted by phosphate-sodium dodecyl sulfate buffer. Arrows indicate the positions of three reference proteins. Seven chromatographic fractions correspond to different sizes of aggregates (F1–F7).

lected peaks, the SDS in all SE-HPLC fractions was removed by precipitating the protein fraction with 15% (w/v) trichloroacetic acid. The residue was washed twice with 1 ml of acetone and dried. Dry protein extracts were dissolved in a 1 M Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% pyronin, and 5% (v/v) β-mercaptoethanol.

Proteins collected from the various fast-protein liquid chromatography peaks and total reduced proteins from barley grains were fractionated in a 13% SDS-PAGE using the method of Laemmli (1970) modified by Payne and Corfield (1979) and Montembault et al (1983).

Statistical Analysis

Analysis of variance was performed using a Stat-ITCF computer package. Genotype and nitrogen level were treated as fixed effects. Differences among genotype, nitrogen level, and their interaction means for each characteristic were determined by the Newman-Keul homogeneity test.
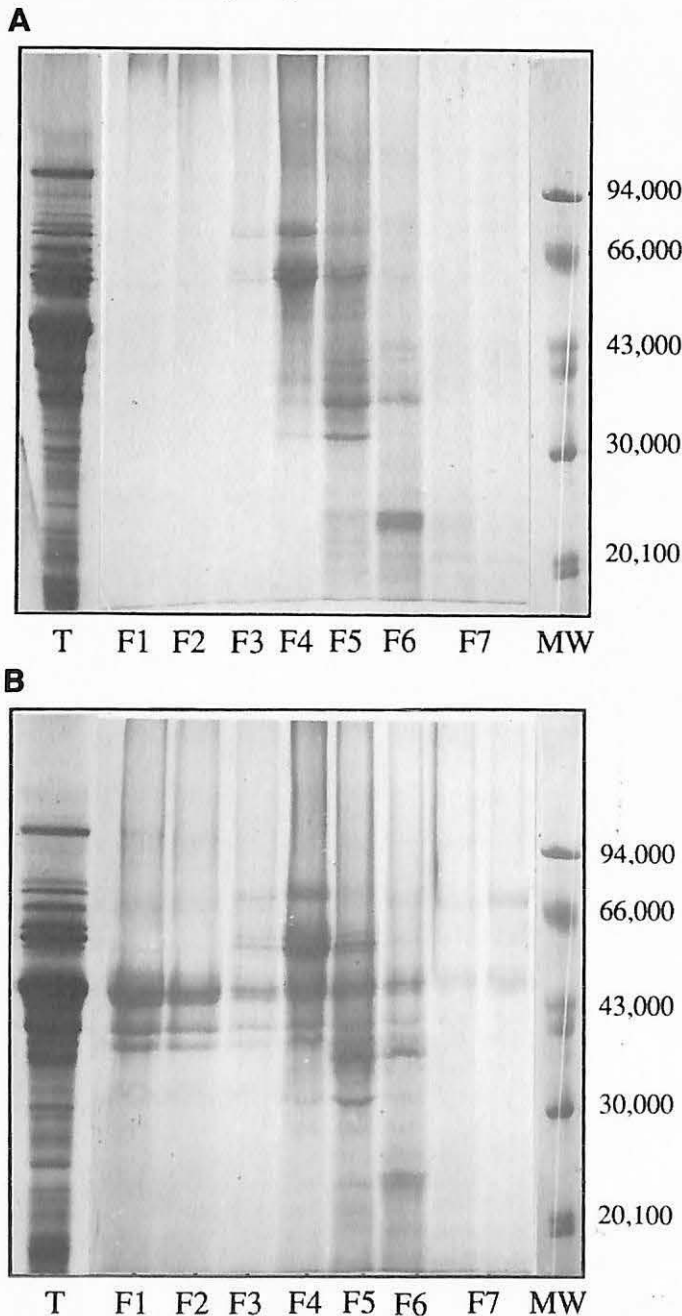

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of unreduced (**A**) and reduced (**B**) protein fractions (F1–F7) collected from size-exclusion high-performance liquid chromatography. T = total reduced proteins; MW = molecular weight standards.

RESULTS

SE-HPLC Elution Patterns

Figure 1 shows the typical elution pattern of unreduced storage proteins extracted by phosphate-SDS buffer from barley grains. Seven chromatographic fractions corresponding to different sizes of aggregates were separated from phosphate-SDS extracts (F1–F7). The percentages of the main fractions were calculated from the area of the chromatograms. The molecular weights were: F1 >1,000,000; F2 350,000–1,000,000; F3 130,000–350,000; F4 60,000–130,000; F5 20,000–60,000, F6 5,000–20,000; and F7 <5,000.

Characterization of Barley SE-HPLC Fractions

The polypeptide composition of the protein fractions contained in the seven chromatographic peaks was analyzed by SDS-PAGE with both unreduced and reduced proteins (Fig. 2). The first peak (F1), which elutes at the void volume, was composed of B- and D-hordein subunits that migrate only upon reduction. The intermediate aggregates (F2) were predominantly made up of B-hordein subunits that are also soluble only upon reduction. F3 also contained B-hordein subunits soluble upon reduction, with some C hordeins. F4 contained the highest proportion of C-hordein subunits, with still some B hordeins. However, in contrast to F1, F2, or F3, which were essentially composed of aggregated forms of B hordeins (soluble only upon reduction), F4 probably contained B hordeins that were mainly free or monomeric forms, so named by Millet et al (1991) because these fractions can be observed without any reducing agent. Interestingly, the percentage of F4 and the percentage of C-hordein subunits estimated by densitometric scanning of SDS-PAGE gels (*results not presented*) were linearly related ($r = 0.85$), whereas no correlation was found between aggregated fractions F1 or F2 and the percentages of B- or D-hordein subunits. The percentage of F4 was moderately correlated with total protein content ($r = 0.59$), but it did not correlate with the percentages of the other SE-HPLC fractions. The F1-to-F4 ratio was also highly correlated with the soluble nitrogen content ($r = 0.82$).

Of the remaining fractions, F5 and F6 were less rich in the major B- or C-hordein subunits and contained increasing amounts of fast-moving (possibly albumin-like) components. F7 did not seem to contain much protein material and was not studied further.

F_i (insoluble residue after extraction with the phosphate-SDS buffer) should correspond to the fraction containing highly aggregated proteins, which is referred to as gel protein in previous studies. In SDS-PAGE, this gel protein fraction consisted essentially of B- and D-hordein bands that migrated only upon reduction (Van den Berg et al 1981, Smith and Lister 1983). From the patterns presented in Figure 2, it is also clear that the D-hordein band is absent from all SE-HPLC peaks, suggesting that almost all the D hordeins (and presumably a significant proportion of the B hordeins) are in the F_i residue.

TABLE I
Range and Mean Values of Genotype (G) and Nitrogen Level (N) for Protein Content, Percentage of Chromatographic Fractions (F1–F6), Soluble Nitrogen (N_S), and Insoluble Residue (F_i)

| Characteristic | Range | | Mean |
	G^a	N^b	$G \times N^c$
Protein content	13.5–10.7	13.6–11.4	12.2
F1	10.8–6.1	9.5–8.7	9.0
F2	12.7–8.9	11.1–10.3	10.7
F3	5.3–4.6	5.0–4.8	4.9
F4	25.4–16.6	21.2–18.8	19.7
F5	16.7–13.9	15.3–14.8	15.0
F6	28.9–22.3	26.7–24.8	26.1
F1/F4 ratio	0.6–0.3	0.5–0.4	0.5
N_s	1.8–1.4	1.6–1.5	1.6
F_i	28.5–15.8	27.1–16.7	20.6

[a] $n = 8$.
[b] $n = 6$.
[c] $n = 48$.

Factor Analysis

Protein content and SE-HPLC data (percent of all fractions and F1-F4 ratio) obtained from the eight cultivars grown under six nitrogen treatments are summarized in Table I. The diversity of genotypes and nitrogen levels were reflected in very wide ranges for most chromatographic measurements.

Analysis of variance was used to determine the respective influence of genotype, nitrogen level, and their interaction on protein content, percentage of each chromatographic fraction, soluble nitrogen, and F_i. The results concerning percentage of variability assignable to genotype, nitrogen level, replicates, residue, and F test are presented in Table II. These results clearly indicate that the genotype effect is significant for all characteristics; variances for genotypes are generally much larger than those for nitrogen level. The variance ratio, which expresses the ability of a criterion to discriminate between genotypes, indicates that genotype has a greater effect than nitrogen level on SE-HPLC parameters; this is also suggested by the ranges in values shown in Table I. The results clearly show that the interaction of genotype and nitrogen level is not significant. All of the fractions F1-F6 are essentially genotype-dependent. Interestingly, some of these fractions (F3 or F5 and, to a lower degree, F1 or F2) are better able to discriminate between genotypes than others (F4). Total protein content and F_i are significantly influenced at the same level by genotype and nitrogen. The large percentage of variability assignable to residue for F_i could be caused by the method of evaluating this fraction, as well as by the variability of residue relative to protein content.

To determine whether spring and winter barley, or two-rowed and six-rowed cultivars, show the same variability, the variances were separately analyzed for each group and the genotype effect was resolved (*results not shown*). Spring barley appeared to be much more influenced by nitrogen level than by genotype, as suggested by the high percentage of variability assignable to the effects of nitrogen level, genotype, and their interaction. In particular, F1 of spring barley is clearly cultivar-dependent, whereas F4 is strongly influenced by nitrogen level. Similarly, no significant effect is observed for F1 among two-rowed barleys; but again, nitrogen treatments have a significant effect on F4. Bénétrix (1993) also showed chromatographic parameters for winter barley are essentially cultivar-dependent, but the variation attributed to nitrogen level is larger in magnitude than the variation of genotype for protein content. All characteristics for the six-rowed barleys are essentially cultivar-dependent.

In addition, mean squares for all genotypes were split into mean squares for each group (variability within each genetic group) and mean squares between groups (variability of one group compared to the variability of another). Variability within groups was more predominant in F1 for winter barley (83.2%) than it was in F1 for spring barley (15.7%). In contrast, the variability between groups was more predominant for F4 (50.9%) than it was for F1 (1.1%), indicating that differences in F4 between winter and spring barleys are more important than differences within each of these groups. The variability within the six-rowed barleys was predominant for most traits, but no distinction between the two-rowed and six-rowed barleys could be made.

Effect on Protein Content and SE-HPLC Fractions

Nitrogen level. In our study, the experimental growing conditions were intended to approximate those of regular barley cultivation. This resulted in a relatively small range of total protein values, much smaller than that usually obtained in hydroponic experiments. The mean value and standard deviation of protein content and some SE-HPLC fractions for each of the six nitrogen treatments (pooled genotypes) are shown in Figure 3. Although a small range of nitrogen values may tend to reduce environmental effects and enhance genotypic effects, the Newman-Keul's test clearly indicated that high-level nitrogen treatment (N6) as well as late treatment (N5) result in a significant increase in total grain nitrogen over that of treatments N1-N4. The percentages of F1, F4, and F_i increased when crops were supplemented with increased levels of nitrogen fertilizer. High-level nitrogen treatments (N5 and N6) resulted in significantly higher percentages of total protein
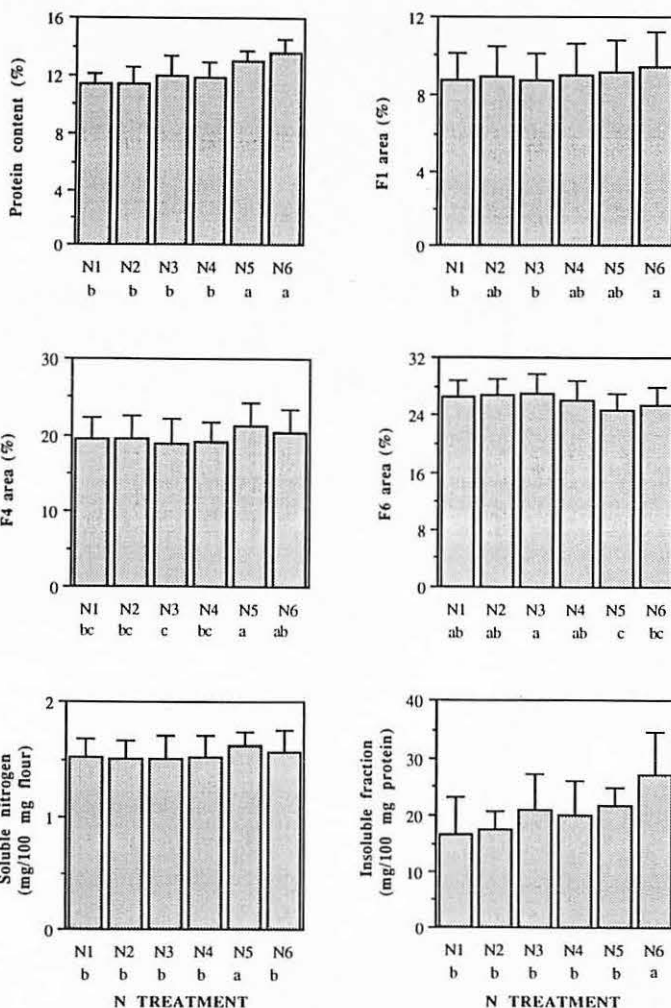
Fig. 3. Main effect of nitrogen nutrition (treatment levels N1-N6) as determined by the Newman-Keul homogeneity test for: protein content; percentage of chromatographic fractions F1, F4, and F6; soluble nitrogen; and insoluble residue. Mean values with the same letter are not significantly different for each characteristic. Vertical bars represent standard deviation from means.

TABLE II
Percentage of Variability and Variance Ratio for the Influence of Genotype (G) and Nitrogen Level (N) Determined by Analysis of Variance on Protein Content, Percentage of Chromatographic Fractions (F1-F6), Soluble Nitrogen (N_S), and Insoluble Residue (F_i)[a]

Characteristic	G	N	$G \times N$ Interaction	Replicate	Residue	σ^2_G/σ^2_N[b]
Protein content	38.8**	31.3**	12.7 ns	0.0 ns	17.2	1.2
F1	83.9**	2.9**	5.6 ns	0.2 ns	7.4	28.9
F2	74.3**	5.0**	8.6 ns	0.0 ns	12.1	14.9
F3	49.9**	2.9 ns[c]	20.8 ns	0.0 ns	26.4	Large[d]
F4	68.5**	7.0**	13.0 ns	0.0 ns	11.5	9.8
F5	73.1**	2.3 ns	11.1 ns	0.0 ns	13.5	Large
F6	69.0**	8.1**	11.5 ns	0.0 ns	11.4	8.5
F1-F4 ratio	86.5**	1.0 ns	5.2 ns	1.0*	6.3	Large
N_S	66.3**	5.3*	13.7 ns	0.0 ns	14.7	12.5
F_i	24.0**	20.2**	24.2 ns	1.3 ns	30.3	1.2

[a] ** and * = F-test significance at the 0.01 and 0.05 level of probability for the percentage of variability assignable to different traits.
[b] Ratio of variance assignable to G to variance assignable to N.
[c] Not significant.
[d] Large number that cannot be expressed because σ^2_N is not distinguishable from zero (value for the F-test under nitrogen level was not significant).

and F_i than that of all other treatments. F4 (rich in C-hordein subunits) appeared to be more sensitive to the amount of nitrogen applied than it was to the growth stage during application (e.g., N1–N4). In contrast, the percentage of F6 (possibly albumin-like proteins) tended to decrease when nitrogen was applied late. F3 and F5 were not significantly affected by nitrogen level.

Mean values of genotypes. The mean value of protein content and some SE-HPLC fractions for each of the eight cultivars (pooled nitrogen levels) are shown in Figure 4. The Newman-Keul's test was used in testing homogeneity of means to establish homogeneous groups. This test failed to support the hypothesis that these characteristics have similar mean values. The cultivars differed in their protein content, but groups defined by Newman-Keul's test strongly overlap. For instance, Karl has a very low protein content, while Plaisant is particularly rich in proteins. In F1, a very significant differentiation of cultivars is attained (one level per variety in the Newman-Keul's test). Flamenco, and to a lesser extent Triumph and Clarine, show the highest percentages of F1, whereas Pirate presents very low percentages. However, no clear discrimination on the basis of a genetic group can be observed. Comparison of means for F2 shows roughly the same results as for F1. A small difference is observed in F3 among the various cultivars but, interestingly, the percentages of F3 tend to be higher for most of the spring barleys, the highest values being reached with Karl. Significant differences among distinct genetic groups are indicated for F4. Spring barley can be clearly separated from winter barley, excepting Clarine, and have percentages of F4 lower than 20%. Three of the winter cultivars have percentages of F4 that are higher than 20% of the total soluble proteins. The cultivars are well distinguished with regards to fraction F5, but no group really stands out. Spring

cultivars tend to have greater amounts of F5 than the winter cultivars; Pirate, which is particularly rich in F5, is the exception. Plaisant shows very low percentages of F6, whereas Karl presents high percentages of this fraction. Soluble nitrogen content is higher for the winter barley than for spring; again, Pirate is the exception. F_i is the highest for Pirate, which is clearly distinguished from the other varieties using the Newman-Keul's test.

Stability of genotypes. The success of breeding for quality depends not only on improvements to the potential quality level of genotypes, but also on the stability of this quality expression. In wheat, it has been suggested that the stability of aggregate composition in response to changes in the nitrogen level could indicate the stability of quality expression (Scheromm et al 1992). Regression analyses of the genotype response to an environmental index have frequently been used by plant breeders to evaluate stability (Peterson et al 1992).

In the present study, the variances and standard deviations of various parameters of protein composition were used to evaluate the stability of barley genotypes in response to nitrogen fertilization (Fig. 4). The effect of increased nitrogen levels on protein content differs according to genotype. For instance, Triumph, Flamenco, and Pirate have a larger standard deviation across nitrogen levels than do Plaisant or Karl. The same statistical approach was applied to all SE-HPLC fractions. On the basis of the percentage of F1, Flamenco and Pirate appear unresponsive to nitrogen treatments, whereas Triumph is strongly responsive. The obvious differences in F4 show that Triumph and Pirate are much more susceptible to changes than are Karl and Traill. In contrast, the percentage of F_i shows that Pirate appears highly susceptible to nitrogen treatments, whereas Karl is quite insensitive.

These different behaviors become more obvious when plotting protein content or percentage of F4 against the nitrogen nutrition index (Fig. 5). The nitrogen nutrition index is a concept used to determine trait stability. It was calculated for N1–N6 by regressing genotype means on fertilization means, as in Peterson et al (1992). For F4, the slope of the regression line is much less steep for Karl than it is for Triumph, indicating that the former has a more stable behavior. Therefore, Karl can tolerate higher nitrogen levels without an excessive increase in protein content or F4. However, Triumph can retain the same protein characteristics only when nitrogen is applied very early (sowing or tillering stage).
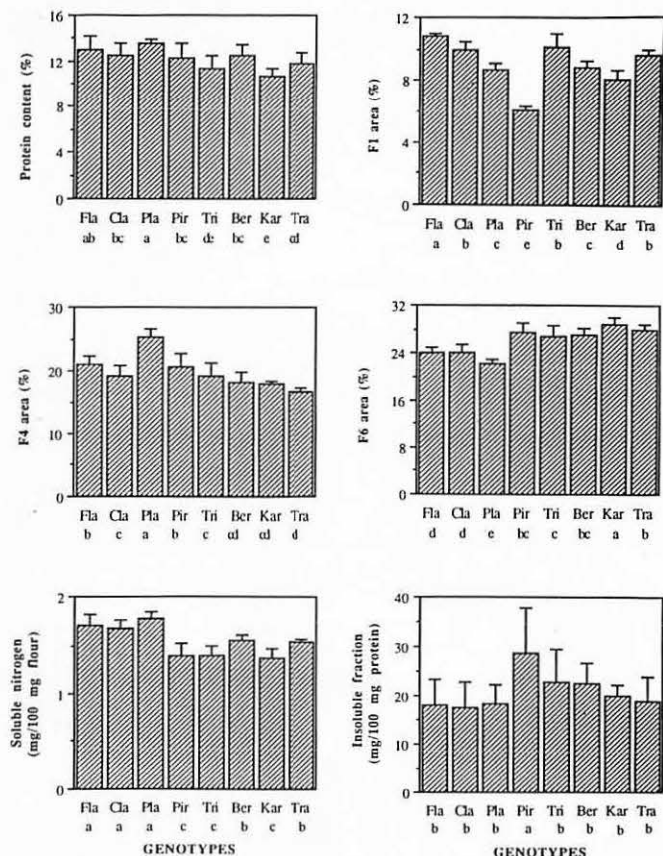

Fig. 4. Main effect of genotype as determined by the Newman-Keul homogeneity test for: protein content; percentage of chromatographic fractions F1, F4, and F6; soluble nitrogen; and insoluble residue. Fla = Flamenco, Cla = Clarine, Pla = Plaisant, Pir = Pirate, Tri = Triumph, Ber = Bérénice, Kar = Karl, Tra = Traill. Mean values with the same letter are not significantly different for each characteristic. Vertical bars represent standard deviation from means.
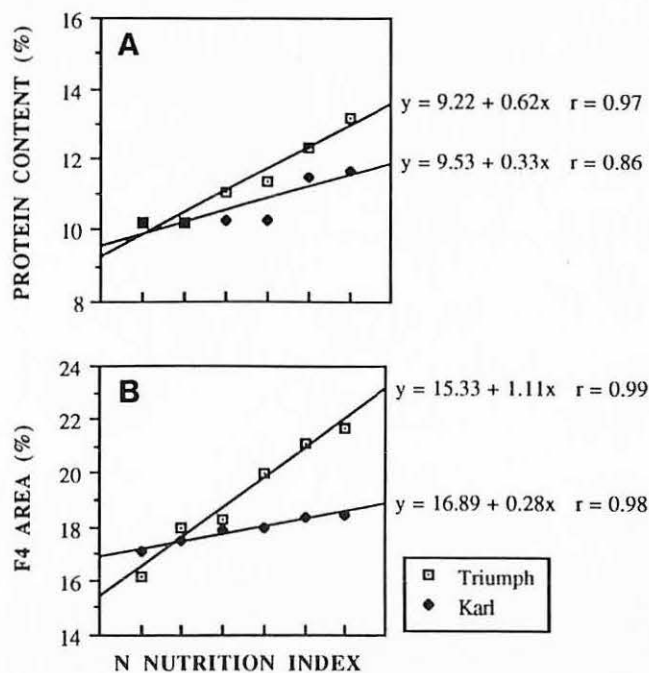

Fig. 5. Correlation with nitrogen nutrition index. (**A**) grain protein content (db). (**B**) F4 area (%).

DISCUSSION

Nitrogen Fertilization

Several studies on wheat have found that the variation attributed to nitrogen nutrition was much lower than the variation assignable to the other environmental factors (Dachkevitch and Autran 1989, Peterson et al 1992). In the present study, conducting the experiment under controlled conditions not only permitted better reproducibility among genotypes, but also reduced the effects of environmental factors other than nitrogen fertilization. Despite the relatively small range of nitrogen values obtained from the various fertilizer treatments, it is clear that F_i and total protein content were most influenced by nitrogen treatments; F4 was also influenced, but to a lesser extent (Table III). In actual growing conditions of malting barley, nitrogen is moderately applied at sowing (Mahaut 1992). It is the recommended method to avoid late applications that impair malting quality. Our study shows that late treatments cause an increase of total protein content and a specific increase in F_i and F4. This suggests that, in addition to the possible influence of a lower starch content, the negative effect of excess grain proteins on malting performance results primarily from excessive amounts of F_i or F4, which corroborates recent reports by Janes and Skerritt (1993).

Comparison of Cultivars

The analysis of variance (Table II) showed that protein characteristics (protein content and aggregate composition) were largely cultivar-dependent. Although it is possible that environmental effects were reduced as a result of the small range of nitrogen values, the genotypic influence seems particularly important in winter and six-rowed cultivars. In contrast, in spring and two-rowed barleys, both the protein content and composition of aggregates are affected by the time and rate of nitrogen application. The largest differences between cultivars, as expressed by the coefficients of variation (Table III), were found in F1, F4, and F_i, but the Newman-Keul's homogeneity test shows that, to the extent that this approach permits, F4 is the best criterion for distinguishing between winter and spring barleys. No clear distinction between two-rowed and six-rowed barleys could be achieved using characteristics for SE-HPLC fractions or protein content.

Individual Characteristics of Cultivars

There are some interesting relationships between the average level or stability of the protein attributes of the various cultivars and their malting quality potential.

Winter barley. Flamenco, a winter cultivar that is totally unsuitable for malting purposes, is particularly rich in total protein. This trait is highly sensitive to nitrogen levels. In addition, Flamenco has a high level of F4. The cultivar Pirate has a very low percentage of aggregated fractions (F1–F3), but it has a distinctly large amount of F_i. Pirate is well known to have reduced malting yields, so this characteristic might be related to an excessive proportion of insoluble proteins. Clarine is the best of the malting, two-rowed, winter barleys, and it has lower amounts

of F4 and higher amounts of F_i than the other winter barleys. Another interesting example is Plaisant, the only six-rowed winter barley that is traditionally used by French maltsters. This cultivar has a high protein content and a very high percentage of F4. It is not really responsive to nitrogen treatments. In fact, Plaisant has a low malting potential because it has to be treated with gibberellic acid to give a rapid and homogeneous germination (Delatte, *personal communication*). This is in agreement with its high F4 content.

Spring barley. Although Triumph has been replaced to a great extent by new spring barleys, it still remains a quality reference cultivar for the French maltsters. It has a relatively low protein content and a low percentage of F4. On the other hand, because it appears very responsive to nitrogen treatments, it demonstrates that early nitrogen applications do not impair quality potential. Among the two American malting cultivars, Traill has a high total protein content and a high percentage of F1, but it has a very low percentage of F4. Karl contains a low percentage of total protein at various nitrogen levels. Because its low level of F4 is also very tolerant to high and late applications, it is likely that high-nitrogen fertilizers can be applied to increase its grain yield without affecting its malting quality.

An important question is whether a given nitrogen treatment can promote conditions conducive to a high malting score of a barley by optimizing the protein composition of its grain. Although the genotype-nitrogen interaction in this study was not found to be significant (Table II), it is not easy to answer this question accurately. For instance, the lowest level of application (150 kg of nitrogen per hectare) clearly resulted in low percentages of total proteins, F_i, and F4. However, in treatments N2–N4, there were no clear differences caused by the various dates of application (early tilling, stem extension, heading). The highest level treatments, N5 and N6, which are likely to be detrimental to malting quality, have different effects on the protein composition. N6 gives much higher proportions of F_i but lower proportions of F4, whereas the opposite is true for N5. Barley with a good quality potential as a result of a low proportion of F_i (such as Clarine) might therefore tolerate high nitrogen levels if a treatment similar to N5 were used, but it might be impaired by a treatment similar to N6. In contrast, barley with a good quality potential as a result of a low proportion of F4 (such as Triumph), which consists essentially of C-hordein subunits, might tolerate a treatment similar to N6 but might be impaired by a treatment similar to N5.

Physicochemical Basis of the Observed Effects

The individual characteristics of cultivars give us a better insight into the functional effect of the main hordein groups in the malting process. High F4 is frequently associated with barley well known to give difficulties in the malting process. All the spring barleys have lower mean percentages of F4. Because higher percentages of total proteins are associated with an excess of F4, it is possible that the negative role of excess protein in malting actually results from an excess of F4. Further, because F4 consists essentially of C-hordein subunits (and is significantly correlated with the percentage of C hordeins in the grain), the hypothesis of Millet (1991) can be expanded. C hordeins are very hydrophobic storage proteins: because of their amino acid composition (they are devoid of cysteine) they can only be involved in hydrophobic interactions (Shewry and Miflin 1983). However, Skerritt and Janes (1992) demonstrated that C hordeins were poorly modified in malting, thus it can be suggested that large amounts of C hordeins may constitute a limiting factor in grain hydration and hydrolysis of substrates during steeping and malting. If our results can be confirmed on a larger number of cultivars grown in actual field conditions, it might be desirable to screen barley cultivars on the basis of percentage of F4. Such a method could help eliminate samples that would impair uniformity of steeping and malting, including winter barleys that should be used only for animal feed.

The effect of the most insoluble hordein fractions should not be neglected either. It should be recognized that, in using a phosphate-SDS buffer, the major aggregate fractions are centrifuged

---

TABLE III
Coefficients of Variation (%) and Mean Values of Genotypes (*G*) and Nitrogen Levels (*N*) for Protein Content, Percentage of Chromatographic Fractions (F1–F6), Soluble Nitrogen (N_S), and Insoluble Residue (F_i)

Characteristic	Mean	Coefficient of Variation	
		G	*N*
Protein content	12.2	25.9	27.6
F1	9.0	57.1	12.5
F2	10.7	37.7	11.6
F3	4.9	17.2	0.5
F4	19.8	47.7	18.0
F5	15.0	20.3	4.3
F6	26.0	31.0	12.6
N_S	1.6	34.4	11.5
F_i	20.6	66.7	72.4

down at the extraction stage. They are considered only as the residue or F_i fraction. In our study, cultivars showed high proportions of either medium-size aggregates (F1 or F2 fractions that contain B- and D-hordein subunits) or large-size protein aggregates (F_i). However, no consistent association can be found between high percentages of F1 or F2 and poor quality barley, because high percentages of F1 are observed in both spring (Triumph) and winter (Flamenco, poor, and Clarine, good) barleys. On the other hand, the effect of F_i might help explain the low malting potential of cultivars such as Pirate. This would be in agreement with previous studies on the SDS-unextractable gel protein fraction (Smith and Simpson 1983, Skerritt and Janes 1992). Hence, poor quality barley samples may be the result of either excess hydrophobic C-hordein-based structures or excess insoluble B- or D-hordein-based structures.

CONCLUSIONS

Although malting quality is a composite of characteristics, resulting from the interaction of a number of factors involving nonprotein components, this study provides some new insights into the relationship between protein aggregates and malting quality.

The composition in B-, C-, or D-hordein subunits of seven SE-HPLC fractions from eight widely varying barley cultivars treated with six different levels of nitrogen fertilization was established. Unlike studies on polypeptides (SDS-PAGE) that give fingerprints of genotypes, investigating the profile of protein aggregates through SE-HPLC appeared to provide an effective means of evaluating the effects of nitrogen fertilization.

The effect of the various times and rates of nitrogen fertilization showed that the main protein fractions of barley do not change in the same way. The F_i and total protein content are significantly influenced by nitrogen fertilization. In contrast, some SE-HPLC fractions (F3 and F5) are essentially cultivar-dependent, and some others (F4, a C-hordein-rich fraction) are significantly influenced by nitrogen fertilization. Higher levels and later applications of nitrogen, considered detrimental to malting quality, are always associated with higher proportions of F4.

Considering the characteristics of the different types of cultivars (winter, spring, two-rowed, six-rowed) in this study, F4 emerges as the best criterion for separating winter and spring barley. Because spring barleys are largely preferred by maltsters, it follows that the presence of F4 may partially explain the origin of differences between cultivars with regard to malting quality. However, because there is also a significant effect of nitrogen fertilization, quantitation of F4 (as well as the most insoluble hordein fractions) might be more useful for checking barley loads in malting plants and for selecting samples that are suitable for malting purposes than it would be for early screening of barley breeding lines.

These results provide an approach for understanding the basis of the physico-chemical or functional properties of hordeins in malting quality. They further support the hypothesis that high levels of C-hordein subunits impair malting quality by limiting water diffusion during the steeping phase.

LITERATURE CITED

BAXTER, E. D. 1981. Hordein in barley and malt—A review. J. Inst. Brew. 87:173-176.

BAXTER, E. D., and WAINWRIGHT, T. 1979. Hordein and malting quality. J. Am. Soc. Brew. Chem. 37:8-12.

BÉNÉTRIX, F. 1993. Etude biochimique, ultrastructurale et agronomique de la qualité d'une orge: Rôle des hordéines. Thèse de Doctorat. Ecole Nationale Supérieure d'Agronomie: Montpellier, France.

BIETZ, J. A. 1983. Separation of cereal proteins by reversed-phase high-performance liquid chromatography. J. Chromatogr. 225:219-238.

CORKE, H., and ATSMON, D. 1988. Effect of nitrogen nutrition on endosperm protein synthesis in wild and cultivated barley grown in spike culture. Plant Physiol. 87:523-528.

DACHKEVITCH, T., and AUTRAN, J.-C. 1989. Prediction of baking quality of bread wheats in breeding programs by size-exclusion high-performance liquid chromatography. Cereal Chem. 66:448-456.

EL-NEGOUMY, A. M., NEWMAN, C. W., and MOSS, B. R. 1979. Amino acid composition of total protein and electrophoretic behavior of protein fractions of barley. Cereal Chem. 56:468-473.

GRIFFITHS, D. W. 1987. The ratio of B to C hordeins in barleys as estimated by high performance liquid chromatography. J. Sci. Food Agric. 38:229-235.

JANES, P. W. and SKERRITT, J. H. 1993. High performance liquid chromatography of barley proteins: Relative quantities of hordein fractions correlate with malt extract. J. Inst. Brew. 99:77-84.

LAEMMLI, J. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680-685.

LÁSZTITY, R. 1984. The Chemistry of Cereal Proteins. CRC Press: Boca Raton, FL.

LUNDH, G., and MacRITCHIE, F. 1989. Size-exclusion HPLC characterization of gluten protein fractions varying in breadmaking potential. J. Cereal Sci. 10:247-253.

MAHAUT, B. 1992. Quelles sont les qualités que recherchent les malteurs? Perspec. Agric. 175:72-76.

MARCHYLO, B. A., and KRUGER, J. E. 1984. Identification of Canadian barley cultivars by reversed-phase high-performance liquid chromatography. Cereal Chem. 61:295-301.

MIFLIN, B. J. 1987. The biology and genetics of cereal storage proteins. Pages 1-2 in: Protein Evaluation in Cereals and Legumes. CEC Programme of Coordination of Agricultural Research on Plant Productivity. V. Pattakou, ed. Commission of the European Communities: Luxembourg.

MILLET, M. O. 1991. Etude du caractère agrégatif des protéines de l'orge. Relations avec la qualité malticole et brassicole. Thèse de Doctorat. Université des Sciences et Techniques du Languedoc: Montpellier, France.

MILLET, M. O., MONTEMBAULT, A., and AUTRAN, J.-C. 1991. Hordein composition differences in various anatomical regions of the kernel between two different barley types. Sci. Aliments 11:155-161.

MONTEMBAULT, A., AUTRAN, J.-C., and JOUDRIER, P. 1983. Varietal identification of barley and malt. J. Inst. Brew. 89:299-302.

PALMER, G. H., and BATHGATE, G. N. 1976. Malting and brewing. Pages 237-324 in: Advances in Cereal Science and Technology, Vol. 1. Y. Pomeranz, ed. Am. Assoc. Cereal Chem.: St Paul, MN.

PAYNE, P. I., and CORFIELD, K. G. 1979. Subunit composition of wheat glutenin proteins, isolated by gel filtration in a dissociated medium. Planta 145:83-88.

PETERSON, C. J., GRAYBOSCH, R. A., BAENZIGER, P. S., and GROMBACHER, A. W. 1992. Genotype and environment effects on quality characteristics of hard red winter wheat. Crop Sci. 32:98-103.

RIGGS, T. J., SANADA, M., MORGAN, A. G., and SMITH, D. B. 1983. Use of acid gel electrophoresis in the characterisation of B hordein protein in relation to malting quality and mildew resistance in barley. J. Sci. Food Agric. 34:576-586.

SCHEROMM, P., MARTIN, G., BERGOIN, A., and AUTRAN, J.-C. 1992. Influence of nitrogen fertilization on the potential bread-making quality of two wheat cultivars differing in their responses to increasing nitrogen supplies. Cereal Chem. 69:664-670.

SHEWRY, P. R., and MIFLIN, B. J. 1983. Characterization and synthesis of barley seed proteins. Pages 143-205 in: Seed Proteins, Biochemistry, Genetics, Nutritive Value. W. Gottschalk and H. P. Müller, eds. Martinus Nijhoff/Dr. W. Junk Publishers: The Hague, The Netherlands.

SHEWRY, P. R., FAULKS, A. J., PARMAR, S., and MIFLIN, B. J. 1980. Hordein polypeptide pattern in relation to malting quality and varietal identification of malting grain. J. Inst. Brew. 86:138-141.

SHEWRY, P. R., WOLFE, M. S., SLATER, S. E., PARMAR, S., FAULKS, A. J., and MIFLIN, B. J. 1981. Barley storage proteins in relation to varietal identification, malting quality, and mildew resistance. Pages 596-603 in: Barley Genet. Proc. Int. Symp., 4th. Edinburgh University Press: Edinburgh.

SHEWRY, P. R., FRANKLIN, J., PARMAR, S., SMITH, S. J., and MIFLIN, B. J. 1983. The effects of sulphur starvation on the amino acid and protein composition of barley grain. J. Cereal Sci. 1:21-31.

SKERRITT, J. H., and HENRY, R. J. 1988. Hydrolysis of barley endosperm storage proteins during malting. II. Quantification by enzyme- and radio-immunoassay. J. Cereal Sci. 7:265-281.

SKERRITT, J. H., and JANES, P. W. 1992. Disulphide-bonded 'gel protein' aggregates in barley: Quality-related differences in composition and reductive dissociation. J. Cereal Sci. 16:219-235.

SLACK, P. T., BAXTER, E. D., and WAINWRIGHT, T. 1979. Inhibition by hordein of starch degradation. J. Inst. Brew. 85:112-114.

SMITH, D. B., and LISTER, P. R. 1983. Gel forming proteins in barley grain and their relationship with malting quality. J. Cereal Sci. 1:229-239.

SMITH, D. B., and SIMPSON, P. A. 1983. Relationships of barley proteins soluble in sodium dodecyl sulfate to malting quality and varietal

identification. J. Cereal Sci. 1:185-197.

SMITH, D. B., LISTER, P. R., and HANSON, P. R. 1986. Discrimination of barley varieties by electrophoresis of endosperm proteins extractable into a mixture of sodium dodecyl sulfate, 2-mercaptoethanol and dimethylformamide. J. Cereal Sci. 4:107-116.

VAN DEN BERG, R., MUTS, G. C. J., DROST, B. W., and GRAVELAND, A. 1981. Proteins from barley to wort. Proc. Congr. Eur. Brew. Conv. 47:461-469.

WESENBERG, D. M., HAYES, R. M., STRANDRIDGE, N. N., BURGER, W. C., GOPLIN, E. D., and PETR, F. C. 1976. Registration of 'Karl' barley. Crop Sci. 16:73.

ZOSCHKE, M. 1970. Effect of additional nitrogen nutrition at later stages on protein content and quality of barley. Pages 345-356 in: Improving Plant Protein by Nuclear Techniques. IAEA: Vienna.

CEREAL CHEMISTRY.

AN INTERNATIONAL JOURNAL SINCE 1924