NOTE

High-Lysine Barley Screening with Fluorometry and Endoproteolytic Assays

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ABSTRACT

2-(*N*-Morpholino)ethanesulfonic acid-buffered sodium dodecyl sulfate extracts of barley meal were assayed with two fluorometric methods, using either fluorescamine or *o*-phthalaldehyde as the fluorochrome. Both methods, especially fluorescamine, showed a good agreement with the lysine content but a less strong correlation with protein content. In a third assay, digestion of meal samples with endoproteinase Lys-C, hydrolyzing polypeptides at the carboxyl end of lysyl residues, resulted in an increase of amino terminals. This increase was detected with ninhydrin. Ninhydrin absorbances showed a linear correlation with lysine content. The protein content of the digested or undigested extracts could be reliably estimated from the absorbances at 215 and 225 nm.

We have been working on low-cost, simple techniques to detect nutritionally best breeding lines of barley with high protein content rich in lysine. Formerly, we described a method for screening high-lysine barleys with the bicinchonic acid (BCA) assay (Smith et al 1985) of meal extracts (Ahokas and Naskali 1988). The method also involves measuring ultraviolet (UV) absorbance at 215 and 225 nm to estimate protein content (Waddell 1956). To provide an alternative to the BCA assay, we studied two fluorescence assays using either fluorescamine (Udenfriend et al 1972) or *o*-phthalaldehyde (OPA) (Roth 1971) as the fluorochrome. We also introduce a method employing endoproteinase Lys-C, which specifically hydrolyzes polypeptides at the C side of lysyl residues (Masaki et al 1981, Anonymous 1984). The corresponding increase in amino terminals indicates the lysyl residue level. The digestions were futher studied with gel electrophoresis.

MATERIALS AND METHODS

The 13 barley cultivars, breeding lines, mutants, or accessions were raised under various field conditions (Table I). Meal samples were ground with a Tecator Cyclotec mill to pass a 0.5-mm screen. Moisture was determined with an oven at 130°C for 60 min.

Lysine Determination with Enzymatic Assay

The enzymatic hydrolysis of proteins for the saccharopine dehydrogenase (SDH) determination was a modification from that of Winkler and Schön (1979). Pronase E (Serva) was autodigested for 30 hr at 40°C. Proteinase K (Merck) was used without autodigestion. During this determination, gloves and sterile media and lab ware were used whenever possible. Meal samples (24–25 mg) were hydrolyzed in 10 ml tubes with Teflon-lined screw caps (Kimax) by shaking at 40°C for 40 hr. The hydrolysis mixture (2 ml) contained Tris-HCl, pH 7.0 (100 m*M*) (Sigma), ethanol (10%, v/v), sodium dodecyl sulfate (SDS) (0.006%, w/v) (BDH), pronase E (1 mg of autodigested protein), and proteinase K (50 μg). The proteolytic activity was removed from the hydrolysates by ultrafiltration with Centricon-10 microconcentrators (Amicon) (mol wt cutoff of 10 kDa). SDH determinations from ultrafiltrates were performed principally according to Nakatani et al (1972). The reaction mixture (3 ml) contained 8 μmol of α-ketoglutarate (Sigma), 0.26 μmol of reduced β-nicotinamide adenine dinucleotide (Boehringer), 0.4 units of SDH (Sigma), and 400 μl of ultrafiltrate, with a final Tris-HCl (pH 7.0) content of 80 m*M*. The decreases in the absorbance at 340 nm were evaluated by measuring immediately after mixing and then after a 30-hr incubation in the dark at 25°C. The blanks were incubated without SDH and ultrafiltrate. The concentration of added lysine in the range studied, 17 to 257 nmol per sample, correlated perfectly to the decrease in absorbance at 340 nm when incubated with SDH under the above conditions ($r = 1.000$).

Lysine Determination with TNBS

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) method for lysine was according to Kakade and Liener (1969) as mentioned by Ahokas (1982).

Protein Determination

Determinations of Kjeldahl N were run by a contract with Viljavuuspalvelu Oy (Helsinki) on coded, duplicate samples of 1 g with a Kjeldatherm combuster and Vapodest IV distiller (Gerhardt). Protein was extracted from barley meal with SDS-containing 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH buffer of pH 6.8, the supernatants of which were used for the BCA assay (Pierce) and for UV determination at 215 and 225 nm as described previously (Ahokas and Naskali 1988). Tris buffer is not compatible with the fluorochromes used.

Fluorometric Lysine Assay with Fluorescamine and OPA

With fluorescamine, the supernatants of the SDS-MES extracts (Ahokas and Naskali 1988) were used for fluorescence determination essentially according to Böhlen et al (1973). Fluorescamine (Sigma) was freshly dissolved in acetone (0.3 mg/ml) and dispensed with a small glass-and-Teflon dispensor. Fifty microliters of the supernatant was pipetted into 1.45 ml of 50 m*M*

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potassium phosphate buffer (pH 8.0) into which 500 μl of fluorescamine in acetone was dispensed and vortexed vigorously. The fluorescence was measured after 2 min with a TKO 100 DNA fluorometer (Hoefer Scientific Instruments) having an exitation maximum of 365 nm and a photodetector maximum of 460 nm. Blank samples contained buffer but no meal extract. Before analyzing each set of samples, the fluorometer was standardized with an Ovalene block no. 2 (Starna). Freshly prepared OPA reagent (Fluka) was similar to that of Goodno et al (1981). Twenty-five microliters of the supernatant was pipetted into 2 ml of OPA reagent and vortexed and measured with the fluorometer as above after 2 min.

Lysine Assay with Endoproteinase Lys-C

The digestion was carried out in sterile, 10-ml tubes with Teflon-lined screw caps (Kimax). Meal samples of 12–13 mg were extracted at 60° C for 20 min with 1.335 ml of sterile 75 m*M* sodium potassium phosphate buffer (pH 8.0) containing 0.3% SDS, preceded and followed by a 15-sec vortexing. After the extracts cooled to room temperature, sterile distilled and deionized water (2.615 ml) was added to each. After vigorous vortexing, a 1-ml sample was transferred to a new tube to serve as an undigested reference. A 0.2-unit aliquot of endoproteinase Lys-C (analytical grade, Boehringer) in 50 μl of water was then added to the extracts. The digestion and reference tubes were incubated for 12 hr at 27° C with shaking. Blanks containing buffer and buffer plus enzyme were run simultaneously. The digestion mixtures were centrifuged for 15 min at 12,000 × *g* in Eppendorf tubes, and the supernatants were stored frozen at −20° C until used. Both digested samples and undigested references were assayed by the ninhydrin method of Yemm and Cocking (1955) as well as by the UV protein, BCA, and fluorescamine procedure described above.

Three blanks were usually included in each series of determinations. Arbitrary units were calculated on an absorbance/dry weight basis or on fluorescence/dry weight basis.

TABLE I
Description of Barley Material

Barley	Special Characteristics	Lysine (μg/mg, dry weight) TNBS[a]	SDH[b]	Kjeldahl Nitrogen (mg/g dry weight)
CI 3947, Hiproly	Hulless, two-rowed	4.73	3.53	28.90
CI 4362, Hiproly Normal	Hulless, two-rowed	3.18	2.90	23.75
Risφ 13 (mutant in cultivar Bomi)	Hulled, two-rowed	3.62	3.26	17.70
Risφ 1508 (mutant in cultivar Bomi)	Hulled, two-rowed	3.24	3.27	14.60
Bomi	Hulled, two-rowed	2.09	2.39	13.25
HA 86-141 (Risφ 1508*2/Glacier//Crypt/2*Risφ 1508)	Hulled, six-rowed	3.18	3.51	17.20
HA 86-182 (CI 2229/Risφ 1508)	Hulless, two-rowed	2.91	2.75	21.10
Risφ 56 (mutant in cultivar Carlsberg II)	Hulled, two-rowed	2.72	2.70	14.05
Carlsberg II	Hulled, two-rowed	2.26	2.36	13.05
HOR 2608, Noire 2R Montpellier	Hulled, two-rowed, black	4.00	3.39	29.75
HA 22	Hulled, two-rowed	2.41	2.78	16.90
HA 86-H3-35 (PI 391137/2*Adorra)	Hulled, two-rowed	2.82	2.88	21.35
HA 86-H3-67 (PI 406276/2*Adorra)	Hulled, two-rowed	3.20	3.06	25.85

[a] Determined with 2,4,6-trinitrobenzenesulfonic acid.
[b] Saccharopine dehydrogenase determination.

TABLE II
Correlation Coefficients[a] Between Various Assays

Method	No. of Determinations	Lysine by SDH[c]	Kjeldahl Nitrogen	Protein by UV[d]	BCA[e]	Fluorescamine	OPA[f]
Lysine by TNBS[b]	2	0.928	0.705	0.684	0.965	0.960	0.909
Lysine by SDH[c]	2	···	0.568	0.523	0.869	0.911	0.907
Kjeldahl nitrogen	2		···	0.959	0.836	0.673	0.527
Protein by UV[d]	5			···	0.804	0.675	0.488
BCA[e]	5				···	0.929	0.835
Fluorescamine	5					···	0.956
OPA[f]	5						···

[a] Significance: 0.1%, $r = 0.801$ (df = 11); 1.0%, $r = 0.684$; 5.0%, $r = 0.553$.
[b] 2,4,6-Trinitrobenzenesulfonic acid.
[c] Saccharopine dehydrogenase.
[d] Ultraviolet absorbance.
[e] Bicinchonic acid assay.
[f] *o*-Phthalaldehyde assay.

TABLE III
Some Correlation Coefficients[a] of Endoproteinase Lys-C Digested and Undigested References

Method	Endoproteinase Lys-C Digested: Ninhydrin	UV[b] Protein	BCA[c]	Fluorescamine	Undigested Reference: Ninhydrin	UV[b] Protein	BCA[c]	Fluorescamine
Kjeldahl nitrogen	0.530	0.979	0.926	0.690	0.492	0.952	0.874	0.579
Lysine by TNBS[d]	0.924	0.618	0.881	0.949	0.841	0.613	0.952	0.932
Lysine by SDH[e]	0.924	0.461	0.761	0.880	0.901	0.460	0.872	0.915

[a] Based on the means of two or three determinations of each of the barleys. Significance: 0.1%, $r = 0.801$ (df = 11); 1.0%, $r = 0.684$; 5.0%, $r = 0.553$.
[b] Ultraviolet absorbance.
[c] Bicinchonic acid assay.
[d] 2,4,6-Trinitrobenzenesulfonic acid.
[e] Saccharopine dehydrogenase.

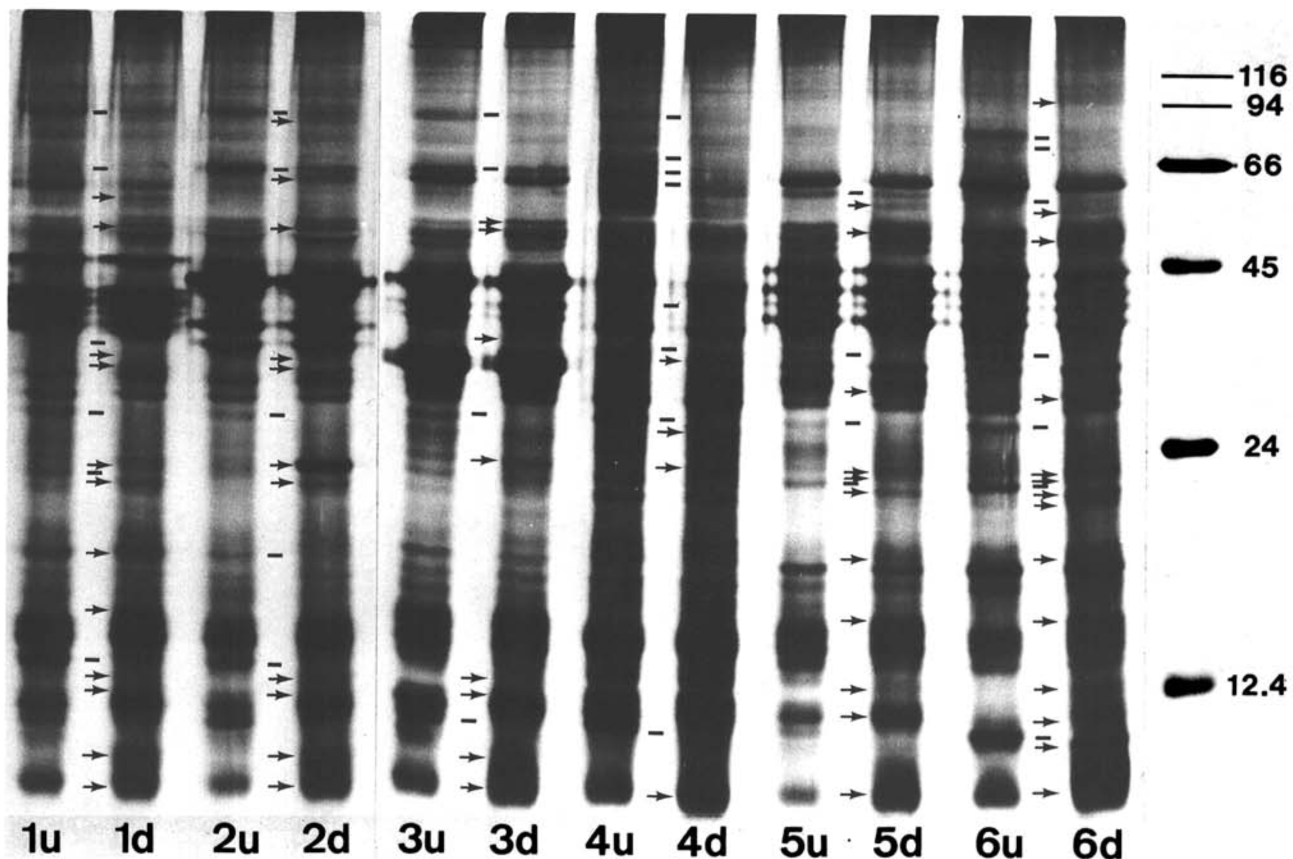

Fig. 1. Sodium dodecyl sulfate-polyacrylamide slab gel. Undigested reference (**u**) and digested (**d**) supernatants from the endoproteinase Lys-C assay run side by side. Equal amounts of protein (according to the ultraviolet determination) in equal volumes were loaded in each well after the addition of glycerol (final concentration 8.7%, v/v), heating in boiling water for 2 min, and β-mercaptoethanol was added subsequently (final concentration 5%, v/v). The separation gel was made from 9% acrylamide according to Laemmli (1970). The gel was fixed and stained with the silver procedure (Bio-Rad). The material from the left to the right: (**1**) HA 86-H3-35, (**2**) HA 86-H3-67, (**3**) HA 86-182, (**4**) HA 86-141, (**5**) CI 4362, and (**6**) CI 3947 followed by molecular weight markers, kDa. The amount of endoproteinase Lys-C contained in these tracks is not visible when coelectrophoresed on the gel. Dashes refer to protein bands that decreased or disappeared, arrows indicate bands that increased or appeared as a result of the endoproteinase Lys-C treatment.

RESULTS AND DISCUSSION

The two methods, TNBS and SDH determination, were used as lysine reference. The TNBS method is based on amino group staining prior to an acid hydrolysis and extraction with diethylether. The SDH method employs an enzymatic hydrolysis and enzymatic determination in the hydrolysate.

Comparisons between the fluorometric methods are presented in Tables II and III. There is a close correlation between the estimates for lysine, i.e., between TNBS and SDH, between TNBS and BCA, and between BCA and fluorescamine, between TNBS or SDH versus fluorescamine, and between TNBS or SDH versus OPA (Table II). UV measurements accurately estimated the nitrogen content (Table II). The SDH method tends to underestimate lysine in the high-protein barleys when compared with the TNBS determinations (Table I). This may be because of competitive inhibition of the SDH enzyme by other amino acids.

With the endoproteinase Lys-C digestion method, both TNBS and SDH showed good agreement with the ninhydrin determinations of the digested samples (Table III). Only the fluorescamine determination could be regarded as an accurate technique for lysine estimation with the undigested reference dissolved in a phosphate-buffered·SDS solution (Table III). Run without the reference samples used here, the endoproteolytic assay combined with UV estimation of protein content may provide a useful basis for the isolation of high-lysine barleys. Some other methods for the α-amino group determination than ninhydrin might work as well.

The effect of the endoproteinase Lys-C could be monitored with SDS-polyacrylamide gel electrophoresis of the digested and undigested extracts. Generally, the background staining of the tracks increased, especially on the low molecular weight half, due to the digestion. The disappearance of some common protein bands, together with some bands specific for a cultivar, were observed in two independent runs after a digestion with a different enzyme lot (Fig. 1). Additional bands of lower molecular weight also appeared following the digestions (Fig. 1).

There is little or no endogenous proteolytic activity in resting grain at pH 8 (Mikola 1983). Moreover, the treatment at 60° C in the presence of SDS would probably control such activity to a large extent. Endoproteinase Lys-C is evidently a serine proteinase not inhibited by egg or soybean trypsin inhibitor (Anonymous 1987). Meal from the cultivar Bomi did not inhibit endoproteinase Lys-C activity with tosyl-glycyl-prolyl-lysyl-4-nitroanilide acetate (Boehringer) as the substrate when measured at 405 nm. It is not, however, certain that all barley varieties, or microorganisms infesting the grains, would lack inhibitor to this enzyme.

Endoproteinase Lys-C increases the number of amino terminals in relation to the number of internal lysyl residues. The decrease in the correlation of BCA versus lysine (TNBS and SDH) due to digestion (Table III) supports our hypothesis that BCA is fairly insensitive for measuring the concentration of amino terminals (Ahokas and Naskali 1988).

The methods above can be extended to single grain samples by grinding with a miniature drill (Ahokas 1988, Ahokas and Naskali 1988). The embryo and part of the endosperm can then be saved for germination.

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