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Relationship between Alcohol-Soluble Proteins Extracted from Maize Endosperm by Different Methods

Jacques Landry,[1] Jerrold W. Paulis,* and Dennis A. Fey

The glutelin fraction of maize protein is retained in the residual meal after extraction of salt-soluble proteins and zein and is solubilized only after disruption of its disulfide bonds. Using varied solvent systems, different researchers have fractionated reduced glutelin into components with different properties; like zein, some of these polypeptides are soluble in aqueous ethanol. In this study, the compositions of the fractions extracted from glutelin with a solvent consisting of 63% ethanol containing sodium acetate and mercaptoethanol were compared to those of proteins obtained by sequential two-step procedures consisting of 55% 2-propanol with mercaptoethanol followed by 55% 2-propanol containing mercaptoethanol and sodium acetate or by a pH 3 lithium chloride solution containing mercaptoethanol. The relationships of these proteins and similar alcohol-soluble proteins are discussed.

According to Osborne and Mendel (1914), glutelin cereal grains are defined as proteins not extractable by aqueous solutions of salt or alcohol and soluble in dilute alkali, which may cause glutelin degradation, including cleavage of disulfide bonds (Paulis et al., 1969). Maize glutelins can be fractionated into three subgroups by selective extraction (Landry and Moureaux, 1970). These subgroups have been termed as follows: G_1 or alcohol-soluble glutelins, which are isolated in aqueous alcohol in the presence of 2-mercaptoethanol (ME); G_2 or salt-soluble glutelins, which are extracted with aqueous salt solution and ME, buffered at pH 10; G_3 or zeanins, which are solubilized by sodium dodecyl sulfate (SDS) and ME at pH 10. G_2 has an amino acid composition between that of zein and of salt-soluble proteins (Landry and Moureaux, 1970).

Three similar reduced glutelin fractions were distinguished by Paulis and Wall (1971) on the basis of their solubility in 6 M guanidine hydrochloride and in 63% ethanol. By comparing the amino acid composition of Landry-Moureaux and Paulis-Wall fractions, Misra et al. (1976) have related guanidine hydrochloride soluble and ethanol-soluble glutelins to G_1 and guanidine hydrochloride soluble and ethanol-insoluble glutelins to a mixture of G_2 and G_3. Furthermore, Paulis and Wall (1977a,b) isolated alcohol-soluble reduced glutelins (ASG) in the presence of ethanol, sodium acetate (NaOAc), and ME. These glutelins were further divided into water-insoluble (WI) and water-soluble (WS) fractions. By extracting G_2 successively with acidic and alkaline solutions, Landry and Moureaux (1981) have shown that only acid-soluble G_2 has an amino acid composition close to that of WS-ASG. The coextraction of alcohol- and water-soluble constituents with aqueous alcohol in the presence of reductant was also observed with 1-propanol (1-PrOH) by Wilson et al. (1981) and with ethanol or 2-PrOH, NaOAC, and ME by Landry and Moureaux (1981), but Melcher and Fraij (1980) observed only small amounts of water-soluble protein in this extract.

Thus, there is considerable evidence that the fractionation of corn glutelins is largely dependent on the solvents and experimental conditions used. We have now isolated zein, G_1, some G_2 fractions, WI-ASG, and WS-ASG and have compared their amino compositions, relative molecular weights (M_r), electrophoretic behaviors at pH 3.5, and apparent pI's. These results now enable us to better define these diverse alcohol-soluble proteins.

Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604.

[1]Present address: Laboratoire de Chimie biologique, I.N.R.A., I.N.A.P.G., 78850 Thiverval, Grignon, France. J.L. was the recipient of a NATO fellowship while a visiting scientist in Peoria.

MATERIALS AND METHODS

Preparation of Proteins. Seeds from a normal inbred, W64A^{Ht}, were supplied by Illinois Foundation Seeds Inc., Champaign, IL. This grain was dry-milled by a conventional procedure at room temperature (RT); the endosperm used for the protein extractions was from the air-aspirated first and third break grits (Brekke et al., 1973). These fractions (horny portion of endosperm) were combined and rapidly ground in a Udy cyclone hammermill through a 0.024 in. mesh screen in the presence of dry ice to lower the temperature and thereby minimize protein denaturation. To defat the meal, 550–850 g was placed in a 5 × 75 cm glass column and eluted with ca. 6 L of anhydrous acetone and then with 2 L of diethyl ether at 4 °C (Landry, 1979).

Albumins, globulins, and nonprotein nitrogen were extracted by stirring 1010 g of defatted endosperm meal with 14 L of 3% NaCl at 4 °C for 2 h. The residues were removed by continuous centrifuging at 4 °C with an International No. 2 centrifuge in a 10-in. perforated basket with filter cloth at 2500 rpm. The insolubles were rinsed with 2 L of 3% NaCl and 40 L of H_2O, at 4 °C. Ammoniacal saturated $AgNO_3$ solution gave almost no precipitate for Cl^- after water washing, indicating that most NaCl had been removed from the residue. The meal residues were lyophilized to dryness.

To extract alcohol-soluble proteins, 102 g of NaCl-extracted residue was successively stirred for 1 h at RT with 1 L of 90% 2-PrOH to solubilize α-zein (αZ), 2 times at RT for 2 h each time and then overnight with 500 mL of 55% 2-PrOH to extract remaining zein (Z), and once for 2 h and then overnight at RT with 400 and 300 mL of 55% 2-PrOH–1.2% ME for G_1. In a second extraction scheme (without prior removal of αZ), 432 g of NaCl-extracted residues was successively stirred 3 times for 1, 2.5, and 1.5 h at RT with 2, 1.5, and 2 L of 55% 2-PrOH, respectively, to extract zein (Z) and then stirred overnight twice with 2 and 1.5 L of 55% 2-PrOH–1.2% ME to extract G_1. Half of these last extracted residues (196 g) was stirred overnight at RT with 800 mL of 0.84% LiCl–0.06 N sodium formate–0.24 N formic acid (pH 3) buffer containing 1.2% ME for G_2-pH 3 glutelin (sequence A, Figure 1), and the

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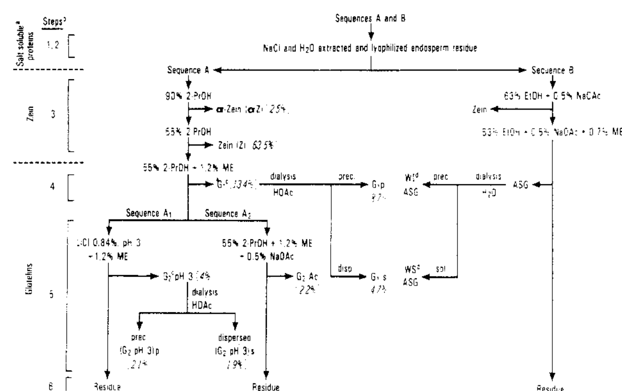

Figure 1. Isolation of protein fractions from maize endosperm. Nitrogen percentages are in italics. Concentrations are given in w/w for alcohol, w/v for salt, and v/v for ME. [a]Protein fractions as defined by Osborne and Mendel (1914). [b]Steps as defined by Landry and Moureaux (1981). [c]Protein fractions as defined by Landry and Moureaux (1970). [d]Protein fractions as defined by Paulis and Wall (1977a,b).

other half was stirred with 1 L of 55% 2-PrOH–1.2% ME–0.5% NaOAC for 3 h at RT for G_2-Ac glutelin (sequence A_2, Figure 1).

The suspensions from both extraction schemes were filtered through a medium-grade sintered glass funnel; the residues were washed with ca. $^1/_4$ volume of extractant. A 2-fold molar concentration of acrylonitrile to mercaptoethanol was added to the G_1, G_2-pH 3, and G_2-Ac filtrates and stirred for 1 h at RT to alkylate the protein sulfhydryls and prevent their reoxidation. In the case of G_2-pH 3 filtrate, solid LiOH was added to the extract to raise the pH to 8 for alkylation. The reaction was stopped by acidifying with HOAc (ca. pH 3).

Zein extracts were purified by the procedure of Landry (1979). The first extract of G_1 was purified by precipitation overnight at 4 °C with a 5 to 1 (v/v) solution of 3% LiCl to the extract, followed by evaporation to $^1/_3$ volume. An equal volume of *t*-BuOH and 5 mL of HOAc was then added to the suspension to solubilize the protein. The solution was successively dialyzed against concentrated HOAc for 1 h and 1 M HOAc for 5 h. A portion of the dialysate (110 mL) was placed on a 5 cm × 40 cm column containing 120 g of Sephadex LH-20 equilibrated with 50% *t*-BuOH–1% HOAc solvent and eluted at 70 mL/h with that mixture.

In the second extraction scheme, the alkylated G_1 extract was reduced $^1/_3$ by evaporation, solid $(NH_4)_2SO_4$ was added to 10% concentration, and protein was allowed to settle for 5 h at 4 °C. The precipitated protein was removed by centrifugation at 1000*g* for 15 min and dialyzed against 50% HOAc and then 5% HOAc and, finally, against several changes of 2% HOAc for 3 days before the soluble G_1s and insoluble G_1p protein were separated by centrifuging the suspension at 1000*g* and lyophilized.

The alkylated G_2-pH_3 extract was precipitated by addition of $(NH_4)_2$ SO_4 to a final concentration of 40% and stored overnight at 4 °C. Three phases separated: crystals at the bottom, an intermediate turbid solution, and a top layer containing some propanol. The turbid center layer was siphoned off and glacial HOAc added to dissipate the turbidity. This layer next was dialyzed against 5% HOAc and then against 2 changes of 2% HOAc for 4 days. Alkylated G_2-Ac extract was dialyzed directly, like G_2-pH 3. Both G_2-pH 3 and G_2-Ac dialysates were lyophilized.

WS-ASG and WI-ASG were isolated from the endosperm meal as shown in Figure 1 and by the procedure of Paulis and Wall (1977a,b).

Analytical Methods. Aliquots of extracts and portions of weighed dried materials were assayed for nitrogen by a semimicro Kjeldahl method.

Samples of protein (10–50 mg) were refluxed in 6 N HCl (2 mL/mg of sample), and the liberated amino acids were quantitatively determined with a Dionex D-300 amino acid analyzer. The levels of methionine and cystine in some samples were determined after conversion to methionine sulfone or cysteic acid by performic acid (Moore, 1963).

Proteins (1.5 mg) were reduced by heating at 37 °C overnight in 0.1 mL of 0.125 M HCl buffer (pH 8.9) containing 5.1% SDS, 5% ME, and 10% glycerol for reduced zeins (R) and without ME in the same buffer for native zein (N). Sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) was performed following the procedure of Laemmli (1970) but using pore-gradient gels. Slab gels (1.5 mm thick) were prepared in a Bio-Rad Model 220 vertical gel apparatus. A gradient 10–15% polyacrylamide (% T) resolving gel that contained 4% Bis and 15-mm stacking gel (5% T, 3% C) was prepared by using 0.015% flavin monucleotide as a polymerization catalyst. Electrophoresis was performed at 20 mA (constant current) while the tracking dye traveled through the stacking gel (ca. 1.5 h), and at 30 ma while the dye traversed the resolving gel (ca. 1.5 h). Gels were stained overnight with 0.024% Coomassie Brilliant Blue R250 in MeOH–HOAc–H_2O (3:1:12 v/v/v) containing 6% trichloroacetic acid (TCA) and destained for a few minutes with MeOH–HOAc–H_2O (4:1:8 v/v/v).

The protein for acidic polyacrylamide gel electrophoresis (PAGE) were reduced with 1% ME in 8 M urea overnight at RT, alkylated for 1 h with acrylonitrile, and acidified with aluminum lactate buffer (Paulis and Wall, 1977a,b). For electrophoresis, a 2 × 125 × 260 mm polyacrylamide gel (5% T, 5% C) was prepared with 0.0017 M aluminum lactate–0.05 M lactic acid–8 M urea, pH 3.5, buffer, 0.07% ammonium persulfate (w/v), and 0.14% Temed. The gel was preelectrophoresed with 2 L of 0.0008 M aluminum lactate–0.05 M lactic acid–4 M urea, pH 3.5, buffer in the wells of an LKB Multiphor for 5 h at 13 W (constant power) at 4 °C. Samples, 200–300 μg of protein contained in 10 μL, were placed in slots in the gel. Electrophoresis was then carried out lengthwise with the same buffer used for preelectrophoresis in the wells but at 6.5 W (constant power) for 16 h at 4 °C. The protein bands were fixed by soaking the gels overnight in 12.5% TCA and then photographed on a glass plate placed over a black background, with illumination from a perpendicular light box. The gels were next washed for 30 min in EtOH–HOAc–H_2O (50:16:134) and stained overnight with 0.03% Coomassie Brilliant Blue R250 in MeOH–HOAc–H_2O (5:1:5) and destained for 4 h in MeOH–HOAc–H_2O (4:1:8).

The previously prepared alkylated protein solutions were also used for isoelectric focusing (IEF) on Whatman No. 1 paper wicks. The IEF polyacrylamide gel slab (2 × 125 × 260 mm) was 5% T and 3% C containing 0.05% ammonium persulfate (w/v), and 5% pH 3.5–10 ampholines in 6 M urea. IEF was carried out at 13 W (constant power) for 4 h at 4 °C using the LKB 2117 Multiphor with saturated aqueous aspartic acid and 0.1 M lysine for the anodic and cathodic solutions, respectively.

RESULTS

Extraction of Proteins. The defatted horny endosperm meal contained 2.2% N (as-is basis). The amounts of proteins isolated under various conditions and expressed as percentage of total nitrogen of horny endosperm are reported in Figure 1. Of the total N the α-zein accounted for only 2.5%, the remaining zein from three successive

Table I. Amino Acid Composition of Endosperm and Its Protein Fractions[a]

	defatted endosperm	α-zein	zein, 1st extr	zein, 2nd extr	WI-ASG	G_1	G_1p	G_1S	WS-ASG	G_2-Ac	(G_2-pH 3)s	(G_2-pH 3)p
Asx	54	51	50	50	29	21	28	6	6	0	7	44
Thr	34	29	30	30	29	32	32	42	38	45	43	42
Ser	57	59	61	61	52	49	52	51	38	38	47	51
Glx	195	210	210	212	190	238[b]	225[b]	214[b]	169	211[b]	184[b]	219[b]
Pro	104	96	95	99	127	136	124	217	243	261	260	165
Gly	43	18	19	20	43	72	79	82	71	70	77	72
Ala	119	139	136	141	105	110	130	83	55	55	56	82
Cys	8	7	10	2	42	nd[c]	nd	nd	46	nd	(5)	nd
Val	47	41	40	41	37	40	34	62	72	73	72	58
Met	16	9	13	10	65	51[d]	37[d]	6[d]	9[d]	2[d]	nd	nd
Ile	35	39	37	37	25	15	14	15	20	18	18	29
Leu	153	192	190	190	146	113	125	100	106	101	97	106
Tyr	34	35	37	37	37	50	56	29	17	17	15	24
Phe	42	52	51	51	33	24	31	17	9	9	8	19
Lys	12	0	0	0	2	0.6	0	0	1	0	8	7
His	21	11	10	11	22	19	12	51	74	74	77	52
Arg	24	12	13	12	17	24	22	26	27	26	28	30

[a] Expressed as number of residues per 1000. [b] Glx and (carboxyethyl)cysteine were not well resolved in hydrolysates of cyanoethylated proteins. Approximate values of Glx can be obtained by subtracting 45 (mean number of Cys residues of WI- and WS-ASG) from these figures, except for (G_2-pH 3)s, where the presence of Cys indicated that these residues were not all cyanoethylated. [c] Not determined. [d] Values are only estimations based on HCl-hydrolyzed proteins.

extracts accounted for 63.5%, and G_1 accounted for 13.4%. Yields of precipitated G_1 (G_1p) and soluble G_1 (G_1s) obtained by dialysis of extract against 2% HOAc were 8.7% and 4.7%, respectively.

After extraction of zein and G_1, the G_2 extracted by aqueous 2-PrOH in the presence of ME and NaOAC (G_2-Ac) represented 2.2% of the total protein, whereas G_2 removed at pH 3 (G_2-pH 3) in the presence of ME and LiCl constituted 4%. The G_2-pH 3 was further separated by dialysis against 2% HOAc into soluble, (G_2-pH 3)s, and precipitated, (G_2-pH 3)p, fractions, accounting for 1.9% and 2.1%. Therefore, the sum of proteins extracted in sequences A_1 and A_2 reached 83.4% and 81.6%, respectively.

Amino Acid Analysis. Amino acid compositions (expressed as number of residues per 1000) of horny endosperm protein and zein fraction and of various glutelin fractions are given in Table I. The compositions of α-zein and other zein samples appear identical. Compared to zein, reduced and cyanoethylated G_1 contained less aspartic acid or asparagine, alanine, isoleucine, leucine, and phenylalanine but more proline, glycine, methionine, tyrosine, histidine, and arginine. The same differences are also found when WI-ASG's amino acid composition is compared to zein's.

The composition of G_1p differs from that of WI-ASG in content of glycine, alanine, isoleucine, leucine, histidine, tyrosine, and arginine, but both are high in methionine. The amino acid compositions of G_1s, WS-ASG, and G_2 fractions resemble each other. Compared to the composition of zein and G_1p, they are poorer in aspartic acid, alanine, and phenylalanine but are richer in glycine, proline, valine, histidine, and arginine. The composition of (G_2-pH 3)p differs from those of other G_2 fractions mainly by a higher content of aspartic acid or asparagine, phenylalanine, tyrosine, isoleucine, and alanine and lower amounts of proline and histidine. (G_2-pH 3)s and (G_2-pH 3)p, in contrast to other G_2 fractions, contained some lysine.

SDS–PAGE. The molecular weight distribution of protein fractions, as determined by SDS–PAGE, is shown in Figure 2 and 3. Native α-zein and zein consisted of the same two subunits (Z_1 and Z_2) of M_r 22 000 and 20 000 and of their oligomers (dimers, trimers, and so on). In contrast, reduced zein was made up mainly of both Z_1 and Z_2 components and lacked oligomeric forms. Two faint bands, migrating at ca. M_r 43 000, and two additional bands with M_r 10 000 and 16 000 also were detected.

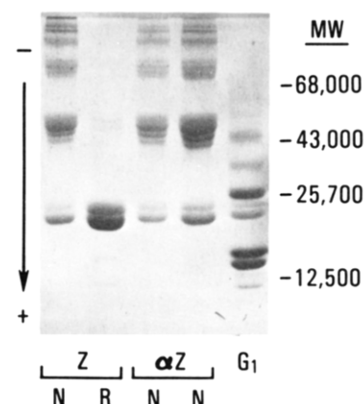

Figure 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of (no ME) native (N) and (plus ME) reduced (R) zein, native (N) α-zein (300 and 150 μg) and alkylated–reduced G_1 glutelins. Protein standards: bovine serum albumin (68 000), ovalbumin (43 000), α-chymotrypsin (25 700), and cytochrome c (12 500).

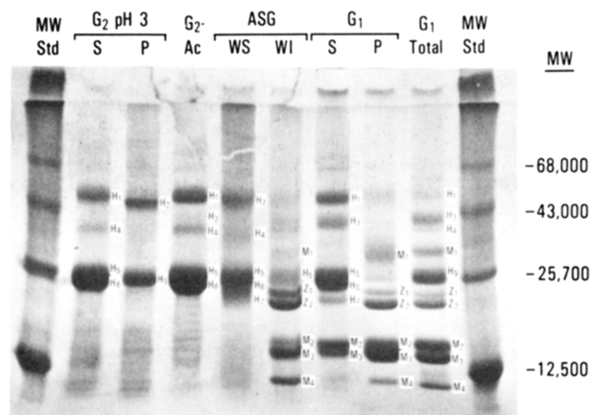

Figure 3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of alkylated–reduced high-histidine (H) and high-methionine (M) glutelin fractions, WI-ASG, G_1, total, and G_1p containing zein-like bands (Z). Same standards as in Figure 2.

The pattern of G_1 appears more complex (Figure 3). It

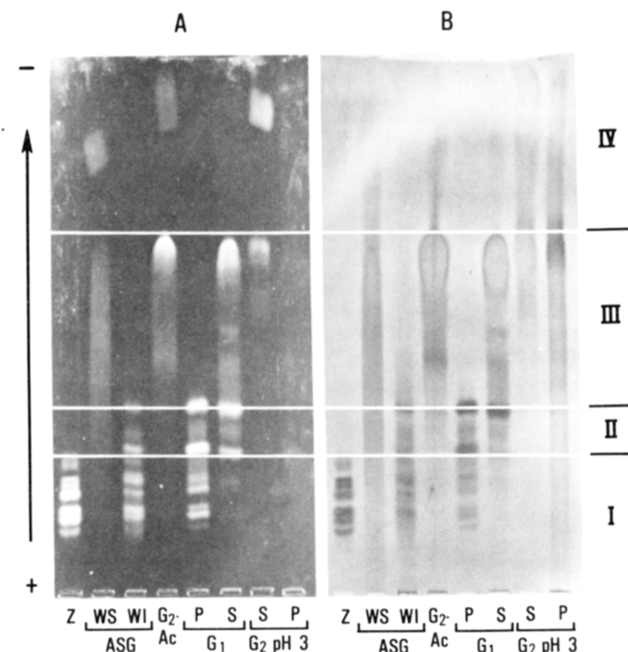

Figure 4. Acidic urea–polyacrylamide gel electrophoresis of alkylated–reduced zein and glutelin fractions. (A) Gel fixed with TCA; (B) the same gel stained with Coomassie Blue after (A).

contains three major bands (M_r 14 000, 16 000 and 25 000) and five minor bands (M_r 10 000, 20 000, 22 000, 32 000 and 47 000). To better distinguish bands in each glutelin fraction shown in Figure 3, bands of M_r lower than 67 000 were designated by M (high methionine), Z (high leucine) and H (high histidine), respectively, similar to components characterized by others in the same fractions, which were rich in these amino acids (Paulis and Wall, 1977a,b; Wilson et al., 1981; Esen et al., 1981; Gianazza et al., 1977). They were numbered according to their relative mobilities. On the basis of the separations in Figure 3, it appears that (i) the pattern of G_1p corresponds to that of G_1 free of G_2-Ac, (ii) WS-ASG, G_2-Ac, and (G_2-pH 3)s give very similar patterns, whereas that from (G_2-pH 3)p differs by the lack of bands H_1, H_3, and H_4 and by a narrower band H_5. A band H_6 very close to H_5 also was visible in the pattern of WS-ASG, which suggests that the broad band observed in patterns of G_1s, (G_2-pH 3)s, and G_2-Ac corresponds to the unresolved bands H_5 and H_6. On the other hand, G_1s also contained a band with mobility intermediate between those of Z_1 and Z_2. This band, which remained soluble after dialysis in 2% HOAc, therefore differs from zein. It is also present in WS-ASG and is designated H_7.

Acidic Urea–PAGE. Some reduced and alkylated zein and glutelins that appear similar in mobility by SDS–PAGE may differ greatly in mobilities when separated by charge during PAGE in aluminum lactate buffer, as shown in Figure 4. The patterns were revealed by TCA fixation (Figure 5) and by staining with Coomassie Brilliant Blue R250 in a mixture of MeOH–HOAc–H_2O (Figure 5).

Zein was resolved into nine bands that had the slowest mobilities (region I). None among these bands are present in either G_2-Ac, G_2-pH 3, or WS-ASG. Some bands that exhibit the same mobilities as zein are detected in patterns of G_1p and WI-ASG, which contain several additional bands (region II). G_1s contains components having mobilities similar to those of G_1p in region II, plus some bands, streaking material, and a spot constituting region III. The region III spot is characteristic of WS-ASG, G_2-Ac, (G_2-pH 3)s, or (G_2-pH 3)p. Every fraction is characterized by differences in the relative prominence of

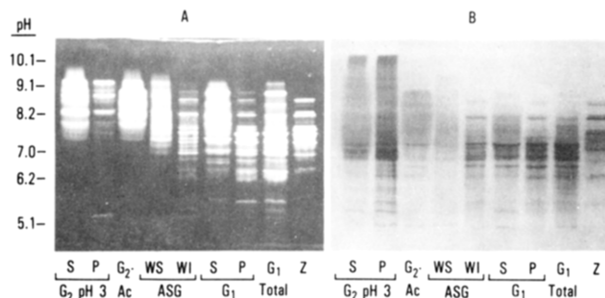

Figure 5. Isoelectric focusing (pH 3.5–10) of alkylated–reduced zein and glutelin fractions. (A) Gel fixed with TCA (overnight); (B) another gel, fixed in TCA (30 min) with Coomassie Blue.

different bands or streaking material as detected by TCA fixation or staining. In addition, patterns of WS-ASG, G_2-Ac, and (G_2-pH 3)s contain a TCA-precipitated spot of high mobility (region IV). This spot is at the same general position for each fraction but shows some variation in mobility due to a temperature gradient in gel.

Isoelectric Focusing (IEF). Zein and glutelin fractions exhibit complex IEF patterns (parts A and B of Figures 5). Between pHs ranging from about 6.5 to 8.9 on the gel, 20 zein bands were visualized by TCA precipitation and 15 by staining.

IEF patterns confirmed the previous observations from Figures 3 and 4 that G_1p and WI-ASG are similar and G_1s, WS-ASG, G_2-Ac, and (G_2-pH 3)s are related. However, IEF patterns of (G_2-pH 3)s and (G_2-pH 3)p differ from those of other fractions by having additional subunits with p*I*'s either higher than 9.3 or ranging from 7.0 to 7.6 that are detected only by staining with Comassie Brilliant Blue R250 (Figure 5). Finally, (G_2-pH 3)p differs from (G_2-pH 3)s by the absence of streaking material characteristic of other G_2 fractions.

DISCUSSION

The various protein fractions identified in this investigation have been isolated from horny endosperm only. Zein recovered from horny endosperm was similar to zein isolated from whole endosperm (Melcher and Fraij, 1980; Gianazza et al., 1977; Tsai, 1980) and from whole grain (Landry, 1979) of same cultivar; it exhibits the same amino acid composition (Melcher and Fraij, 1980; Gianazza et al., 1977; Landry, 1979), the same polymorphism as judged by SDS–PAGE in the native and reduced state (Landry, 1979; Tsai, 1980), and the same charge heterogeneity as detected by acidic urea electrophoresis (Landry, 1979) as do the other zeins. Similarities such as these can be extended to properties of zein isolated from endosperm of other maize cultivars (Paulis and Wall, 1977a,b; Paulis, 1981). Extraction with 90% isopropyl alcohol removed a portion of zein referred to here and earlier as α-zein (Turner et al., 1965).

Characterization of fraction G_1 indicates that it contains some proteins identical with those of G_2 (Figures 3 and 4). On the basis of solubility in dilute acetic acid, G_1 separated into G_1s and G_1p. The amino acid composition of G_1p is very close to the compositions of the polypeptides Z13.5 and Z9.6 reported by Gianazza et al. (1977), which correspond in M_r to M_2 plus M_3 and to M_4, respectively. Z_1 and Z_2 subunits would represent a small percentage of G_1. G_1s, as judged by SDS–PAGE, is made up of some components belonging to G_1p (M_2 plus M_3) and G_2-Ac.

Since WI-ASG is practically free of H components, and since WS-ASG does not contain appreciable amounts of M protein, dialysis of ASG against water is a very efficient way to fractionate the alcohol-soluble components of reduced glutelin. The M components specific to WI-ASG

could then be isolated by cryoprecipitation at −20 °C in alcoholic solution, as described by Melcher and Fraij (1980).

G_2 glutelins and WS-ASG have amino acid compositions with some similarities to those of prolamins; these proteins have low amounts of lysine and aspartic acid or asparagine and high contents of glutamic acid or glutamine and proline. The apparent pI's of G_2 glutelins and WS-ASG range from pH 7.3 to beyond pH 10.1; therefore, their content of acidic residues must be very low. G_2 glutelins and WS-ASG also are distinguished from any other protein fractions by a very high content of histidine. WS-ASG also has been shown to have a very unusual N-terminal amino acid sequence (Esen et al., 1982).

In G_2 glutelins and WS-ASG, the most prominent component (H_5) has an M_r of 25 000. Two additional components, H_6 and H_7, having M_r's close to that of H_5, also exist; H_6 and H_7 also are detected in reduced-soluble (RS) proteins separated by two-dimensional electrophoresis by Wilson et al. (1981). However, fractions 4 and 5 (M_r 27 500), isolated from ASG by Esen et al. (1981), had amino acid compositions resembling those of our ASG fractions (Table I) but having a lower histidine content (55 or 60 residues per 1000 instead of 74 for G_2Ac). These comparisons all indicate that ASG is a subset of several components rich in histidine that vary in size and amino acid composition.

G_2 glutelins are extracted in the presence of reductant and at room temperature by salt solutions but not by water or aqueous alcohols (Landry and Moureaux, 1970); they display extractability traits of globulins. The solvents used to extract WS-ASG and G_2-Ac consist of an alcohol solution containing acetate, suggesting that these proteins also have solubility properties similar to those of prolamins. This dual solubility is consistent with the amino acid composition of G_2 glutelins. The solubility of G_2 glutelins in aqueous alcohol is also enhanced at high temperature (60 °C) (Landry, 1979; Lee et al., 1976); furthermore, the effect of 60° C temperature reextraction is evidenced by the presence of G_2 glutelins in ASG isolated by Esen et al. (1981) without NaOAc. Reciprocally, the coextraction of G_1s (which is similar to WS-ASG) with G_1p (this investigation) could be due to incomplete elimination of salt after washing the meal with water.

The coextraction of some G_2 glutelins with zein and G_1 when alcoholic extraction is performed in the presence of salt or at high temperature and the existence in G_2 glutelins and WS-ASG of components with size close to those of zein subunits would be responsible for the presence of additional bands found occasionally in SDS–PAGE patterns of zein. For example, bands designated as Z_1a by Lee et al. (1976) and as Z by Tsai (1980) would be component H_5 from G_2 glutelins. Likewise, a band designated as Z22 and found between two subunits of zein [Z23 (Z_1) and Z21 (Z_2)] by Soave et al. (1976) could also originate from G_2 glutelins (H_7), and the M_r 26 000 component detected in zein extracted with 63% EtOH–0.6% NaOAc–0.5% ME by Melcher and Fraij (1980) could correspond to traces of H_5 and H_6.

Possibly, the extractabilities of WS-ASG by salt and reductants in aqueous or alcoholic medium are dependent upon some of the treatments of the meal before extraction. The absence of significant amounts of WS-ASG in alcohol-soluble glutelins isolated by Melcher and Fraij (1980) in the presence of NaOAc could be due to extracting the salt-soluble proteins at room temperature and suggests an insolubilization. This phenomenon also occurs to some extent when extraction with NaCl is performed at 10 °C instead of 4 °C (Landry and Moureaux, 1981). On the basis of facts, isolation of protein bodies in isotonic solution at room temperature, as described by Miflin et al. (1981), could promote the insolubilization of WS-ASG, which they termed RS-proteins. Despite data reported by Miflin et al. (1981), doubt remains about the absence of RS-proteins in protein bodies, especially since Vitale et al. (1982) have detected M_r 28 000 subunits in them. Due to the uncertainty of M_r estimation by SDS–PAGE, it is not possible to ascertain whether these M_r 28 000 subunits correspond to G_1 polypeptides (M_r 32 000) or to the G_2 component (M_r 25 000).

The intrinsic properties of these G_2 glutelins and WS-ASG are similar to those of RS-proteins of Wilson et al. (1981). Moreover, these authors noted that RS-proteins cannot be distinguished by IEF from zein and G_1 unless the reduced sample is alkylated. This observation is further supported by comparing data of Figure 5 with those of Soave et al. (1976) and Righetti et al. (1977), who found similar IEF patterns for zein, total ASG, and WI-ASG when they were only reduced. Wilson et al. (1981) have assumed that IEF differences between pyridylethylated RS-proteins and zein are related to the increase of positive charge of polypeptides. Such a hypothesis is inconsistent with the present data because cyanoethylation, which introduces neutral groups, produced the same effect as did pyridylethylation. Therefore, alkylation of reduced polypeptides appears a prerequisite to better assessment of the heterogeneity of alcohol-soluble proteins by IEF. Alkylation of reduced proteins is essential for studies of maize genetics when such studies are based on IEF of all alcohol-soluble proteins.

CONCLUSION

Three unique subsets of alcohol-soluble proteins can be extracted from maize endosperm under various experimental conditions. All these alcohol-soluble proteins have been termed *zein* [The term of *zein* used by Wilson and co-workers is written in italics to avoid confusion with the name "zein" as it is employed in this study and which corresponds to *zein-1* of Sodek and Wilson (1971).] (1 plus 2) or total *zein* by Wilson et al. (1981) and have been subdivided into *zein* and RS-proteins. The designation of protein mixtures of different complexities by the same word is obviously inappropriate and results in confusion. For example, *zein-2*, which is defined by Sodek and Wilson (1971) as zein and G_1, was later described as consisting of zein, G_1, and RS-proteins by Wilson et al. (1981). Such broad definitions may overestimate zein's polymorphism, as emphasized by Landry (1979), Landry and Moureaux (1981), and Paulis (1982). High-leucine protein with M_r 20 000 and 22 000 can be considered similar or identical with zein and takes into account zein's polymorphism.

The terms Landry and Moureaux (1981) proposed for the three unique subsets of maize alcohol-soluble proteins are zein, zein-like, and prolamin-like. Thus, zein is defined as proteins extracted at room temperature with aqueous EtOH or 2-PrOH from endosperm meal after lipids, salt-soluble nitrogen, and salt are removed.

G_1 glutelins, extracted after zein by aqueous EtOH or 2-PrOH in the presence of mercaptoethanol, contain three kinds of components. The first type consists of polypeptides having properties identical or very similar with those of zein. The second type comprises unique components that like zein are soluble in aqueous alcohol after reduction and are insoluble in water; however, most have M_r's lower than zein and are richer in sulfur residues. The third type has components similar to that in WS-ASG that were shown to be high in proline (Esen et al., 1981). The

major polypeptides in G_1 and WI-ASG differ from zein and WS-ASG and can be classified as a water-insoluble, high-methionine, M_r 14000 and 10000 protein. Extraction with 63% EtOH plus 0.5% NaOAc plus 0.7% ME, followed by dialysis against water, is a simple method to effectively separate the high-proline–high-histidine and the high-methionine bands as WS-ASG and WI-ASG fractions. This is verified by WS-ASG and WI-ASG having one of the highest proline–highest histidine and highest methionine content, respectively, compared to those of the other protein fractions.

WS-ASG and acid-soluble G_2 glutelins have properties very different from zein and WI-ASG, as demonstrated in this and other investigations. After glutelin disulfide bonds are broken with reductant, WS-ASG or G_2-Ac glutelins become soluble in water; aqueous alcohol, neutral, acidic, or basic salt solutions, and dilute HOAc. They are high-proline–high-histidine proteins with predominant M_r 25000. According to Osborne–Mendel solubility schemes, these polypeptides could be classified as either albumins, globulins, prolamins, or glutelins. They are commonly classified as glutelins because they are soluble only after disulfide bond disruption. The term "reduced–soluble proteins", used by Wilson et al. (1981) for these subunits, is not specific enough to define the solubility of this fraction. Likewise, the term "prolamin-like" (Landry and Moureaux, 1981) stresses only the resemblance of G_2 subunits to prolamins. The term WS-ASG best describes this protein as being water soluble, alcohol soluble, and associated by disulfide bonds in the water glutelin protein.

The presence of cereal proteins with multiple solubility characteristics demonstrates once again that there are no clear-cut boundaries between Osborne and Mendel (1914) protein classes. The combined data from amino acid analyses, SDS–PAGE, IEF, and acidic PAGE have been useful in classifying these proteins according to their physical characteristics. On the basis of these data, a defined system for naming the alcohol-soluble glutelins is one using WS-ASG and WI-ASG. There is still need for total sequence analysis to establish the precise nature of the differences in these proteins.

Registry No. 2-PrOH, 67-63-0; EtOH, 64-17-5; ME, 60-24-2; LiCl, 7447-41-8; sodium acetate, 127-09-3.

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A New Procedure for Specific Determination of β-Amylase in Cereals

Paul R. Mathewson* and Bradford W. Seabourn

A new method for specific determination of β-amylase in cereals has been developed. This method utilizes a commercially available colorimetric substrate consisting of *p*-nitrophenyl oligosaccharides. Results are linear with β-amylase concentration over a wide range of activity. Tests in which increasing amounts of cereal α-amylase were added to a constant amount of β-amylase showed no significant increase in absorbance using this substrate. Under the same conditions, the commonly used Bernfeld assay for β-amylase activity showed an increased response. Using a new substrate, we observed a constant response for wheat samples with increasing degree of sprouting. This substrate was also found to be useful for the determination of fungal α-amylase activity.

The amylase enzymes in cereal grains play vital roles in the growth and development of the plant as well as in processing plant material for food use. In cereals, α-amylase has received considerable attention due to its dextrinogenic activity on starch and the detrimental results in terms of food processing. Since the saccharogenic activity of β-amylase is much less detrimental to processing grains, this enzyme has been viewed as relatively unimportant. However, there are other situations, physiological studies in particular, in which the ability to measure β-

U.S. Grain Marketing Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Manhattan, Kansas 66502, and Department of Grain Science and Industry, Kansas State University, Manhattan, Kansas 66506.

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