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Physicochemical Properties of Maize Glutelins As Influenced by Their Isolation Conditions

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The influence of various parameters (presence of sodium acetate in alcohol solutions, pH, concentration and nature of reductant, and blocking of disulfide bonds) upon the distribution and the quality (amino acid composition and electrophoretic mobility at pH 3.5) of G_1-, G_2-, and G_3-glutelins in maize grain was investigated. G_2-Glutelins were divided into two fractions by extraction at pH 3 and 10; acid-soluble G_2-glutelins were also coextracted with G_1-glutelins when sodium acetate was added to alcohol solution containing 2-mercaptoethanol. The possible contamination of zein by G_1- and G_2-glutelins is discussed. Maize proteins can be classified as alcohol-soluble (zein and G_1-glutelins and acid-soluble G_2-glutelins) and alcohol-insoluble proteins (salt-soluble proteins, acid-insoluble G_2- and G_3-glutelins, and insoluble glutelins). The extension of such a classification to grain proteins of other cereals is examined.

Maize grain proteins can be separated into five fractions by a selective extraction method (Landry and Moureaux, 1970). Thus the successive contact of six media with a grain meal results in the release of salt-soluble proteins and zein, referred to as fractions I and II, and of three glutelin subgroups, denoted by G_1, G_2, and G_3 and corresponding to fractions III, IV, and V. Numerous investigations concerning the distribution and the composition of these five protein fractions as well as their accumulation in developing grain, their disappearance in endosperm during germination, their localization in histological parts, and the influence of genetic factors upon their accumulation rate afforded a further insight into the physicochemical and biological properties of glutelins.

However, literature data show that the six media described by Landry and Moureaux (1970) do not always lead to isolation of well-delineated protein fractions (Landry, 1979a; Landry and Moureaux, 1980). The variations in resolution seemed to be due to slight alterations in experimental conditions which can modify efficiency and selectivity of a given medium. For example, 0.5% sodium acetate in aqueous ethanol and 2-mercaptoethanol (2ME) is sufficient to cause simultaneous extraction of fractions III and IV (Paulis and Wall, 1977), whereas without salt only fraction III is isolated. Therefore it appears necessary to assess the validity of the selective extraction method previously developed (Landry and Moureaux, 1970) by examining the possibilities of cross contamination of protein fractions.

The present paper reports the results of such a study. Several sequences of solvents were investigated to determine the influence of various experimental methods upon the distribution and the nature of glutelin subgroups. Glutelin subgroups were identified both by amino acid composition and by electrophoretic behavior on starch gel at pH 3.5, since similar amino acid compositions of two extracts may hide differences in their protein compositions. The data presented here and in other investigations demonstrate the existence of several protein sets in maize grain, each containing constituents with unique physicochemical properties.

MATERIALS AND METHODS

Maize samples were seeds from a normal hybrid (INRA 260). Samples were designated I_1, I_2, and I_3 indicating three different harvest years.

Extraction of Proteins. General Scheme. The experimental conditions for protein extraction (Landry and Moureaux, 1970, 1980) are briefly summarized. Meal was defatted at −10 °C with anhydrous acetone and then with diethyl ether. Samples (3.5 g) were suspended in 35 mL of extractant. The solid material was isolated from extractants; the duration (minutes) and number of extractions and the identification of protein fractions are reported in Table I for two extraction sequences, A_0 and D_0; "A" and "D" refer two extraction schemes tested previously (Landry and Moureaux, 1970).

Specific Extraction Schemes of Protein Groups. The diverse sequences differ from the sequences A_0 or D_0 by alterations of experimental conditions at one or several steps. Thus, at step 1, for sequences D_3 and D_4, salt extraction was performed at 10 °C. At step 3, for sequence D_4, an additional extraction (step designated by 3s) was made with 55% 2-PrOH plus 0.5% sodium acetate (60 min). At step 4, for sequence D_4, extraction was carried out in the presence of sodium acetate (0.5% w/v). At step 5, for sequences A_2, A_3, D_1, and D_4, glutelins were successively extracted at step 5a with a pH 3 buffer (0.04 M citric acid plus 0.02 M Na_2HPO_4) plus 0.6% 2ME for 60, 30, and 30 min and at step 5b with a pH 10 buffer (0.125 M boric acid plus 0.02 M NaOH) plus 0.6% 2ME for 60, 30, and 30 min. At step 6, for sequence A_2, phenol–acetic acid–water (1:1:1 w/v/v) without 2ME was used. On the

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Table I. Protein Extraction Procedures for Sequences A_0 and D_0

step	sequence	extractant	time of extraction, min	fraction	protein groups
1	A_0, D_0	NaCl, 0.5 M (4 °C)	60, 30, 30	I	albumins globulins
2	A_0, D_0	water (4 °C)	15, 15		
3	A_0	EtOH, 60%[a] (20 °C) and then at 60 °C	30, 30 30	II	zein
	D_0	2-PrOH, 55% (20 °C)	60, 30, 15		
4	A_0	EtOH, 60%, + 2ME, 0.6% (v/v) (20 °C)	30, 30		
	D_0	2-PrOH, 55%, + 2ME, 0.6% (v/v) (20 °C)	30, 30	III	G_1-glutelins
5	A_0, D_0	NaCl, 0.5 M, pH 10, + 2ME, 0.6% (v/v) (20 °C)	60, 30, 30	IV	G_2-glutelins
6	A_0, D_0	$NaDodSO_4$, 0.5% (w/v), pH 10, + 2ME, 0.6% (v/v) (20 °C)	60, 30, 15	V	G_3-glutelins
	A_0, D_0				insoluble

[a] All aqueous alcohol concentrations in this study are expressed in weight by weight.

other hand, at steps 4, 5a, 5b, and 6, for sequences A_3 and D_2, the concentration in 2ME was only 0.1%. Regarding sequence A_1, extraction was performed by percolation through a column, filled with 4 g of meal dispersed in 44 g of ball glass, with 160 mL of 0.5 M NaCl (step 1), 82 mL of H_2O (step 2), 230 mL of 60% EtOH at 20 °C and 130 mL at 60 °C (step 3), 95 mL of 60% EtOH plus 0.6% 2ME (step 4) and 230 mL of 0.5 M NaCl plus pH 10 buffer plus 0.6% 2ME (step 5).

Preparation of Specific Protein Fractions for Electrophoretic Analyses. Albumins were extracted with water and precipitated by adding solid ammonium sulfate to 80% saturation. Globulins were extracted after albumins with 0.5 M NaCl and precipitated by ammonium sulfate (80% saturation). Protein pellets of albumins and globulins were taken up in 0.1 M NaCl and dialyzed against water until free of salt. The supernatant (albumins) and precipitate (globulins) after dialysis were dried by lyophilization.

Zein was isolated on purified as described elsewhere (Landry, 1979a). Zein contains two major species, having mean molecular weights of 45 000 and 22 000. Reduced zein has two major subunit fractions, of molecular weights 22 000 and 24 000. Gel filtration of native zein gives two main fractions, D (dimeric) and M (monomeric). Native fraction D has a mean molecular weight of 45 000, whereas reduced D contains 22 000 and 24 000 molecular weight subunits; native and reduced fraction M contains only the monomeric subunits (Landry, 1979a; Landry and Sallantin, 1978).

G_1-, G_2-, and G_3-glutelins were always extracted with the above-described media (sequence D_0, Table I) regardless of meal treatment.

G_3-Glutelins, before electrophoresis, were free of $NaDodSO_4$ by anion-exchange chromatography (Weber and Kuter, 1971).

Protein fractions were reduced by 16 h with a 100-fold molar excess of reductant [either 2ME or tributylphosphine (TBP)] overestimated cystine. Sulfhydryls were alkylated with a 2-fold excess of blocking agent [acrylonitrile or propylene oxide (PO)].

Glutelin subgroups were also isolated from a meal, free of salt-soluble nitrogen and zein, and treated first with TBP then with PO. This is a slight modification of the method of MacClaren and Sweetman (1966) for preparation of reduced and S-alkylated keratin. Thus, 1 g of meal previously treated with 0.5 M NaCl, water, and 55% 2-PrOH was stirred in a mixture of 1 mL of 50% (w/w) aqueous 1-PrOH and 0.031 mL of TBP (0.125 mmol). After 1 day, PO (0.018 mL, 0.25 mmol) was added, and the solution was stirred for 1 more day.

Analytical Determinations. Amino acid analyses were performed with a Phoenix Model K 8000 automatic analyzer according to the procedure of Spackman et al. (1958) on protein hydrolysates (6 N HCl; 105 °C; 24 h). No corrections were made for destruction of amino acids or for incomplete hydrolysis.

Starch gel electrophoreses were carried out in gels at pH 3.5 containing 6 M urea, 0.0011 N aluminum lactate, 0.053 N lactic acid, and 12% starch (Landry, 1979a).

RESULTS

Distribution of Protein Groups. The distribution of protein groups in samples I_1, I_2, and I_3, as determined by the use of nine sequences of five or six media is shown in Table II.

In all extraction sequences studied, the same media were used for the same steps, namely, 0.5 M NaCl at step 1, water at step 2, aqueous alcohol at step 3, the same alcohol solution used at step 3 but with 2ME at step 4, salt solution with 2ME at step 5, $NaDodSO_4$ solution containing 2ME, buffered at pH 10 (except sequence A_2), at step 6.

The diverse extraction sequences differ from sequences A_0 and D_0, used as the reference, as follows: (1) solvents percolated through a mixture of meal and ball glass in sequence A_1; (2) salt sodium at step 1 is at 10 °C in sequences D_3 and D_4; (3) sodium acetate is added to the aqueous 2-propanol at steps 3 and 4 in sequence D_4; (4) acidic and alkaline buffers are successively used at step 5 in sequences A_2, A_3, D_1, and D_2; (5) phenol plus acetic acid plus water, without 2ME, is used at step 6 in sequence A_2; (6) a lower concentration of 2ME is used at steps 4, 5, and 6 in sequences A_3 and D_2. Full details for all extraction sequences are included under Materials and Methods.

Sequences D_0, D_1, and D_3. The three sequences are very similar since the same media were employed at the same steps. The only differences are pertinent to extraction of G_2-glutelins performed in sequence D_1 with acidic and alkaline buffers and to that of salt-soluble proteins carried out in sequence D_3 at 10 °C.

As shown in Table III, the amounts of zein plus G_1-glutelins extracted with identical operating conditions is constant in three sequences. This observation confirms that the three samples have the same protein distribution

Table II. Nitrogen Proportions of Solubility Groups[a]

step	fraction[b]	N groups[b]	samples: I_1 A_0[e]	I_1 A_1	I_1 A_2	I_1 A_3	I_1 D_0[e]	I_2 D_1	I_2 D_2	I_3 D_3	I_3 D_4
1	I	SSP + NPN[c]	19.0	22.0[f]	19.0	19.0	19.0	18.2	18.3	17.5[g]	17.5[g]
3	II	zein	40.0	43.7[f]	40.0	40.0	38.0	43.6	43.7	44.0	42.1
3s											2.4[h]
4	III	G_1-glutelin	8.5	6.0[f]	8.9	7.8[i]	11.5	6.9	6.2[i]	6.7	11.7[h]
5	IV	G_2-glutelin	8.0	9.7[f]			10.0			6.6	1.0
5a					6.1[j]	3.9[i,j]		7.3[j]	4.1[i,j]		
5b					2.9[k]	5.1[i,k]		3.2[k]	6.3[i,k]		
6	V	G_3-glutelin	18.0		(16.1)[l]	17.2[i]	18.0	13.8	14.1	18.8	19.5
		insoluble P[d]	6.5	18.6	7.0	7.2	3.5	7.0	7.3	6.6	6.0

[a] Expressed as percent of total recovered nitrogen. [b] Fraction and nitrogen groups as defined from Table I. [c] Salt-soluble proteins plus nonprotein nitrogen. [d] Proteins. [e] From Landry and Moureaux (1970). [f-h,j-l] Extraction conditions other than those specified in Table I. [f] Percolation. [g] Higher temperature extraction. [h] Presence of sodium acetate in 2-propanol solution. [i] Reduction of 2ME concentration at 0.1%. [j] pH 3 buffer. [k] pH 10 buffer. [l] Phenol-acetic acid-water mixture without 2ME.

Table III. Protein Distribution in Grains I_1, I_2, and I_3 according to the Sequences D_0, D_1, and D_3[a]

step	protein	I_1, D_0	I_2, D_1	I_3, D_3
1	salt soluble	19	18.2	17.5
3 + 4	zein + G_1	49.5	50.5	50.7
5	G_2-glutelins	10	10.5	6.6
6	G_3-glutelins	18	13.8	18.8
	insoluble	3.5	7	6.6

[a] Data from Table II and expressed as percent of total recovered nitrogen.

since the accumulation of G_2-glutelins and of basic proteins (salt-soluble proteins, G_3-glutelins, and insoluble proteins) is parallel to that of zein plus G_1-glutelins (Landry and Moureaux, 1970). Indeed, grain I_2 is very similar to I_1. Its lower content in G_3-glutelins and higher content in insoluble proteins may be attributed to sample effect or to experimental conditions. On the other hand, grain I_3 appears different from I_1 since it contains less salt-soluble proteins, more insoluble proteins, and much less G_2-glutelins. But on the above background, the low amount of G_2-glutelins in sample I_3 must be attributed to the higher temperature (10 °C) used to extract its salt-soluble proteins. Other data also support this assumption.

Sequences A_0, A_1, and D_0. Sequences A_0 and A_1 possess identical media. Compared to sequence A_0, sequence A_1 shows that percolation increases the amounts of nitrogen extracted at steps 1, 3, 3 + 4, and 5. Comparing sequences A1 and D_0, only the proportions of nitrogen isolated at steps 1 and 3 are higher in A_1; those of steps 3 + 4 and 5 are the same, indicating the similarity in the exhaustivity of extractions of zein plus G_1-glutelins and of G_2-glutelins between both sequences.

Sequences of A_2 and D_1. In the sequences A_2 and D_1, acidic and alkaline buffers were used to steps 5a and 5b, respectively. Irrespective of sequences, G_2-glutelins can be separated into acid-soluble and acid-insoluble fractions, corresponding to 0.7 and 0.3, respectively, of the total nitrogen released at steps 5a and 5b. This is higher in sequence D_1, confirming the better exhaustivity of an extraction of G_2-glutelins when zein and G_1 glutelins are isolated with aqueous 2-propanol.

Sequences A_2, A_3, D_1, and D_2. Sequences A_3 and D_2 differ from A_2 and D_1 by the use of lower concentration of 2ME, if only the five first steps are taken into consideration. When A_3 and D_2 are compared to their corresponding sequences A_2 and D_1, lower 2ME concentration results in a decrease of the amount of nitrogen extracted at steps 4 and 5a but does not alter the amount of total nitrogen isolated at steps 5a and 5b. Since all G_2-glutelins are extractable at pH 10, the exhaustivity of acidic extraction increases at a higher 2ME concentration.

Comparison of sequences D_1 and D_2 shows that reducing the ME concentration slightly increases the amount of proteins extracted at step 6. The same would hold for sequences A_2 and A_3 if phenol plus acetic acid plus water, without 2ME (sequence A_2), is as efficient as $NaDodSO_4$ with 0.6% 2ME. But it is probable that the extra nitrogen isolated at step 6 corresponds to nitrogen not released at step 4, since G_1-glutelins are soluble in $NaDodSO_4$ solution. If so, it would be confirmed that a high 2ME concentration in the extractant promotes selectivity.

Finally comparing the amounts of insoluble proteins in sequences A_0, A_2, and A_3, it can be noted that phenol plus acetic acid plus water, without 2ME (sequence A_2) is not a better extractant than $NaDodSO_4$.

Sequences D_3 and D_4. Sequence D_4 differs from D_3 by an additional extraction (step 3s) of nitrogen with aqueous 2-propanol including sodium acetate and by the use at step 4 of the extractant with sodium acetate also added. Including sodium acetate in the aqueous 2-propanol solution does not markedly increase zein extraction since total amounts of nitrogen isolated at steps 3 and 3s are the same in both sequences D_3 and D_4. But at step 4 in sequence D_4, the same solution of sodium acetate in aqueous 2-propanol, with 2ME added, extracts nearly twice the nitrogen solubilized by sequence D_3. This additional nitrogen extracted when sodium acetate is present seems to originate from G_2-glutelins. Indeed, only 1% of total nitrogen was extracted at step 5, sequence D_4.

Amino Acid Analysis. Amino acid compositions of some protein extracts, designated by extraction sequence and step, are given in Table IV. Glutelins in extracts $A_{2.5b}$ and $A_{3.5a}$ appear to be identical but differ from those of $A_{2.5b}$, whereas those in extracts $A_{3.5b}$ correspond to a mixture of proteins in extracts $A_{3.5a}$ and $A_{2.5b}$. There is a close similarity between total G_2-glutelin composition (extract $A_{0.5}$) and that of mixtures of acidic and alkaline extracts. These observations confirm that G_2-glutelins can be selectively fractionated and that the separation is dependent on 2ME concentration.

Extract $D_{4.3s}$, isolated by aqueous 2-propanol and sodium acetate, has an amino acid composition similar to that of zein. However, significant differences from zein in basic amino acids, aspartic acid or asparagine, glycine, leucine, and phenylalanine, suggest that extract $D_{4.3s}$ is a mixture

Table IV. Amino Acid Composition of Some Prolamin and Glutelin Fractions[a]

extracts[b]	$A_{2.5a}$	$A_{3.5a}$[c]	$A_{2.5b}$	$A_{3.5b}$	$(A_{2.5})$[d]	$(A_{3.5})$	$A_{0.5}$[e]	$D_{4.3S}$	$D_{4.4}$	$D_{4.5}$	$D_{4.6}$
Asx	11	12	55	35	25	25	22	58	24	82	100
Thr	47	47	46	44	46	45	46	37	37	36	49
Ser	46	46	65	59	52	53	52	64	50	62	68
Glx	179	180	200	202	186	193	198	201	195	262	144
Pro	249	246	127	173	209	204	195	109	178	72	60
Gly	74	76	88	88	78	83	78	43	77	100	88
Ala	57	60	75	65	63	63	69	128	104	69	100
Val	71	70	64	63	69	66	65	39	42	50	56
Met	5	3	8	8	6	6	8		23	11	15
Ile	17	21	32	25	22	24	22	28	17	24	36
Leu	94	92	86	83	91	87	92	161	124	56	91
Tyr	24	24	15	16	21	20	26	36	42	22	28
Phe	13	13	23	21	16	17	21	46	22	22	41
Lys	6	6	32	21	14	15	11	5	1	37	49
His	77	71	62	65	72	67	62	16	34	47	27
Arg	32	32	21	32	28	32	30	19	24	47	49

[a] Expressed as number of residues per 1000 residues. [b] Extracts are denoted by the sequence and the step. [c] Extracts underlined are obtained with lower concentration of 2ME. [d] The composition of extracts in parentheses corresponds to that calculated from a mixture of extracts isolated at pH 3 and then at pH 10. [e] From Landry and Moureaux (1970).

of proteins, some originating from germ (Landry and Moureaux, 1980).

Extract $D_{4.4}$, released by 2-propanol plus sodium acetate, contains 2 and 3 times as much arginine and histidine, respectively, as do G_1-glutelins (sequence D_0). Moreover, its composition is identical with that of the alcohol-soluble glutelins, isolated by Paulis and Wall (1977) with the same extractant, which contain a water-soluble fraction rich in histidine. The amino acid analyses, therefore, demonstrate that sodium acetate induces G_2-glutelin extraction at step 4.

The proteins of extract $D_{4.5}$, like those isolated at step 5 (extract $A_{0.5}$), are rich in histidine but have a composition different from those of total G_2-glutelins (extract $A_{0.5}$) or their fractions (extracts $A_{2.5a}$ and $A_{2.5b}$). The composition of extract $D_{4.6}$ agrees with that of G_3-glutelins (Landry and Moureaux, 1970).

Starch Gel Electrophoresis. Figure 1 shows electrophoretic patterns of salt-soluble proteins, zein, and glutelin subgroups. G_1-Glutelins have more fast-migrating components than does zein. G_2-Glutelins contain the two fastest components detected in glutelins. G_3-Glutelins exhibit tailing from the origin to the slowest G_2-glutelin band and have two bands similar to zein and one band between the fastest G_1-glutelin band and the slowest G_2-glutelin band.

G_1-Glutelin turbidity increased with extract age. The data of Figure 1, patterns 2 and 3, show that this turbidity is due to precipitation of certain proteins; a sensitivity of G_1-glutelins to precipitation was also observed by Melcher and Fraij (1980).

Figure 2 shows the electrophoretic patterns of glutelin subgroups isolated by different extraction procedures are those of zein and salt-soluble proteins (as well as of their subfractions). The slowest components are among the zeins, and the fastest are globulins. Patterns of zein or its fractions are similar, irrespective their native or reduced state, indicating that the electrophoretic separation depends mainly on polypeptide charge. To facilitate comparisons, we divided the set of diagrams in Figure 2 into three regions. Region I, of low mobility, has all bands of zein and its fractions. Region II, of intermediate mobility, ranges from the fastest zein band (excluded) to the fastest G_3-glutelin band (excluded). Region III consists of the fastest G_3-glutelin and all faster bands. G_1-Glutelins, reduced (TBP) and alkylated (PO) before their extraction from meal (sample 17), have numerous components ranging from the slowest band of region I to the fastest band of region II. When these glutelins are extracted from a TBP-treated meal and are then alkylated with PO (sample 15), they have a similar pattern, but the fastest band of region II is absent. Subsequent extraction by aqueous 2-propanol after treatment of the meal with PO releases the fastest glutelin band of region II and one of higher mobility (sample 14). G_2-Glutelins, whether reduced by 2ME (sample 19) or reduced by TBP and alkylated with PO (sample 13), consist of one band in region III. G_3-Glutelins exhibit tailing and have several bands with mobilities identical with that of the zein or G_1-glutelin band.

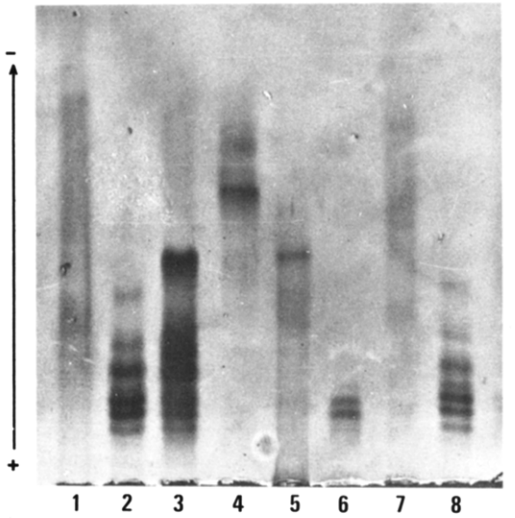

Figure 1. Starch gel electrophoresis (6 M urea, pH 3.5) of all the protein groups isolated by sequential extraction. Origin at the bottom. Samples: 1 = native salt-soluble proteins; 2 = G_1-glutelins, 7-day-old turbid solution; 3 = G_1-glutelins, 2-day-old solution, slightly turbid; 4 = G_2-glutelins, 2-day-old solution, slightly turbid; 5 = G_3-glutelins, 2-day-old solution; 6 = native zein; 7 = reduced (2ME) and alkylated (acrylonitrile) salt-soluble proteins (sample 1); 8 = alkylated (acrylonitrile) G_1-glutelins.

DISCUSSION

As previously reported (Landry and Moureaux, 1970; Sodek and Wilson, 1971; Misra et al., 1975; di Fonzo et al., 1977), G_1- and G_2-glutelins resemble zein and salt-soluble proteins, respectively, in amino acid composition and electrophoretic behaviors. Incomplete extraction of protein groups could explain this resemblance. However, the percolation extraction sequence (A_1), as compared to se-

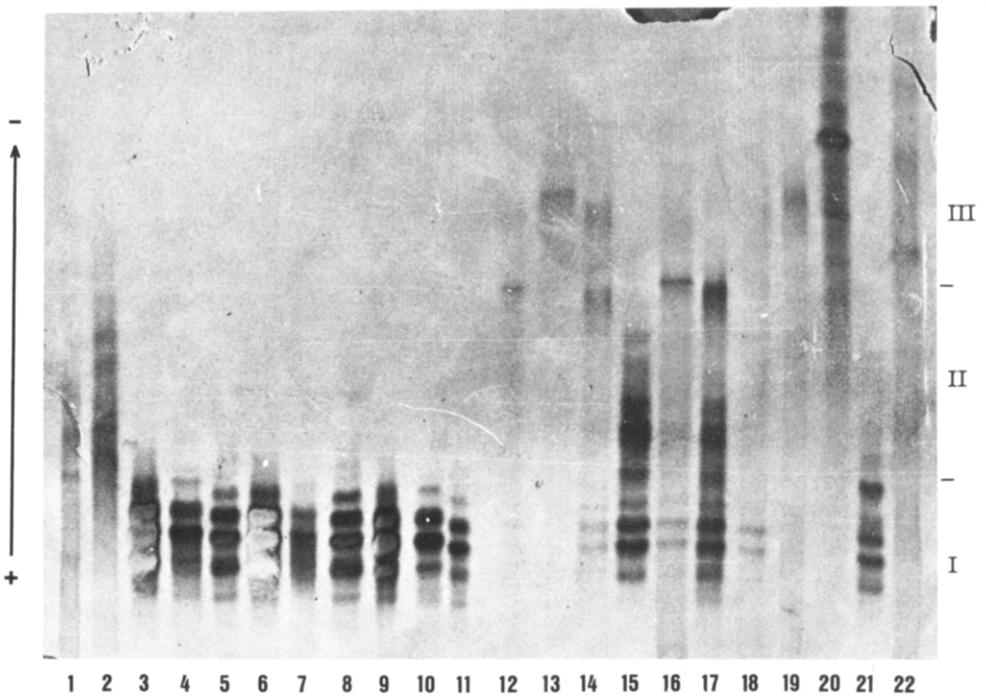

Figure 2. Starch gel electrophoresis (6 M urea, pH 3.5) of all the protein groups, isolated by sequential extraction, and of some subgroups. Origin at the bottom. Samples: 1 = reduced (2ME) albumins; 2 = reduced (2ME) globulins; 3 = reduced TBP and alkylated (PO) total zein; 4 = native total zein; 5 = reduced (2ME) total zein; 6 = zein: reduced (TBP) and alkylated (PO) fractiion D; 7 = zein, native fraction D; 8 = zein, reduced (2ME) fraction D; 9 = zein, reduced (TBP) and alkylated (PO) fraction M; 10 = zein, native fraction M; 11 = zein, reduced (2ME) fraction M; 12 = G_3-glutelins extracted after sample 13; 13 = G_2-glutelins extracted after sample 14; 14 = G_1-glutelins reduced (TBP), alkylated (PO), and extracted after sample 15; 15 = G_1-glutelins reduced (TBP), extracted, and then alkylated (PO); 16= G_3-glutelins extracted after sample 17 and G_2-glutelins; 17 = G_1-glutelins reduced (TBP)and alkylated (PO) before their extraction; 18 = reduced (2ME) G_2-glutelins; 19 = reduced (2ME) G_2-glutelins; 20 = reduced (2ME) and alkylated (acrylonitrile) globulins; 21 = reduced (2ME) G_1-glutelins; 22 = reduced (2ME) and alkylated (acrylonitrile) albumins.

quence A_0, shows that using larger volumes of solvent at each step will only slightly increase the nitrogen released by salt, alcohol, or salt with 2ME. The sequential extraction method, therefore, does not lead to artificial fractionation of glutelins, but its selectivity is dependent on the experimental conditions used. The fact that G_1- and G_2-glutelins can be solubilized in alcoholic media suggests that some operating conditions can promote the coextraction of these subgroups with zein. Before studying these possibilities and their consequence upon zein heterogeneity, we will review the extractability of glutelins and the properties of their subgroups, as indicated by the present and previous results.

Extractability of Glutelins. Many authors, as reviewed for maize by Wall and Paulis (1978), have suggested that interpolypeptide disulfide bonds make cereal glutelin poorly soluble, since glutelin extraction requires reducing and occasionally alkylating agents. Indeed, maize glutelins are rich in cysteine. Misra et al. (1976a) proved that fractions III–V correspond to the glutelin fractions defined by Paulis and Wall (1971), so, the number of cysteinyl residues in G_1-, G_2-, and G_3-glutelins can be estimated as 40, 45, and 25 per 1000, respectively. But, as shown here, 2ME cannot extract all glutelins. Thus, half of acid-insoluble G_2-glutelins, which are not extracted with 0.1% 2ME (sequences A_3 and D_2), are solubilized at pH 10 without any increase in 2ME concentration. Moreover, G_3-glutelins, having an amino acid composition similar to that of salt-soluble proteins (though richer than them in hydrophobic residues and with lower cysteine content than other glutelin subgroups), appear to be nonextractable by 2ME in a saline or alcoholic medium or by their combination.

The inability to extract G_3-glutelins with such media may be related to noncovalent interpolypeptide bonds. An aqueous solution of phenol and acetic acid (Synge, 1957) or of $NaDodSO_4$ may solubilize glutelins by cleaving some of these bonds. Complete solubilization and partial dissociation of aggregates would depend on the nature and concentration of components, when they do not contain reducing agent. Likewise, complete solubilization of aggregates, when the components of the medium are in low concentration, or their complete dissociation would depend on cleavage of intrapolypeptide bonds. Such a point of view is consistent with the observations of Kobrehel and Bushuk (1978), Wasik et al. (1979), and Huebner and Wall (1980) concerning the solubilization and the dissociation of wheat glutenin.

Properties of G_1-Glutelins. The total amount of maize proteins extracted by alcohol without salt (step 3 + 4) is virtually independent of experimental conditions (Table II). In contrast, the ratio of zein and G_1-glutelins varies as emphasized in the study of their accumulation in developing grain (Moureaux and Landry, 1972). The use of aqueous 2-propanol does not seems to lead to exhaustive extraction of zein; this is also suggested by the very slow electrophoretic bands in G_1-glutelins, which may be identical with or closely related to those of zein. These subunits could be termed zein-2 (if zein, as extracted by aqueous alcohols, is referred to as zein-1) to indicate the typical extractability. The fastest bands in G_1-glutelins, however, differ distinctly from zein in electrophoretic mobility at pH 3.5, in their high content of methionine, in molecular weight (Misra et al., 1976b; Gianazza et al., 1977; Paulis and Wall, 1977), and by their precipitation from alcohol solution at −20 °C (Melcher and Fraij, 1980). These high-mobility proteins might be called zein-like proteins to emphasize their similarity to zein-1 in amino acid composition. Thus the term G_1-glutelins, or fraction III, indicates the mixture of zein-2 and zein-like proteins si-

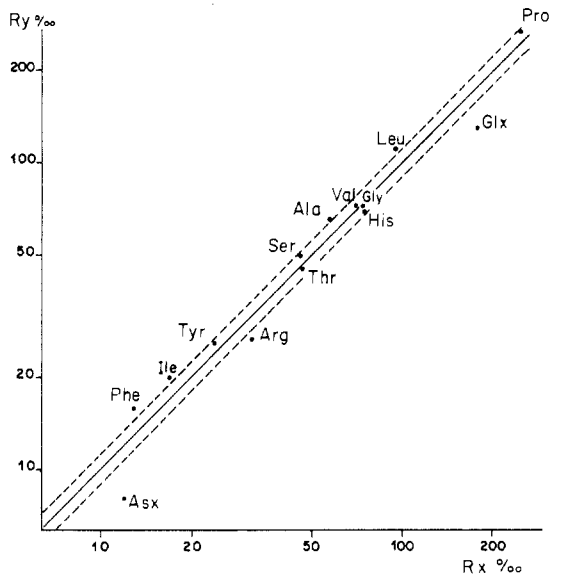

Figure 3. Amino acid composition of acid-soluble G_2-glutelins (x), as isolated from extract $A_{2.5a}$, and of the water-soluble fraction of alcohol-reduced glutelins (y), as isolated by Paulis and Wall (1977). Results are expressed in number of residues (R) per thousand and are plotted on a logarithmic scale. In case of composition identity the dots theoretically fall on the first bisector and are virtually located with the range delineated by dashed parallels representing the limits of relative errors (±10%).

multaneously extracted by aqueous alcohol in the presence of 2ME.

Properties of G_2-Glutelins. G_2-Glutelins, isolated at pH 10, can be fractionated on the basis of their extractability at pH 3.

Acid-soluble G_2-glutelins exhibit some general characteristics of cereal prolamins. They are rich in proline and in glutamic acid or glutamine and are poor in lysine and aspartic acid or asparagine. Moreover, they may be extracted both by acidic and alcoholic media. Indeed, as shown in Figure 3, amino acid compositions of acid-soluble G_2-glutelins and water-soluble alcohol-soluble glutelins (Paulis and Wall, 1977) are nearly identical. Consequently, the acid-insoluble G_2-glutelins are not removed by alcoholic extractants. Moreover, since they are not released at step 5 in sequences D_3 and D_4, they may only have been insolubilized as a result of the higher temperature initially used to extract salt-soluble proteins. Because of their extractability and amino acid composition, especially their relatively high lysine content, acid-insoluble G_2-glutelins may be regarded as being similar to G_3-glutelins. Their insolubility at low pH might also explain the apparent lability of the fastest G_2-glutelin band (Figure 1, sample 4), which is absent in G_2-glutelins in Figure 2 (samples 13 and 19).

Thus, fraction IV contains both acid-soluble and -insoluble G_2-glutelins, rich in histidine, which might be called prolamin-like and glutelin-like, respectively. Both terms are explicit enough to show that the amount of G_2-glutelins isolated at step 5 depends on conditions used earlier to extract salt-soluble and alcohol-soluble proteins. Nevertheless, the extraction conditions specified for steps 5a and 5b in sequences A_2 and D_1 constitute an additional step in the isolation of glutelin subgroups.

Properties of G_3-Glutelins. Starch gel electrophoresis pattern of G_3-glutelins at pH 3.5 consists of a few bands and tailing from the origin, suggesting the occurrence of ill-defined species. Indeed, Misra et al. (1976b) found no evidence for size separation of G_3-glutelins by NaDodSO$_4$–polyacrylamide gel electrophoresis. In contrast, di Fonzo et al. (1977) found that G_3-glutelins, as extracted from immature and mature grains, exhibit many bands upon NaDodSO$_4$–polyacrylamide gel electrophoresis, several of which have the same mobilities as albumin and globulin components. Such bands may reflect both nonexhaustive extraction of salt-soluble proteins and more complete dissociation of G_3-glutelin complexes.

The nature of G_3-glutelins can be surmised, however, from studies concerning their evolution in developing (Landry and Moureaux, 1976) and germinating (Moureaux, 1979) grain. G_3-Glutelins exist at the earliest stages of grain development, before zein accumulation, so they may consist of membrane proteins from cell organelles such as mitochondria or ribosomes. This hypothesis lends support to the existence of nonconvalent bonds in glutelins since hydrophobic interactions stabilize these membranes and their multimeric enzymes. There is also a close parallel between the decrease in the amount per grain of salt-soluble proteins and the increase of G_3-glutelins, observed during grain maturation. Likewise, the increase of salt-soluble proteins during the imbibition phase of germination is closely paralleled by the decrease of G_3-glutelins. Moreover, in vitro conversion of salt-soluble proteins into G_3-glutelins upon thawing of previously frozen grains (Moureaux and Landry, 1972) suggests noncovalent aggregation of salt-soluble proteins.

Therefore, G_3-glutelins include membrane-bound proteins (which may be assumed to be true glutelins), some naturally associated salt-soluble proteins, and some proteins altered during extraction.

A small amount of nitrogen remains insoluble after all these extraction procedures. This residue consists mainly of proteins from previously defined groups becoming insoluble due to interaction with lipids, carbohydrates, or polyphenols via oxidation processes.

G_1- and G_2-Glutelins as Possible Contaminants of Zein. The present data confirmed and extended the earlier observations that maize proteins extracted by alcoholic media include zein, G_1-glutelins, and acid-soluble G_2-glutelins. However, depending upon experimental conditions, alcohol extracts will contain these three protein fractions in variable proportions. For example, zein-2, as isolated from nondefatted endosperm (Sodek and Wilson, 1971), appears to be a mixture of G_1- and G_2-glutelins, since its amino acid composition is similar to that of extract $D_{4.4}$. In that study (Sodek and Wilson, 1971), the presence of lipids may have enhanced that extraction of G_2-glutelins by aqueous 2-propanol and 2ME without sodium acetate. Likewise, the very high histidine content detected by Gianazza et al. (1977) in the mixture of two main zein components extracted with aqeous 2-propanol and 2ME suggests a contamination of the sample by G_2-glutelins.

The presence of salt or the use of particular experimental conditions can also promote coextraction of G_1- and G_2-glutelins with zein by aqueous alcohol solutions where 2ME is absent. Thus zein, extracted by Bietz et al. (1979) with 0.5% sodium acetate in 63% ethanol at room temperature, also contains low molecular weight (12 600) subunits, characteristic of G_1-glutelins (zein-like proteins). These subunits are also present in proteins extracted with 63% ethanol at 60 °C (Lee et al., 1976). Moreover, one main component in this extract is rich in histidine, proline, and glycine, indicating the presence of acid-soluble G_2-glutelins.

Thus, these comparisons by NaDodSO$_4$ electrophoresis and amino acid analyses indicates that zein has a polymorphism due to the presence of variable amounts of G_1-glutelins (zein-2; zein-like) and G_2-glutelins (acid sol-

uble). With these considerations in mind, zein (zein-1) can be defined as protein material extracted at room temperature by an aqueous alcohol, free of reductant and salt, from a maize meal deprived of lipids and salt-soluble proteins. The absence of salt in the alcohol solution requires its complete removal by a prerequisite extraction step involving exhaustive meal washing with water, even though only minute amounts of nitrogen are solubilized.

Classification of Maize Proteins. The foregoing data demonstrate that maize grain proteins can be divided into two categories on the basis of their extractability in alcohol media. Thus zein and G_1- and acid-soluble G_2-glutelins are extracted by aqueous alcohols with reducing agent and salt whereas the other protein fractions are not extracted by such a medium. Such a differentiation resembles very closely that previously established (Landry and Moureaux, 1980) where, according to their location in grain, zein and G_1- and G_2-glutelins were grouped in the category of endosperm-specific proteins. As a first approximation, all these proteins can, therefore, be considered as extractable in alcohol medium and, consequently, as prolamins. However, such an extension introduces a useless and premature complexity, in the case of physicochemical study, since some subunits belonging to zein and G_1- and G_2-glutelins have similar molecular weights and isoelectric points (Gianazza et al., 1977; Misra et al., 1976b; Paulis and Wall, 1977; Soave et al., 1975). Such a view explains why, in previous studies of zein (Landry and Sallantin, 1978; Landry, 1979a), only zein-1 was taken into consideration.

Proteins of Other Cereal Grains. The use of the solvent sequence D described under Materials and Methods has permitted assessment of some characteristics of grain proteins from sorghum (Jambunathan and Mertz, 1973; Guiragossian et al., 1978; Paulis and Wall, 1979) and from pearl millet (Nwasike et al., 1979) by isolation of three glutelin subgroups analogous to those from maize. These investigations indicate that the classification for maize proteins is also true for proteins of sorghum and pearl millet.

For barley, however, the same solvent sequence proved unsuitable (Landry et al., 1972) since alcohol-soluble proteins are only partially extracted at steps 3 and 4, and since only minute amounts of nitrogen are released by salt with 2ME at step 5. It was found, however, that these problems can be reduced when the extractions at steps 3 and 4 are performed either at high temperature (60 °C) by aqueous alcohols or at room temperature by a mixture of aqueous alcohols and acetic acid and when the extraction at step 5 was carried out by acetic acid and 2ME. Thus, alcohol media, under suitable operating conditions, can extract barley proteins analogous to all alcohol-extracted proteins of maize. These proteins include α-, β-, γ-, δ-, and ϵ-hordeins (Waldschmidt-Leitz et al., 1961), hordein-like material (Laurière et al., 1976), and prolamin-like material. In this mixture, (δ+ϵ)-hordeins, like zein (zein-1), is the fraction poorest in basic amino acids, nonextractable by aqueous dilute acetic acid. The prolamin-like barley fraction, like maize acid-soluble G_2-glutelins, is the fraction richest in basic amino acid and is extracted by more complex medium and by dilute acids (Landry, 1979b; Landry et al., 1972). Since barley and wheat prolamins and certain glutelins have analogous physicochemical properties, it may also be possible to extend this classification to wheat grain proteins.

Therefore, it appears possible to isolate from any cereal (except rice) grains two main sets of proteins. One is extractable by an alcohol solution containing reducing agent and acid, and the other is not extracted by such a medium. The first set consists of prolamins, as defined by Osborne, and of other proteins more and less closely related to prolamins in physicochemical properties. Thus, all proteins in this set could be regarded as alcohol-soluble proteins. Their isolation in bulk by one medium may be the best way to assess the influence of agronomic factors on protein accumulation in grain and on its nutritional quality, since the alcohol-soluble proteins make up most of the storage proteins. However, the total set of alcohol-soluble proteins include many discrete proteins which, because of their similar solubilities or sizes, would be difficult to completely separate and characterize. Thus, more detailed studies must be carried out on less complex mixtures, isolated on the basis of differences in protein extractability by a sequence of several media.

The foregoing observations, therefore, show that the procedure of sequential extraction of maize glutelins, as described by Landry and Moureaux (1970), provides an appropriate background for the development of new fractionation methods, which may be more or less selective, according to the purpose for studying the cereal protein.

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Glycoalkaloids of Wild, Tuber-Bearing *Solanum* Species

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The total glycoalkaloid (TGA) contents and individual glycoalkaloid compositions of foliage from 16 wild, tuber-bearing *Solanum* species of potential use in potato breeding were determined by using a combination of analytical procedures. A wide range of TGA contents was found (0.2–39.0 mg/g dry weight). Most of the species had higher TGA levels than *Solanum tuberosum*, cv. Katahdin (2.6 mg/g dry weight). Great diversity in individual glycoalkaloid composition was found among the species. Most species contained only α-solanine and α-chaconine, but glycoalkaloids not usually associated with commercial cultivars were found in several species. Glycoalkaloids (probably solamargine and solasonine) not previously found in any tuber-bearing *Solanum* species were found in *Solanum berthaultii* (PI 265858). This report also provides glycoalkaloid data on several previously unanalyzed species. The data emphasize the need for careful evaluation of glycoalkaloids as a potato quality factor in breeding programs which exploit wild *Solanum* species.

The total glycoalkaloid (TGA) levels and individual glycoalkaloid compositions of current potato cultivars do not represent a toxicological or teratogenic hazard. However, the widespread exploitation of wild, tuber-bearing *Solanum* species in potato breeding programs suggests the need for caution in the future. Some species contain much higher TGA levels than are found in *Solanum tuberosum* (Schreiber, 1963; Osman et al., 1978). There is also widespread occurrence among these wild species of glycoalkaloids which are not commonly associated with commercial varieties [for a review, see Schreiber (1968)]. Since TGA levels and individual glycoalkaloid compositions are largely under genetic control (Sanford and Sinden, 1972; McCollum and Sinden, 1979), and glycoalkaloids have been associated with undesirable potato flavor (Sinden and Deahl, 1976), mammalian toxicity and teratogenicity [for a review, see Kuč (1975)], careful consideration of glycoalkaloid contents in the parental species is highly desirable. The need for this was illustrated in the late 1960s when a Gr. Tuberosum cultivar Lenape, which had *S. chacoense* in its ancestry (Akeley et al., 1968), was withdrawn from commerce because of its high TGA content (Zitnak and Johnston, 1970; Burton, 1974). This problem might have been avoided if the TGA content of *S. chacoense*, which is very high (Schreiber, 1963; Tingey et al., 1978), had been considered. Most of the current information on the glycoalkaloid contents of wild potato species was published prior to 1965. Since then, improved methodology has facilitated more sensitive, accurate analyses which have already led to new discoveries. Whereas commercial varieties of *S. tuberosum* were thought to contain only the solanidine glycoalkaloids α-chaconine and α-solanine, Shih and Kuč (1974) demonstrated the presence of the tomatidenol glycoalkaloids α- and β-solamarine in leaves and incubated slices of the cultivar Kennebec. In addition, Osman et al. (1976) reported the presence of a new glycoalkaloid, commersonine, in certain lines of *S. chacoense* and *S. commersonii*.

We have analyzed 16 wild, tuber-bearing *Solanum* species for TGA and individual glycoalkaloid composition, using a combination of highly sensitive, accurate techniques. This report provides an update on previous literature, some of which may be misleading, and also presents glycoalkaloid data on several species of potential use in breeding which were previously unanalyzed.

MATERIALS AND METHODS

Germ Plasm. The wild, tuber-bearing *Solanum* accessions were obtained from the Potato Introduction Station, Sturgeon Bay, WI. The materials were grown at Freeville, NY, and treated in the same manner as previously described (Raman et al., 1979).

Total Glycoalkaloid Content. Freeze-dried leaf powders (0.2–1 g) were homogenized in 5% acetic acid (~40 mL/g of powder) with a Polytron homogenizer operating at full speed for 90 s. Extracts were filtered and the filter was washed twice with 5-mL aliquots of the extraction medium. For determination of the contents of ammonia-precipitable glycoalkaloids in extracts, aliquots (100–500 mg of powder) were precipitated by adding

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