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Comparative Efficiencies of Isopropyl and *tert*-Butyl Alcohols for Extracting Zeins from Maize Endosperm

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Protein of endosperm of maize grains originating from three wild-type inbreds and their *opaque-2* versions were solubilized in diverse extracts (E) by the sequential use of 0.5 M NaCl, water ($E_{1,2}$), alcohol plus a reducing agent (E_3), and salt plus a reducing agent (E_4). Zeins were isolated in extracts E_3 and E_4 obtained by using 55% (w/w) isopropyl alcohol (*i*-PrOH) + 0.2% dithiothreitol (DTT) followed by 0.5 M NaCl + 0.2% DTT buffered at pH 10 or 60% *tert*-butyl alcohol (*t*-BuOH) + 0.2% DTT followed by 0.5% sodium acetate + 0.2% DTT in 30% *t*-BuOH. For a given genotype the percentage of extracted zeins was independent of the nature of the alcohol. The latter had a slight effect on the respective magnitude of E_3 and E_4: E_3 increased at the expense of E_4 when *t*-BuOH was substituted to *i*-PrOH for their isolation. The percentage of the total endosperm nitrogen present in $E_3 + E_4$ was identical to that of fractions $F_{II} + F_{III} + F_{IV}$ isolated according to the classical Landry−Moureaux extraction procedure. SDS-PAGE analysis revealed the presence of all types of zeins (α, β, γ, and δ) in E_3 and F_{III}, residual zeins in E_4 isolated with *t*-BuOH, and streaking only in E_4 and F_{IV} isolated with NaCl at pH 10. The data together with those of the literature were discussed with regard to the influence of procedure on the yield of zeins using alcoholic extraction.

KEYWORDS: Maize; endosperm; proteins; zeins; alcoholic extractants

INTRODUCTION

Zeins, the storage proteins of maize endosperms, are now considered as a set of diverse subunits, referred to as α-, β-, γ-, and δ-zeins (*1*). The classical procedure for their isolation and quantitation consists of extracting them with aqueous ethyl or isopropyl alcohol in the absence and then in the presence of a reducing agent and, eventually, other additives such as sodium acetate (*2*−*4*, *23*, *24*). Aqueous *tert*-butanol (*t*-BuOH) was occasionally employed in the study of zeins. Thus, it was used to better extract and quantitate α-zeins from maize grains (*4*) or to recover them as freeze-dried powder (*5*, *6*). In contrast, aqueous *t*-BuOH has been widely used to extract the sorghum prolamins, kafirins, since Jones and Beckwith (*7*) found that 60% (v/v) *t*-BuOH at room temperature was as efficient as 70% ethyl alcohol at 60 °C in extracting these proteins. Furthermore, Taylor et al. (*8*) have shown that 60% *t*-BuOH + 0.05% (w/v) dithiothreitol (DTT) was a better extractant of kafirins compared to 70% (v/v) isopropyl alcohol (*i*-PrOH) without and then with 0.6% 2-mercaptoethanol (2ME).

The present study was performed to determine whether, and to what extent, the use of *t*-BuOH in lieu of *i*-PrOH was able to improve the extraction of zeins from the endosperm of maize grains.

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MATERIALS AND METHODS

Whole maize grains used for endosperm isolation were from the wild (+) and *opaque-2* versions of three inbred lines: W64A, Oh43, and F2. Sample origin, endosperm isolation by hand-dissection, and defatting of meals with cold hexane were described previously (*9*).

Extraction of Proteins. The overall procedure for extracting protein from duplicate 100-mg samples was previously described (*9*). It involved the successive use off (1) 0.5 M NaCl, (2) water at 4 °C (the combined extracts were referred to as $E_{1,2}$), (3) alcohol plus reducing agent (E_3), and (4) salt plus reducing agent (E_4), leaving a residue referred to as $E_{5,6}$ because it was a mixture of proteins customarily extracted in the presence of detergent (E_5) and insoluble proteins (E_6 by extension).

Two sequences of extractants, differing in the nature of solvents used at steps 3 and 4, were employed. The first, utilized as reference, required the use of 55% (w/w) *i*-PrOH + 0.2% (w/v) DTT followed by 0.5 M NaCl + 0.2% DTT buffered at pH 10; the second used 60% (v/v) *t*-BuOH + 0.2% DTT followed by 0.5% sodium acetate (w/v) + 0.2% DTT in 30% (v/v) *t*-BuOH. The latter extractant was selected by taking into account the extractability properties of γ-zeins. These proteins can be isolated in aqueous or alcoholic medium in the presence of salt and reducing agent (*6*).

The classical procedure of Landry and Moureaux (*2*) was also applied for isolating fractions F_{II} (*i*-PrOH without DTT), F_{III} (*i*-PrOH + DTT), and F_{IV} (NaCl + DTT). The only alteration in the extractants was the substitution of 0.6% 2-mercaptoethanol by 0.2% DTT.

The operating conditions regarding extract isolation were performed as previously described (*9*). The only modification concerned the isolation of E_4 after only a 15 min extraction, considering the few proteins solubilized at this step.

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Table 1. Influence of the Nature of Alcohol on the Extraction of Zeins

	$E_{1,2}$	E_3	E_4	$E_3 + E_4$	$E_{5,6}$
W64A+					
i-PrOH[b]	5.30 (0.28)[d]	71.2 (1.50)	2.80 (0.60)	74.00 (2.10)	20.70 (1.80)
t-BuOH[c]	5.60 (0.14)	73.3 (0.35)	1.30 (0.40)	74.60 (0.70)	19.80 (0.07)
Oh43+					
i-PrOH	5.85 (0.64)	72.0 (1.40)	3.05 (I.06)	75.05 (0.35)	19.15 (0.92)
t-BuOH	5.75 (0.64)	0.85 (0.70)	0.85 (0.70)	76.35 (1.20)	17.85 (1.77)
F2+					
i-PrOH	5.90 (1.40)	72.20 (0.85)	3.20 (1.00)	75.40 (0.14)	18.75 (0.71)
t-BuOH	6.35 (0.49)	75.65 (1.48)	1.15 (0.71)	76.80 (1.40)	18.75 (0.71)
mean +					
t-PrOH		71.80 (1.10)	3.00 (0.70)	74.80 (1.20)	
t-BuOH		74.80 (1.30)	1.10 (0.28)	75.60 (1.20)	
W64A*o2*					
t-PrOH	20.20 (0.90)	38.60 (0.28)	4.80 (0.014)	43.40 (0.42)	35.90 (0.28)
t-BuOH	21.20 (0.28)	40.60 (1.60)	1.50 (0.50)	42.10 (1.10)	36.70 (1.34)
Oh43*o2*					
t-PrOH	17.25 (0.71)	44.40 (0.28)	4.35 (1.34)	48.75 (1.62)	33.95 (1.62)
t-BuOH	16.10 (0.14)	46.75 (1.50)	1.40 (0.00)	49.15 (0.71)	34.75 (0.21)
F2*o2*					
t-PrOH	18.75 (0.92)	34.75 (0.50)	5.05 (1.06)	39.90 (0.71)	41.45 (1.48)
t-BuOH	19.15 (0.78)	39.45 (1.48)	1.40 (0.14)	40.85 (1.34)	40.00 (2.10)
mean *o2*					
t-PrOH		39.20 (0.43)	4.70 (0.08)	44.00 (0.41)	
t-BuOH		42.40 (0.39)	1.43 (0.23)	44.03 (0.45)	

[a] Percent of the total protein recovered. [b] *i*-PrOH: 55% 2-propanol + 0.2% DTT (E_3) then 0.5 M NaCl + 0.2% DTT buffered at pH 10 (E_4). [c] *t*-BuOH: 60% *tert*-butanol + 0.2% DTT (E_3) then 0.5% NaOAc + 0.2% DTT in 30% *tert*-butanol (E_4). [d] Mean and standard deviation from duplicate extractions.

Nitrogen Quantitation. Proteins of extracts were quantitated by ninhydrin assay of α-amino nitrogen released after hydrolysis, using an equimolecular mixture of 17 amino acids and ammonium chloride (Pierce) for calibration and a conversion factor of 1.06 μg of protein for 10 nmol of amino acids. Extracts $E_{1,2}$ and E_4 were hydrolyzed in the presence of 3 M NaOH at 130 °C for 45 min according to the method of Landry and Delhaye (*10*). Alcoholic extracts (E_3, F_{II}, and F_{III}), after removal of alcohol, and $E_{5,6}$ were hydrolyzed in the presence of 1 mL of constant-boiling HCl at 115 °C for 18 h.

Electrophoretic Analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*11*) using precast 4−20% polyacrylamide gradient gels.

RESULTS

Protein Extraction. In a recent study (*9*) zeins were considered to be present in extracts E_3 and E_4, isolated from maize endosperm deprived of lipids and salt-soluble nitrogen by using reducing agent (0.2% DTT) with 55% *i*-PrOH and then 0.5 M NaCl buffered at pH 10.

Table 1 summarizes the protein distribution in endosperm of three maize inbreds and their *opaque-2* versions using both sequences, allowing salt-soluble nitrogen ($E_{1,2}$), zeins ($E_3 + E_4$), and residual proteins (crude glutelins as $E_{5,6}$) to be isolated. For a given genotype the percentage of extracted zeins was seen to be independent of the nature of the alcohol. The latter had a slight effect on the respective magnitudes of E_3 ($P < 0.01$) and E_4 ($P < 0.001$). Thus, E_3 increased at the expense of E_4 when *t*-BuOH butanol was substituted for *i*-PrOH.

Table 2 shows the protein distribution in the endosperm of W64A+ inbred using the classical Landry−Moureaux extraction scheme with operating conditions identical to those above-mentioned for the isolation and quantitation of extracts. The sum ($F_{II} + F_{III} + F_{IV}$) was identical to that of $E_3 + E_4$. These data were compared with those reported for endosperm samples of the same genotype by three teams using a similar but not identical extraction scheme. The yields of F_{II}, F_{III}, and F_{IV} appeared to be dependent on extraction conditions, whereas their sum ($F_{II} + F_{III} + F_{IV}$) was fairly constant as a first approximation.

Table 2. Protein Distribution in Endosperm of W64A+ Maize Using the Classical Procedure of Landry and Moureaux (*2*)[a]

fraction	present study	Soave et al. (*21*)	DiFonzo et al. (*xx*)	Paiva et al. (*22*)	mean[b]
F_I	5.6	6.7	6.5	5.7	6.3 ± 0.5
F_{II}	52.0	39.9	50.7	54.2	48.3 ± 7.5
F_{III}	15.8	29.0	10.6	6.5	15.4 ± 12.0
$F_{II} + F_{III}$	67.8	68.9	61.3	60.7	64.7 ± 4.3
F_{IV}	6.3	6.4	8.8	8.3	7.8 ± 1.3
$F_{II} + F_{III} + F_{IV}$	74.1	75.3	70.1	69.0	71.5 ± 3.3
residue	20.4	18.0	23.4	25.3	22.2 ± 3.8
protein (%)	13.1	11.7	nd[c]	13.3	

[a] Percent of recovered protein. [b] Not including present study (*21*, *22*, *25*). [c] Not determined.

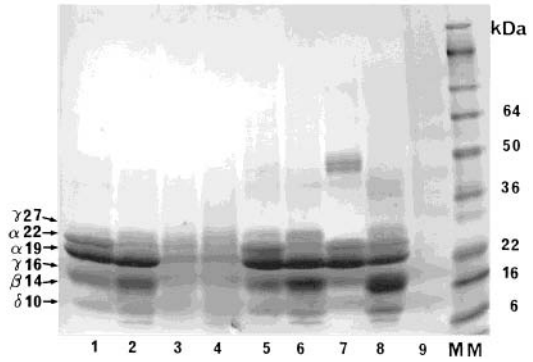

Figure 1. SDS-PAGE of proteins extracted from W64A+ and W64A*o2* endosperms in the presence of *t*-BuOH [lane 1, E_3 (+); lane 2, E_3 (*o2*); lane 3, E_4 (+); lane 4, E_4 (*o2*)], i-PrOH [lane 5, E_3 (+); lane 6, E_3 (*o2*); lane 7, F_{II} (+); lane 8, F_{III} (+)], and salt + reducing agent [lane 9, F_{IV} (+)]. Positions and molecular masses (MM) of protein standards are indicated on the right. Positions of α-, β-, γ-, and δ-zeins are shown on the left.

Electrophoretic Analysis. Proteins extracted in the presence of alcohol and (or) salt plus reducing agent were further characterized by SDS-PAGE analyses (**Figure 1**). Proteins of F_{II}, as isolated with *i*-PrOH in the absence of reducing agent, were made up of two major polypeptides corresponding to α22- and α19-zeins, mainly together with their dimers and a polypeptide corresponding to γ16-zein. All of the extracts isolated with alcohol plus reducing agent in the absence of salt (E_3 and F_{III}) contained polypeptides corresponding to the diverse types of zeins. This was clear with lower amounts of proteins loaded onto the gel (data not shown). The E_4 extracts isolated in the presence of 30% *t*-BuOH and sodium acetate were found to be composed of zeins only: faint bands corresponding to γ27-, α22-, and γ16-zeins and a minor band corresponding to one component of α19-zeins were detected. The electrophoretic pattern of F_{IV} (**Figure 1**) as well as that of E_4 isolated under the same conditions after *i*-PrOH extraction (data not shown) revealed streaking only, but a band corresponding to γ27-zein was detected when extract was concentrated in the presence of dimethyl sulfoxide prior to electrophoresis (data not shown). These observations provided further evidence that proteins constituting E_{IV} extract or F_{IV} fraction are zeins.

DISCUSSION

The results reported here afforded a further insight into the extractibility and the quantitative importance of the diverse zeins

Table 3. Protein Distribution in Wild-Type Endosperm and in Opaque Portion of Two Modified Endosperms According to the Classical Landry–Moureaux Procedure

	N-3 × M-8		M-5 × M-6
	whole endosperm	opaque portion	opaque portion
fraction	+	modified	modified
F_I	5.0	17.0	17.0
$F_{II} + F_{III}$	66.7	24.9	26.2
F_{IV}	9.4	15.5	22.7
$F_{II} + F_{III} + F_{IV}$	76.1	40.4	48.9
residue	18.9	40.7	34.1

[a] From data of Ribeiral (*14*).

present in maize endosperm, especially γ-zeins. First it is worth recalling some features of γ-zeins. These proteins are (i) extracted in the absence or in the presence of alcohol providing that the extractant contains a reducing agent and some salt; (ii) present to more and less significant degrees in the alcoholic extract depending on whether the medium contains added salt or residual from saline extraction; (iii) made up of two subunits rich in histidine of M_r 27000 and 16000 Da, which allow them to be identified from amino acid analysis of extracts isolated using diverse procedures; and (iv) present almost exclusively in fraction F_{IV} (G_2-glutelins) isolated with 0.5 M NaCl + 0.6% 2ME buffered at pH 10 when the Landry−Moureaux extraction scheme (*2*) is rigorously followed; otherwise, some of them are present in F_{III} as evidenced by SDS-PAGE and amino acid analysis. They can be traced in zeins Z_2 of Sodek and Wilson (*3*), in alcohol-soluble reduced glutelins as water-soluble alcohol-soluble reduced glutelins (*12*), and as reduced salt-soluble proteins (*13*).

From the foregoing it is interesting to compare the protein distributions stated above with those of three endosperm samples determined by Ribeiral (*14*) using the original Landry−Moureaux (*2*) procedure for protein extraction and the Kjeldhal method for protein quantitation (**Table 3**). The three samples selected among eight corresponded to the opaque portion of the two modified endosperm *opaque-2* selections (referred to as N-3 × M-8 and M-5 × M-6) and the whole wild endosperm obtained by outcrossing the modified endosperm N-3 × M-8 to a wild pollen source. There is a remarkable identity between the percentages relative to the sum of $E_3 + E_4$ and those relative to the sum $F_{II} + F_{III} + F_{IV}$ when each endosperm of the three wild-type inbreds is compared to the whole endosperm N-3 × M-8, and the endosperm of F2*o2* and Oh43*o2* is compared to the opaque portion of modified endosperm N-3 × M-8 and M-5 × M-6, respectively. This identity was an additional piece of evidence that fraction F_{IV} must be considered as an integral part of alcohol-soluble proteins. It is worth recalling that the kinetics of the relative accumulation of F_{IV} (G_2-glutelins) in developing grain has led Landry and Moureaux, as long ago as 1976, to put it together with F_{II} (zein) and F_{III} (G_1-glutelins) under the term of endosperm-specific proteins.

These observations led to the following conclusions:

(i) The extractability of zeins is not only related to their extent of aggregation, it may be assumed to be also dependent on interactions between polypeptide chains and other components of the endosperm, such as starch. The efficiency of *t*-BuOH would be correlated to a greater power of disrupting these bonds due to a greater swelling of starch or other. The near absence of streakings in the electrophoretic pattern of E_4 proteins when isolated in the presence of *t*-BuOH indicated a better individualization of polypeptides.

(ii) Fraction F_{IV}, when expressed as a percentage of the sum of $F_{II} + F_{III} + F_{IV}$, amounted to 9% (**Table 2**) to 23% (**Table 3**). More generally, it averaged 11 and 13% for 5 (*15*) and 10 (*16*) wild-type inbreds, respectively, and 25% for 5 *o2* inbreds (*15*). Neglecting fraction F_{IV}, as did all workers who used the classical Landry−Moureaux procedure, led zeins to be underestimated by the same percentage (**Table 2**).

On the other hand, γ-zeins, which constitute the bulk of F_{IV} proteins, were quantitated after their isolation using reversed-phase high-performance liquid chromatography of alcohol-soluble proteins. Thus, respective amounts of 12 and 34.5% of the total zeins, on average, were reported by Paulis et al. (*17*) for endosperms of two wild-type inbreds and their *o2* versions. More generally, Dombritz-Kurtzman and Bietz (*18*) found percentages, with respect to solubilized zeins, ranging from 11.8 to 21.1% with a mean of 17.6 ± 3.5% for the hard endosperm of nine inbred, hybrid, and modified maizes, and from 15.4 to 53.6% with a mean of 31.8 ± 10.6% for the soft counterpart. According to data from Ribeiral (*14*) F_{IV} expressed as a percentage of $F_{II} + F_{III} + F_{IV}$ present in the endosperm of hybrids N-3 × M-8 and M-5 × M-4, respectively, amounted to 13.4 and 19.2% for the hard portion and 38.3 and 46.4% for the soft portion. These comparisons suggested that the $F_{IV}/(F_{II} + F_{III} + F_{IV})$ ratio provided a good insight into the extent of γ-zeins with respect to total zeins. They pointed out that aqueous ethyl or isopropyl alcohol without and then with a reducing agent was unable to extract all of the zeins exhaustively. This was true also when alcoholic extractant contained sodium acetate. Thus, Wall et al. (*19*) solubilized 69.8% of the total nitrogen present in the endosperm of W64A inbred (15.3% of protein) with 70% ethanol + 0.5% sodium acetate without and then with 2% mercaptoethanol, whereas Landry et al. (*6*) extracted 81.6% of the total protein of the hard endosperm of W64A+ (13.6% as protein content) by the successive use of 90% *i*-PrOH and 55% (w/w) *i*-PrOH without and then with 1.2% (v/v) 2ME without and then with 0.5% sodium acetate.

In fact, the lysine content (0.9%) of F_{IV} proteins isolated from the endosperm of a commercial hybrid by Landry and Moureaux (*20*) suggested that lysine-free γ-zeins were contaminated by lysine-rich proteins accounting for 2−3% of the total endosperm proteins. Therefore, integrating all of the proteins of F_{IV} into zeins led these proteins as well as γ-zeins to be overestimated. However, the histidine content (4%) of insoluble proteins of the same hybrid suggested that they contained γ-zeins accounting for ∼2% of the total proteins. In other words, the overestimation of zeins, due to the lack of selectivity as for the isolation of F_{IV}, was canceled out by an underestimation of the same extent, due to the lack of exhaustivity in the extraction of γ-zeins. The use of *t*-BuOH as alcoholic extractant allowed a more exhaustive extraction of zeins and, consequently, a better quantitation of these proteins.

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