Note on the Effect of Reducing Agent on Zein Preparation[1]

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ABSTRACT

Zein was extracted from ground endosperms or whole kernels with hot 70% ethanol or isopropanol. However, zein prepared with ethanol or isopropanol contained numerous aggregates on sodium dodecyl sulfate (SDS)-gels unless 2-mercaptoethanol was present during the process. Also, to facilitate maximum zein extraction from the ground samples, the presence of at least 1 m*M* of 2-mercaptoethanol in the alcohol extraction medium was required.

Zein prepared from the mature endosperm of maize inbred W64A has previously been shown to contain two principal components, Z1 (21,800 daltons) and Z2 (19,000 daltons). A mutation at the *opaque*-2 (*o2*) locus suppresses the synthesis of Z1 (Gianazza et al 1976, Jones et al 1977, Lee et al 1976, Misra et al 1976b, Soave et al 1976). Developmental studies of zein accumulation indicate that zein synthesis in the normal inbred starts at 12 days after pollination and continues until about 50 days after pollination. In the *o2* mutant, on the other hand, zein accumulation is not apparent until 16 days after pollination and fails to increase beyond 35 days (Tsai and Dalby 1974).

A rapid method for determining zein content from large numbers of samples was developed by Dalby (1974). This method involves the direct extraction of zein from ground endosperms or whole kernels with 70% ethanol at 60°C for 90 min and the transfer of an aliquot to a filter paper disk. Disks are washed in bulk in (serially) saline, water, ether, acetone, and ether to remove nonzein impurities, and zein content is measured colorimetrically by the method of Jones et al (1975). In order to evaluate the effect of reducing agent on the quantitative extraction of zein, varying concentrations of 2-mercaptoethanol were mixed with the 70% ethanol, 70% isopropanol, or 55% isopropanol for extracting zein according to the procedure of Dalby (1974).

MATERIALS AND METHODS

Preparation of Samples

Maize inbred, W64A, and its homozygous *o2* and *floury*-2 (*fl2*) mutants were grown during 1976 on adjoining plots at the Purdue University Agronomy Farm, West Lafayette, IN. All plants were control self-pollinated. Ear samples were harvested at 12, 16, 22, 28, and 35 days after pollination and at maturity. At each developing stage (with the exception of the mature stage), whole ears were frozen in liquid nitrogen immediately after harvest. Intact kernels were removed from cobs, bulked (at least 20 ears for each stage), and stored at −80°C.

For processing, the embryo was dissected and the endosperms were lyophilized to a constant weight. After lyophilization, samples of endosperms (approximately 20 g) were ground in a Waring Blendor for about 30 sec. A 1-g sample was then powdered in a miniature ball mill (Wig-L-Bug, Crescent Dental Mfg. Co., Chicago, IL) and defatted for 48 hr with *n*-hexane in a Soxhlet apparatus. This powder was the starting material for the extraction of zein and glutelin.

Preparation of Zein and Glutelin

Albumin and globulin were removed from defatted endosperm samples according to a procedure previously described (Tsai 1979). Each 100-mg sample was shaken with 0.5 ml of 5% NaCl in a heavy duty centrifuge table for 30 min at 4°C and centrifuged at 18,000 *g* for 5 min. The washing step was repeated twice. The supernatant containing albumin and globulin was discarded. The residue remaining from the NaCl treatment was suspended in 1 ml of water and centrifuged to lower the salt concentration. The supernatant was discarded and the residue was shaken for 30 min at 60°C with 0.5 ml of 95% ethanol (final ethanol concentration about 70%) containing 0–100 m*M* of 2-mercaptoethanol. After centrifuging at 18,800 *g* for 5 min, the pellet was shaken again with 0.5 ml of 70% ethanol extraction medium for 60 min at 60°C. After incubation, the suspension was centrifuged at 18,800 *g* for 5 min to recover the supernatant. The respective ethanol extracts were combined and regarded as zein.

The residue remaining from the ethanol extraction was resuspended in 1 ml of water and centrifuged. The supernatant was discarded, and the pellet was extracted twice by shaking with 0.5 ml of 0.1*N* NaOH for 30 min at 45°C. After each extraction, the suspension was centrifuged at 18,800 *g* to recover the supernatant. The combined alkali extract was referred to as glutelin. Protein in these fractions was determined colorimetrically by the method of Lowry et al (1951), with bovine serum albumin used as a standard.

SDS-Polyacrylamide Gel Electrophoresis

Extracted zein and glutelin were dialyzed overnight at room temperature against 0.5% SDS with or without 0.1% 2-mercaptoethanol and then characterized on polyacrylamide slab gels as previously described (Tsai et al 1978).

RESULTS AND DISCUSSION

When zein from developing normal 12-day endosperms was extracted with hot 70% ethanol containing 1m*M* 2-mercaptoethanol and dialyzed in 0.5% SDS plus 0.1% 2-mercaptoethanol, both Z1 and Z2 were present (Fig. 1A). The ratio Z1/Z2 appeared to remain constant throughout endosperm development (Figs. 1A-F). However, a Coomassie Blue staining showed that the Z2 component was completely absent in the *o2* endosperm at 12 days although a small amount of protein was found migrating to the position corresponding to Z1 (Fig. 1G). At 16 days after pollination, both Z1 and Z2 were readily identifiable in the mutant, with the Z1 component being suppressed (Fig. 1H). Thereafter, the ratio Z1/Z2 appeared to remain constant.

The component Z (29,000 daltons), which is larger than Z1, was observed in the developing endosperms of both genotypes (Fig. 1). This component was present in the greatest amount at early stages of endosperm development and declined as the endosperm developed; only trace amounts were detected at maturity. However, this component could be detected only if zein was extracted and subsequently dialyzed in the presence of 2-mercaptoethanol (Fig. 2A).

Zein patterns on SDS-gels may vary depending on whether or not the extraction medium and dialysis buffer contain reducing agent. When zein was extracted from the mature normal endosperm with hot 70% ethanol plus 1 m*M* 2-mercaptoethanol and subsequently dialyzed against SDS buffer containing mercaptoethanol, seven components, ie, Z, Z1, Z2, Z3, Z4, Z5, and Z6, were observed (Figs. 2A and 3B). However, if zein was prepared from this endosperm sample in a similar manner without 2-mercaptoethanol, all minor components, ie, Z, Z3, Z4, Z5, and Z6

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disappeared. Concomitantly, at least 19 bands with high molecular weights were identifiable on the gel (Figs. 2B and 3A). A similar observation was made for *o2* (Figs. 3E and F) and another "high-lysine" mutant, *fl2* (Figs. 3I and J). Unlike *o2*, the *fl2* mutant appeared to suppress the synthesis of all zein components in the same relative proportion (Lee et al 1976, Soave et al 1978); however, in addition to these changes, a new minor component, Z1a (24,000 daltons) was induced. The Z1a component was not affected by the reducing agent.

The large molecular weight components appear to represent a different degree of zein aggregation when prepared in the absence of 2-mercaptoethanol. Furthermore, the formation of aggregates can be reversed by dialysis in the presence of 2-mercaptoethanol. Whether or not zein was extracted with 2-mercaptoethanol, dialysis in SDS buffer containing 2-mercaptoethanol prevented aggregation of zein (Figs. 2A and D). On the other hand, the aggregates were present when the extracted zein (extracted with or without the reducing agent) was dialyzed without this reducing agent (Figs. 2B and C). If this preparation was dialyzed again with 2-mercaptoethanol, the aggregates were eliminated (data not shown). The formation of aggregates appeared to be at the expense of all zein components (Figs. 2 and 3). These results agree with previous observations that zein exposed to reducing agents during processing lacks the large molecular weight components as revealed by Sephadex Chromatography (Landry 1965) and agar and starch gel electrophoresis (Turner et al 1965).

The aggregation of zein components is interesting but not surprising. Sequential extraction of endosperm sample with alcoholic solvent and alcohol plus 2-mercaptoethanol yield two zein fractions, $zein_1$ and $zein_2$. Various designations have been given to this alcohol-soluble reduced zein protein: glutelin-1 (Landry and Moureaux 1970), alcohol-soluble glutelin (Paulis and Wall 1971), and $zein_2$ (Sodek and Wilson 1971). $Zein_2$ contains all of the components of $zein_1$ (Z1 and Z2), but in addition it contains the lower molecular weight zein components (Gianazza et al 1976, Paulis and Wall 1977). Amino acid analysis indicates that both zein fractions are similar, although $zein_2$ is rich in sulfur amino acids (Landry and Moureaux 1970, Melcher 1979, Misra et al 1976a, Paulis and Wall 1971, Sodek and Wilson 1971). Because extraction of zein with 70% ethanol plus 1 m*M* mercaptoethanol permits the solubilization of both zein fractions, dialysis without 2-mercaptoethanol (Fig. 2B) conceivably may facilitate the aggregation of $zein_2$ polypeptides that contain cysteine residues. However, preparation of zein (extraction and dialysis) without 2-mercaptoethanol also exhibited a similar pattern of aggregation on SDS gels (Fig. 2C), indicating that a small amount of $zein_2$ fraction was extracted in the absence of this reducing agent. Dialysis of the zein extracted with 70% ethanol with 2-mercaptoethanol revealed the presence of lower molecular weight zein components on SDS gels (Fig. 2D).

Dalby's method (1974) facilitates zein determination for large

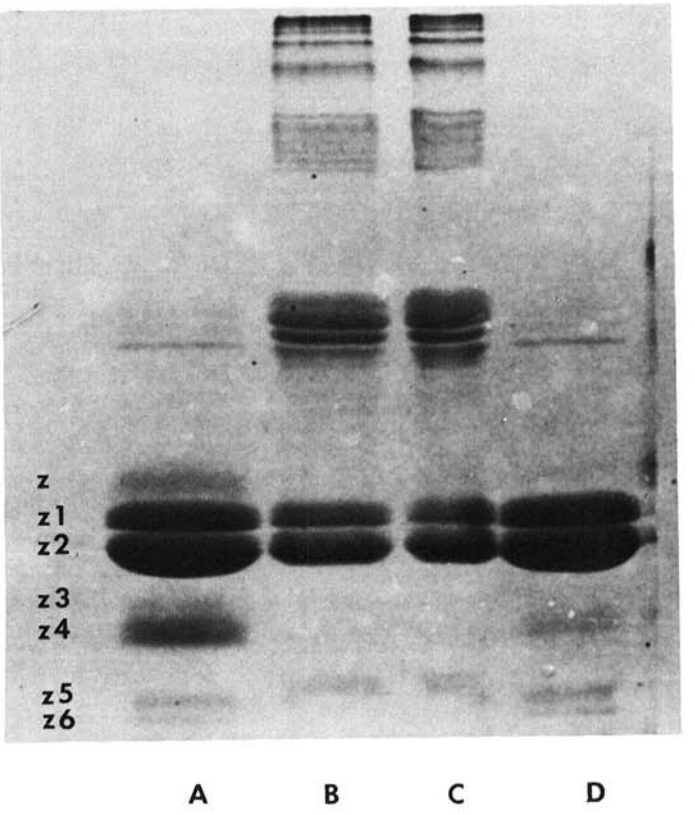

Fig. 2. Effect of reducing agent during dialysis on sodium dodecyl sulfate-gel patterns of zein prepared from the mature normal maize endosperm. **A,** zein extracted with 70% ethanol plus 1 m*M* 2-mercaptoethanol and dialyzed with mercaptoethanol; **B,** zein extracted with 70% ethanol minus mercaptoethanol and dialyzed without mercaptoethanol; **C,** zein extracted with 70% ethanol plus mercaptoethanol and dialyzed without mercaptoethanol; **D,** zein extracted with 70% ethanol minus mercaptoethanol and dialyzed with mercaptoethanol.

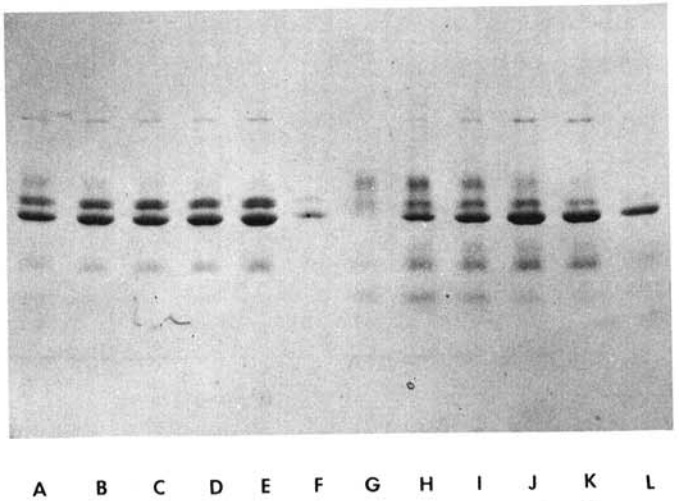

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of zein prepared from developing endosperms of normal maize (**A–F**) and *o2* mutant (**G–K**). Zein was extracted with 70% ethanol containing 1 m*M* 2-mercaptoethanol and subsequently dialyzed with mercaptoethanol. Maize harvested after pollination: **A** and **G,** 12 days; **B** and **H,** 16 days; **C** and **I,** 22 days; **D** and **J,** 28 days; **E** and **K,** 35 days; **F** and **L,** at maturity.

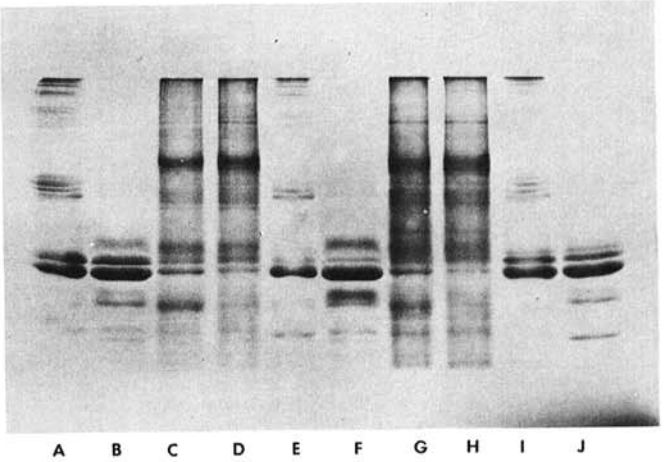

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of zein prepared with and without 2-mercaptoethanol from the mature endosperm of normal maize (**A–D**) and mutants *o2* (**E–H**) and *fl2* (**I,J**). **A, E,** and **I,** zein extracted and dialyzed without 2-mercaptoethanol; **B, F,** and **J,** zein extracted and dialyzed with 2-mercaptoethanol; **C** and **G,** glutelin remaining from ethanol extraction without mercaptoethanol; **D** and **H,** glutelin remaining from ethanol extraction with 2-mercaptoethanol.

TABLE I
Effect of 2-Mercaptoethanol Concentrations on Zein[a] Determination from the Mature Normal Endosperm

Concentration of 2-Mercaptoethanol (m*M*)	Extraction Medium				
	70% Ethanol		70% Isopropanol		55% Isopropanol
	25°C	60°C	25°C	60°C	60°C
0	5.4	7.1	6.4	7.0	7.0
1	5.8	8.1	6.5	8.0	8.1
2	6.1	8.1	6.7	8.1	8.2
10	6.5	8.1	7.3	8.1	8.2
100	7.6	8.1	8.0	8.0	8.2

[a] Percent of sample.

numbers of samples. However, hot 70% ethanol without reducing agent is not effective in total zein extraction because this procedure does not permit the complete solubilization of the $zein_2$ fraction (Landry and Moureaux 1970, Miflin 1978, Paulis and Wall 1971, Sodek and Wilson 1971). Significant amounts of Z1, Z2, Z4, Z5, and Z6 were present in the residue (alkali-soluble) remaining after ethanol extraction (Figs. 3C and G). The presence of 1 m*M* 2-mercaptoethanol in hot alcohol (70% ethanol, 70% isopropanol, or 55% isopropanol) maximized zein extraction; only trace amounts of Z1 and Z2 were detectable in the residue remaining from the extraction with ethanol plus 2-mercaptoethanol (Figs. 3D and H). A further increase in 2-mercaptoethanol concentration did not extract more zein (Table I). Although the extraction of zein with 70% alcohol at 25°C was less effective than those at 60°C, and the extraction appeared to be concentration-dependent on 2-mercaptoethanol, 70% isopropanol containing 100 m*M* 2-mercaptoethanol produced satisfactory extraction at 25°C.

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