Zein, the major alcohol-soluble protein of corn endosperm, is usually extracted with aqueous ethanol or isopropanol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) without β-mercaptoethanol (β-ME), as well as ultracentrifugation, have shown zein to consist of almost equal amounts of dimers and monomers, with lesser amounts of higher molecular weight (mol wt) oligomers. The monomeric polypeptides have molecular weights of 19,000–25,000 (referred to here as 22,000 and 24,000 mol wt) (Turner et al. 1965, Ganchev et al. 1976, Tsai 1980, Paulis 1981). Upon SDS-PAGE in the presence of β-ME, zein appears as only 22,000 and 24,000 mol wt subunits.

Although virtually insoluble in water, zein’s conformation is characteristic of more conventional globular proteins (Danzer et al. 1983). Osmotic studies in dimethylformamide and formamide (Danzer and Rees 1976) show zein to have a number average mol wt of 21,000–24,000. Some zein (β-zein) exists as an aggregate, which dissociates to monomers in formamide, but reaggregates at higher protein concentrations. In contrast, sulfhydryl analysis (Abe et al. 1981) showed that almost all zein cysteine residues form intermolecular disulfide bonds in dimers, polymers, and in protein bodies (Abe et al. 1981). Recently, reversed-phase high-performance liquid chromatography (RP-HPLC) was used to identify corn varieties (Bietz et al. 1986). To further understand the aggregated structure of zein, we used preparative RP-HPLC to fractionate native and reduced and alkylated (R-A) zeins, both solubilized in 8M urea.

Protein Preparation

Zein was isolated from maize inbred W64A (Landry et al. 1983). Samples of native zein for RP-HPLC, IEF, and AU-PAGE were dissolved in 8M urea. Zeins were also reduced in 8M urea with 1% β-ME (16 hr at room temperature), alkylated for 1 hr with acrylonitrile, and acidified with aluminum lactate buffer to form R-A zein (Paulis and Wall 1977). RP-HPLC fractions of native zein were examined both unreduced and after reduction and alkylation.

RP-HPLC

A 250 × 10 mm SynChropak RP-P column (C₁₈) having 300 Å pores was used for preparative RP-HPLC. The column was eluted at room temperature first for 10 min with 42.3% aqueous acetonitrile (CH₃CN) (+ 0.1% trifluoroacetic acid) and then with an 80-min linear gradient from 42.3 to 57.3% CH₃CN (Paulis and Bietz 1986). The column effluent was monitored at 280 nm (absorbance range = 0.4). Protein was injected as two 1.5-ml aliquots (10 mg/ml 8M urea).

Collected peak fractions (Fig. 1) were dried in 1.2 × 15 cm test tubes at room temperature in a Buchler Vortex-Evaporator, and stored at 4°C after analysis. Dried fractions were dissolved in 0.5–2.0 ml of 70% (v/v) aqueous ethanol at 600–1,500 µg/ml. Protein concentrations of fractions were estimated by integration after injection of 30 mg R-A or native zein. Aliquots for SDS-PAGE, AU-PAGE, and IEF were dried in 4 cm³ disposable culture tubes by a stream of nitrogen. Corresponding fractions from replicate zein RP-HPLC fractionations were collected and lyophilized for amino acid analyses.

Analytical Methods

Lyophilized RP-HPLC fractions (equivalent to 1–10 mg of protein) were hydrolyzed by refluxing with 6M HCl (2–10 ml/mg of protein) for 24 hr. Liberated amino acids were determined on a Dionex D-300 amino acid analyzer using a physiological fluids analysis column with lithium buffers. Reported levels of methionine and cysteine include methionine sulfone and cysteic acid. Tryptophan and carboxybethylcysteine (for R-A fractions) were not determined.

Lyophilized native or R-A zein and their RP-HPLC fractions (40–50 µg/5–10 µl sample) were analyzed by SDS-PAGE (Laemmli 1970) using a 10–15% gradient gel (Landry et al. 1983). To analyze native zein, no β-ME was used in samples or gel. β-ME was added to reduce samples of native zein and to R-A zein fractions.

Native and R-A zein RP-HPLC fractions were also analyzed by 2-dimensional PAGE, using ammonium molybdate as an anodic stain.
RESULTS AND DISCUSSION

Isolation and RP-HPLC
Zein was extracted with 55% isopropanol from defatted W64A corn endosperm following salt extraction, and purified after extraction with petroleum ether, ethanol, and 55% tert-butanol by LH-20 chromatography (Landry 1979). RP-HPLC separated native zein (Fig. 1A) into about 17 overlapping peaks; 27 fractions were collected for subsequent electrophoresis and amino acid analysis. Gel filtration chromatography separates similar unreduced zein preparations into peaks corresponding to about 40% monomers, 40% dimers, and 20% higher molecular weight oligomers (Ivanov et al. 1976, Rewa et al. 1978, Rewa and Bruckner 1979, Paulis 1981, Landry and Sallantin 1983, Landry and Guyon 1984, Landry et al. 1986).

R-A zein also yields 15–17 peaks upon preparative RP-HPLC (Fig. 1B). The chromatogram contains more early-eluting protein than does that of native zein. Eighteen R-A zein RP-HPLC fractions were collected for subsequent analysis.

SDS-PAGE
Figure 2A shows SDS-PAGE patterns, under nonreducing conditions, of RP-HPLC fractions of native zein. Initial fractions contained mainly 22,000 mol wt monomers plus some 44,000 mol wt dimers. Later fractions become more enriched in 24,000 mol wt monomers, and dimers of 46,000 and 48,000 mol wt become evident. In late fractions (19–27), monomers diminish, dimers predominate, and trimers, tetramers, and higher molecular weight oligomers appear.

SDS-PAGE under reducing conditions of native zein RP-HPLC fractions (Fig. 2B) shows, as does nonreducing SDS-PAGE (Fig. 2A), that initial fractions contain primarily 22,000 mol wt monomers. Bands corresponding to 24,000 mol wt appear at about fraction 5. These polypeptides are subunits of 46,000 and 48,000 dimers, and of later-eluting higher molecular weight oligomers that contain both 22,000 and 24,000 mol wt subunits.

SDS-PAGE under reducing conditions of RP-HPLC fractions of R-A zein (Fig. 2C) shows that initial fractions (1–4) contain primarily 22,000 mol wt polypeptides, corresponding in elution volume to native 22,000 mol wt zein monomers (Fig. 2B). Later-eluting RP-HPLC fractions (13–18) of R-A zein are also enriched in 22,000 mol wt polypeptides. Polypeptides of 24,000 mol wt occur predominantly in fractions 7–12, which correspond to those RP-HPLC fractions of native zein enriched in dimers (Fig. 2A).

These results show that 22,000 and 24,000 mol wt zein...
polypeptides, in spite of sequence homology (Marks and Larkins 1982), differ significantly in their tendencies to form oligomers. In addition, differences in elution characteristics of native and R-A polypeptides indicate differing surface hydrophobicities. Thus, differences in zein primary structures, though conservative, appear sufficient to significantly alter surface hydrophobicities, and change RP-HPLC elution characteristics of native and R-A polypeptides.

Because some 22,000 mol wt zein monomers have RP-HPLC elution characteristics equivalent to some 22,000 mol wt R-A zeins, while other R-A 22,000 mol wt zeins elute later upon RP-HPLC, intramolecular disulfide bonding may give native 22,000 mol wt zeins conformations having relatively polar surfaces.

Zein polypeptides of 24,000 mol wt, however, differ significantly in reactivity from 22,000 mol wt polypeptides. The 24,000 mol wt polypeptides are primarily dimers and higher molecular weight oligomers, rather than monomers, suggesting that stable conformations are not easily achieved through intramolecular disulfide bonds. Alternatively, surface reactivity that promotes association is significantly greater for 24,000 than for 22,000 mol wt zein polypeptides.

SDS-PAGE reveals that native zein oligomers are more hydrophobic than monomers, and that apparent hydrophobicity increases as molecular size and extent of association increase. Oligomeric zeins are probably stabilized by intermolecular disulfide bonds, because reduction and alkylation changes elution order of polypeptides. Also, oligomeric forms exist in the presence of SDS in native zein (Fig. 2A) but appear as monomers in the

![Figure 2](image-url)

**Fig. 2.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of (A) reversed-phase high-performance liquid chromatography (RP-HPLC) fractions of native zein (Fig. 1A), run under nonreducing conditions; (B) RP-HPLC fractions of native zein, run under reducing conditions; and (C) reduced and alkylated zein (Fig. 1B). Whole zein (Z) was treated the same as fractions for SDS-PAGE.
presence of SDS after reduction (Fig. 2B) or reduction and alkylation (Fig. 2C).

Our results do not totally rule out the possibility, however, that most zeins are monomers in a solvent such as CH$_3$CN, and that oligomers may form in solutions containing SDS or urea through noncovalent interactions. Studies of zein association in the presence both of denaturants and detergents would help resolve this point. Thus, it is conceivable that zeins could associate through noncovalent (e.g., hydrogen) bonds that could remain stable in the presence of SDS and acetonitrile (Fig. 2A). Further studies of zein molecular weight in additional solvents, including those containing acetonitrile, are needed.

These results also demonstrate that zein oligomers do not form randomly, but rather by specific combinations of 22,000 and 24,000 mol wt monomers of different reactivities. Oligomeric zeins adsorb more strongly to the C$_8$ column than do monomers, suggesting that disulfide bonds involved in oligomer formation are in relatively hydrophilic regions of the molecule. Resulting oligomers thus have higher surface hydrophobicities. If such association were noncovalent (e.g., through hydrogen bonds), similar masking of ionic character could increase surface hydrophobicity of oligomers.

**AU-PAGE**

PAGE under acidic denaturing conditions was also used to characterize zeins fractionated by RP-HPLC (Fig. 3). Differences are readily apparent among RP-HPLC fractions of native zein (Fig. 3A). Here, late-eluting fractions (15-27), which contain whole zein (Z) was treated the same as fractions for AU-PAGE. Fractions were those of Fig. 218.

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**Fig. 3.** Acidic urea-polyacrylamide gel electrophoresis (AU-PAGE) of (A) reversed-phase high-performance liquid chromatography (RP-HPLC) fractions of native zein, run under nonreducing conditions; (B) RP-HPLC fractions of native zein, run under reducing conditions; and (C) reduced and alkylated zein. Whole zein (Z) was treated the same as fractions for AU-PAGE. Fractions were those of Fig. 1.
higher molecular weight oligomers (Fig. 2A), appear more heterogeneous than earlier-eluting fractions, which are also heterogeneous and which are enriched in 22,000 mol wt zein monomers (Fig. 2A). Later-eluting fractions also appear more streaked than early fractions.

After reduction and alkylation of RP-HPLC fractions of native zein, AU-PAGE shows less streaking and more distinct bands in later fractions (Fig. 3B). This appears to be due to lower amounts of high molecular weight oligomers in these fractions (see also Fig. 2B). Fractions 1–8 each contain one or two major bands (differing between fractions); later fractions are more heterogeneous, showing charge heterogeneity among proteins of similar hydrophobicity.

Similarly, AU-PAGE of R-A zeins separated by RP-HPLC (Fig. 3C) reveals that zeins of comparable hydrophobicity may differ significantly in distribution of charged amino acids. These results also show that polypeptides with similar charge or mass characteristics may have markedly different surface hydrophobicities. These results confirm the heterogeneity of the zein family, for which more than 100 structural genes exist (Soave and

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Fig. 4. Isoelectric focusing (IEF; pH 6–8) of (A) reversed-phase high-performance liquid chromatography (RP-HPLC) fractions of native zein, run under nonreducing conditions; (B) RP-HPLC fractions of native zein, run under reducing conditions; and (C) reduced and alkylated zein. Whole zein (Z) was treated the same as fractions for IEF. Fractions were those of Fig. 1.
Salamini, 1982). Our results therefore emphasize the
complementary nature of AU-PAGE (based on charge and mass)
and RP-HPLC (based on surface hydrophobicity). AU-PAGE
also reveals (Fig. 3C) that several RP-HPLC fractions (14–17)
of R-A zein have only one major band.

AU-PAGE (Fig. 3A), like SDS-PAGE, reveals unique
differences between monomeric (early eluting; Fig. 2A) and
oligomeric (late-eluting) zeins. These results support our above
interpretation of SDS-PAGE data that zein oligomer formation
is nonrandom; different polypeptides have distinctly different
reactivities and tendencies to associate. AU-PAGE of reduced and
alkylated native zein RP-HPLC fractions also shows that
oligomers contain several components, while late-eluting RP-HPLC fractions of R-A zein are much less heterogeneous (Fig. 3C).
Thus, oligomers differ in composition from late-eluting
monomers.

**IEF**

IEF (pH 6–8) was also used to characterize RP-HPLC fractions
of native zeins, without (Fig. 4A) or with (Fig. 4B) subsequent
reduction and alkylation. IEF was also used to characterize RP-HPLC fractions of R-A zein (Fig. 4C). IEF of native zein fractions
(Fig. 4A) revealed more bands than did AU-PAGE (Fig. 3A).
Again, many bands eluted in a narrow range of surface
hydrophobicity, while many bands similar in isoelectric points
varied widely in hydrophobicity. Later-eluting fractions (Fig. 4A)
are more streaked than earlier fractions because of the presence of
oligomers.

After reduction and alkylation, IEF patterns (Fig. 4B) of native
zein RP-HPLC fractions generally resemble those of unreduced
native zein fractions (Fig. 4A). Later-eluting oligomer fractions,
however, have more components than are apparent upon IEF of
fractions of unreduced native zein (Fig. 4A), further showing that
22,000 and 24,000 mol wt subunits differ both in hydrophobicity
and charge.

IEF patterns of RP-HPLC fractions of R-A zein are shown in
Figure 4C. Fractions 1–4, shown by SDS-PAGE (Fig. 2C) to contain 22,000 mol wt polypeptides, appear similar to IEF patterns
of native zein fractions 1–4 that were reduced and alkylated (Fig.
4B). Later fractions (13–18) differ in IEF pattern (Fig. 4C) from
R-A native zein fractions of similar elution volumes (Fig. 4B),
showing that 22,000 and 24,000 mol wt components of oligomers
differ from 22,000 mol wt monomers. As observed by SDS-PAGE
(Fig. 2) and AU-PAGE (Fig. 3), IEF (Fig. 4) shows that some
RP-HPLC fractions are enriched in specific bands. Components of
similar isoelectric point frequently occur in several fractions of
differing hydrophobicity, and polypeptides having similar surface
hydrophobicities may vary widely in amount and distribution of
ionizable amino acids. IEF combined with RP-HPLC (Fig. 4C)
may reveal more heterogeneity than readily observed by two-
dimensional electrophoresis combining IEF and AU-PAGE (Wall
et al. 1984), since RP-HPLC, based on surface hydrophobicity,
have good resolution and separates proteins by characteristics different
from those of IEF, AU-PAGE, and SDS-PAGE (size and charge).

**Amino Acid Analysis**

Amino acid compositions of various RP-HPLC fractions of
native and of R-A zein are presented in Tables I and II,
respectively. Fractions 2 and 5 of native zein, which contain mainly
22,000 mol wt monomers, differ slightly from fractions 2 and 4 of
R-A zein, which contain mainly 22,000 mol wt monomers. Later-
eluting fractions of native and R-A zeins of similar
hydrophobicities also vary somewhat in composition. Thus,
surface hydrophobicity may not relate closely to total hydrophobicity. These results also support the hypothesis that
22,000 and 24,000 mol wt monomers differ from subunits of native
zein oligomers. This also agrees with different SDS-PAGE, AU-
PAGE, and IEF patterns for different compositions for R-A and
native zeins of similar elution characteristics.

Total contents of nonpolar amino acids (Tables I and II) are
similar among native and R-A zein fractions, irrespective of RP-
HPLC elution volumes. Thus, conservative sequence differences
may significantly change surface hydrophobicity, as revealed by
the RP-HPLC separations. Resulting tendencies to form
oligomers also differ, as shown by electrophoresis (Figs. 2–4) of
resulting fractions.

### CONCLUSION

RP-HPLC, based on differences in protein surface
hydrophobicity, reveals considerably more heterogeneity in zein
than is readily apparent by separations based on size or charge.
RP-HPLC thus essentially adds a third possible dimension to
protein separations. In combination with SDS-PAGE, AU-

**TABLE I**

| Amino Acid Composition of Reversed-Phase High-Performance Liquid Chromatographic (RP-HPLC) Fractions of Native Zein Residues/1,000 Residues |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Acid                | 2   | 5   | 9   | 14  | 16  | 17  | 19  | 21  | 22  | 23  | 25  |
| Glutamine + glutamic acid | 207 | 206 | 220 | 229 | 226 | 209 | 199 | 251 | 241 | 210 | 214 |
| Proline             | 100 | 102 | 69  | 65  | 102 | 61  | 122 | 72  | 87  | 85  | 120 |
| Glycine             | 25  | 23  | 22  | 18  | 24  | 13  | 12  | 15  | 15  | 20  | 16  |
| Alanine             | 145 | 137 | 135 | 149 | 146 | 155 | 143 | 139 | 137 | 161 | 134 |
| Cystine             | 3   | 3   | 2   | 3   | 2   | 3   | 2   | 3   | 2   | 2   | 3   |
| Valine              | 38  | 38  | 40  | 48  | 50  | 38  | 37  | 48  | 51  | 53  | 37  |
| Methionine tr \( ^\) | 4   | 5   | tr  | 4   | 5   | tr  | 6   | 7   | 9   | 6   | 8   |
| Isoleucine          | 27  | 36  | 42  | 39  | 34  | 43  | 43  | 40  | 42  | 37  | 45  |
| Leucine             | 200 | 209 | 221 | 205 | 191 | 210 | 206 | 204 | 187 | 166 | 208 |
| Tyrosine            | 43  | 36  | 38  | 32  | 34  | 37  | 34  | 30  | 30  | 36  | 32  |
| Phenylalanine       | 58  | 58  | 55  | 44  | 45  | 55  | 48  | 45  | 44  | 43  | 48  |
| Lysine              | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 0   | 2   | 0   | 0   |
| Histidine           | 16  | 15  | 11  | 11  | tr  | 6   | 7   | 8   | 10  | 7   | 7   |
| Arginine            | tr  | 10  | 10  | 12  | tr  | 13  | 10  | 9   | 11  | 13  | tr  |
| Nonpolar \( ^\) (sum) | 568 | 568 | 566 | 555 | 570 | 568 | 563 | 551 | 554 | 558 | 592 |

1 Fractions collected from Fig. 1A. Note that similar elution positions in Fig. 1A and 1B do not correspond to the same numbers.

2 tr = Trace.

3 Nonpolar amino acids include proline, alanine, valine, methionine, isoleucine, leucine, and phenylalanine.

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PAGE, and IEF. RP-HPLC reveals major differences in abilities of 22,000 and 24,000 mol wt zeins to form oligomers.

Our studies also suggest specific compositions for zein oligomers. Specific monomers tend to form intermolecular, rather than solely intramolecular bonds. This tendency relates to protein surfaces. RP-HPLC is a highly sensitive probe for revealing such different surface reactivities among zeins.

Differences between RP-HPLC fractions of native and R-A zeins, and analyses of resulting fractions under reducing and nonreducing conditions, suggest that disulfide bonds cause much oligomer formation in zein. The possibility that extensive noncovalent (e.g., hydrogen) bonding facilitates bonding of monomers into oligomers cannot be totally eliminated, however. While SDS affects primarily hydrophobic bonds and urea disrupts hydrogen bonds, the dissociating-denaturing characteristics of RP-HPLC solvents (i.e., acidic aqueous acetonitrile solutions) are largely unknown. Further studies are required to explain fully the relative contributions of covalent and noncovalent bonds to zein oligomer formation.

The maize genome may contain more than 100 zein genes, considerably more than the number of proteins resolved by electrophoresis. Not all of these genes may be expressed. Nevertheless, our results show that RP-HPLC, in combination with electrophoresis, may reveal greater zein heterogeneity than solely intramolecular bonding. Proteins of similar molecular weight and charge may differ significantly in hydrophobic amino acid content and distribution, giving them different conformations and surface hydrophobicities. RP-HPLC also reveals major differences in associative tendencies among each zein class. Clearly, RP-HPLC is a valuable tool for isolation and characterization of zein polypeptides.

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