

# Fractionation of Alcohol-Soluble Reduced Corn Glutelins on Phosphocellulose and Partial Characterization of Two Proline-Rich Fractions

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## ABSTRACT

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Alcohol-soluble reduced glutelin (ASG) from corn endosperm was fractionated by ion exchange chromatography on phosphocellulose. The ASG was dissolved in 0.01 *M* Na-lactate buffer (pH 3.8) containing 60% isopropanol and 0.05 *M* 2-mercaptoethanol and loaded onto phosphocellulose columns equilibrated with the same buffer. Columns were developed with a linear 0.0–0.5 *M* NaCl gradient formed in the starting buffer, yielding two major overlapping peaks (each with shoulders) and four minor peaks. Each peak and subpeak was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by isoelectric focusing. Five

fractions contained essentially a single size component. The two late-eluting fractions were further characterized. Each had the same molecular weight (27,500) and was heterogeneous when analyzed by isoelectric focusing. These fractions were partially soluble in water, rich in proline (26%) and glutamic acid (16%), and deficient in lysine (0.1%) and had threonine as the NH<sub>2</sub>-terminal residue. Polypeptides in these two fractions were similar in physicochemical characteristics to water-soluble ASG, previously described, and appeared to be coded by homologous genes.

Zein, the prolamine of corn, is the major storage protein fraction in corn endosperm. It is soluble in aqueous alcohol solutions (eg, 60–70% ethanol or isopropanol) and constitutes 50–60% of total endosperm protein. Glutelin, the other major storage protein fraction, is extractable with dilute alkali or detergent containing reducing agent and makes up 30–35% of the total protein (Landry and Moureaux 1970, Wall et al 1975). Both zein and glutelin are heterogeneous. Moureaux and Landry (1968) and Paulis et al (1969) independently discovered that some glutelin proteins were soluble in alcohol upon reduction or reduction-alkylation. This fraction is referred to as glutelin-1 (Landry and Moureaux 1970), alcohol-soluble reduced glutelin (ASG) (Paulis and Wall 1971), zeinlike (Misra et al 1975), and zein-2 (Gianazza et al 1976, Sodek and Wilson 1971). ASG and zein contain the same predominant amino acids (Landry and Moureaux 1970, Misra et al 1975, Paulis and Wall 1971, Sodek and Wilson 1971), but amounts of histidine, arginine, proline, glycine, and methionine are higher in ASG, whereas those of leucine, isoleucine, aspartic acid, and phenylalanine are higher in zein.

Gianazza et al (1976) and DiFonzo et al (1977) showed that zein and ASG (or zein-2) had similar isoelectric focusing (IEF) patterns and essentially were made up of the same polypeptides. Gianazza et al (1976) concluded that ASG polypeptides were essentially zein that had formed supramolecular complexes with one another or with nonzein proteins through disulfide bridges and were consequently insoluble in alcohol without reduction. Recently, Paulis and Wall (1977) reported that ASG could be separated into water-soluble and water-insoluble fractions by dialysis against water. They found that the water-soluble ASG had a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile similar to that of zein but was completely different from zein when electrophoresed in polyacrylamide gel containing 8 *M* urea at pH 3.2.

We subjected ASG to ion-exchange chromatography on phosphocellulose, using a buffer containing 60% isopropyl alcohol, and found that fractions characterized by water-solubility and -insolubility could be obtained. Two late-eluting peaks contained proteins similar to water-soluble ASG (Paulis and Wall 1977). This article describes this fractionation and the partial purification and further characterization of the two water-soluble, proline-rich fractions obtained.

## MATERIALS AND METHODS

### Extraction of ASG

Zein was extracted three times (for 2 hr each, by stirring) from 25 g of corn endosperm meal (WF9 × Bear 38) with 60% (v/v) isopropyl alcohol (IPA), using a solvent-meal ratio of 50:1. Then, ASG was extracted from the meal residue with 60% IPA + 0.07 *M* 2-mercaptoethanol (ME) by stirring for 2 hr at room temperature. Again, the solvent-meal ratio was 50:1. This extraction was repeated once more at 60°C for 30 min. The two IPA-ME extracts were combined and dialyzed against water, and the retentate was freeze-dried. The resulting protein powder, containing 15.5% N, was dissolved at a final concentration of 2 mg/ml in 0.01 *M* Na-lactate buffer, pH 3.8, containing 60% IPA and 0.05 *M* ME.

### Fractionation of ASG on Phosphocellulose

Phosphocellulose (medium mesh, Sigma Chemical Co.) was precycled by being treated with 0.5 *M* NaOH and 0.5 *M* HCl (successively, 1 hr each) and was equilibrated with the starting buffer (0.01 *M* Na-lactate, pH 3.8, containing 60% IPA and 0.05 *M* ME). A column (SR 25/100, Pharmacia) with 2.5 × 62-cm bed dimensions was packed by gravity flow. After running three bed volumes of the starting buffer, the ASG sample (~260 mg) was loaded. Loading was followed by washing with three bed volumes of the starting buffer. Then the column was developed with a linear gradient (0–0.5 *M*) of NaCl in the starting buffer. The total volume of gradient was five times the bed volume. All operations were performed at room temperature (23–25°C) and monitored at 280 nm using an ultraviolet monitor (LKB 8300, Uvicord II) with a 0.3-cm flow cell light path. Tube contents under each peak and subpeak were pooled to constitute chromatographic fractions corresponding to individual peaks and subpeaks. Fractions were dialyzed against water and freeze-dried.

### Analytical Procedures

Whole ASG and chromatographic fractions were analyzed in homogeneous (10, 12, and 15%) and gradient (10–15%) gels by SDS-PAGE (Laemmli 1970). After electrophoresis, protein bands were stained with 0.15% Coomassie brilliant blue R-250 in acetic acid/isopropanol/water (10:25:65, v/v) and destained with the same solution minus the dye. Whole ASG and its fractions were also subjected to IEF basically as described by Gianazza et al (1976). The pH gradient was formed by using the broad range ampholyte (LKB, pH 3.5–10) in 5% polyacrylamide gel containing 6 *M* urea. Freeze-dried protein powders were dissolved in 8 *M* urea + 0.35 *M* ME at a final concentration of 5 mg/ml (chromatographic fractions) and 8 mg/ml (whole ASG). The amount of protein

<sup>1</sup>The mention of firm names or trade products does not imply endorsement or recommendation by the USDA over other firms or similar products not mentioned.

applied to each sample track varied from 200 to 320  $\mu\text{g}$ . Electrolyte solutions for IEF were a saturated solution of aspartic acid (anode) and 0.1M lysine (cathode) (Caspers and Chrambach 1977). The IEF gels were fixed and washed in 12.5% (w/v) trichloroacetic acid, stained with 0.15% Coomassie brilliant blue R-250 in acetic acid/ethanol/water (10:45:45, v/v), and destained with an acetic acid/ethanol/water (5:30:65, v/v) solution.

Fractions 4 and 5 were reduced with ME, and resulting cysteine residues were converted to pyridylethyl cysteine with 4-vinylpyridine (Friedman et al 1970). Samples of pyridylethylated fractions 4 and 5 (0.5–0.6 mg) were hydrolyzed for 24 hr at 110°C with 1.0 ml of 6N HCl in ampoules sealed under vacuum. Amino acid compositions were determined in duplicate, using a Glenco amino acid analyzer; relative standard deviations for most amino acids on this analyzer were less than 4%. Percentage recovery of amino acids from the analyzer, based on weight of sample applied (not corrected for moisture), were 78.2% for fraction 4 and 69.1% for fraction 5.

NH<sub>2</sub>-terminal amino acid residues were cleaved from pyridylethylated fractions 4 and 5 by automated Edman degradation, using a Beckman 890C sequencer, and identified by high-performance liquid chromatography (Gates et al 1979).

## RESULTS AND DISCUSSION

All ASG was bound to phosphocellulose under the initial conditions, suggesting that polypeptides in this fraction had a net positive charge at pH 3.8. The "passthrough" fraction contained some nonprotein material absorbing at 280 nm. This fraction was devoid of protein when analyzed by dye-binding (Esen 1978) and showed no detectable protein bands when analyzed by SDS-PAGE. Development of the column with the NaCl gradient yielded an elution profile with two overlapping major peaks (1B<sub>1</sub> and 1B<sub>2</sub>), having shoulders (1A and 1C) on both sides, and four minor peaks (2–5) (Fig. 1). The last peak in the profile was eluted with about 0.2M NaCl.

### Size Heterogeneity of ASG and Its Fractions

When pooled tubes from each peak were analyzed by SDS-PAGE, all ASG bands were found in one or more fractions. The early fractions (1A and 1B<sub>1</sub>) contained primarily a component having a molecular weight of 22,000 (Fig. 2); this component has the same apparent molecular weight and relative elution volume as the 22,000-mol wt zein component (data not shown). Fractions 1B<sub>2</sub> and 1C contained, in addition to bands characteristic of zein, a major 17,000-mol wt component (Fig. 2), and fraction 1C also included a significant amount of a 13,400-mol wt component, the smallest component in the profile of ASG. Furthermore, fractions 1B<sub>2</sub> and 1C had four and five larger size components, respectively, in the 20,000–25,000 range. These components had electrophoretic and chromatographic properties similar to those of the two major size components of zein. They very likely represented those zein components that formed oligomers and polymers through intermolecular disulfide linkages among themselves and/or with ASG components. Two of the bands in the 20,000–25,000 range in fraction 1B<sub>2</sub> and three of these bands in fraction 1C correspond in SDS-PAGE mobility to the 22,000 and 24,000 components of zein (data not shown). This indicated additional size heterogeneity in zein, also observed when chromatographic fractions of zein were analyzed by SDS-PAGE.<sup>2</sup> In unfractionated zein samples, the magnitude of size heterogeneity is obscured. Both fractions 2 and 3 had one low molecular weight component (mol wt 18,000) predominant in their profile. The two late-eluting minor fractions (4 and 5) included one major size component (mol wt 27,500), which is entirely a constituent of ASG and could not be observed in zein by SDS-PAGE even at high sample loads.

### Charge Heterogeneity of ASG and Its Chromatographic Fractions

Whole ASG and its chromatographic fractions displayed extensive charge heterogeneity upon IEF (Fig. 3). ASG included at least 25 charge components, about 16 of which are visible in Fig. 3; most had an apparent isoelectric point (pI) equal to or greater than

7, but 6–8 components had acidic pIs. Absolute pI values were not assigned to components, because the presence of urea tends to make pI determinations unreliable (Righetti and Drysdale 1976). Chromatographic fractions of ASG were also unexpectedly heterogeneous; up to 20 components were apparent in each fraction when faint bands were counted, although three to eight components predominated in each profile (Fig. 3). In general, adjacent peaks included the same components but in different quantities. Neutral and alkaline ASG components occurred in all fractions, but acidic components were observed only in fractions 1B<sub>1</sub>–3. All charge components of ASG appeared in one or more fractions. In addition, chromatography enriched some minor ASG components so that they were detectable in fractions although not in whole ASG.

IEF clearly shows that each ASG component revealed by SDS-PAGE actually contains numerous components differing in charge. For example, fractions 1B<sub>1</sub>, 2, and 3 are essentially homogeneous according to size (Fig. 2), but IEF (Fig. 3) reveals up to 20 components in each fraction. Other IEF experiments (results not shown) suggest that fraction 1A (Fig. 1) may be the least heterogeneous fraction obtained; it was isolated in insufficient quantity for more complete characterization, however. The heterogeneity of ASG subfractions is not surprising; IEF reveals that whole zein and ASG contain 4–5 times as many components as are resolved by SDS-PAGE, which is in agreement with the data of Gianazza et al (1976).

The order of elution of fractions from phosphocellulose was not always consistent with their IEF profiles. For example, IEF

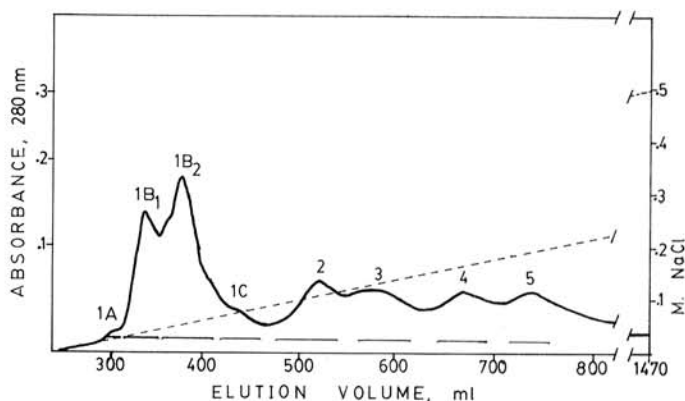


Fig. 1. Chromatographic elution profile of alcohol-soluble reduced glutelin on phosphocellulose. Broken line shows salt gradient. Straight lines along the abscissa indicate tubes pooled to constitute fractions 1A–5.

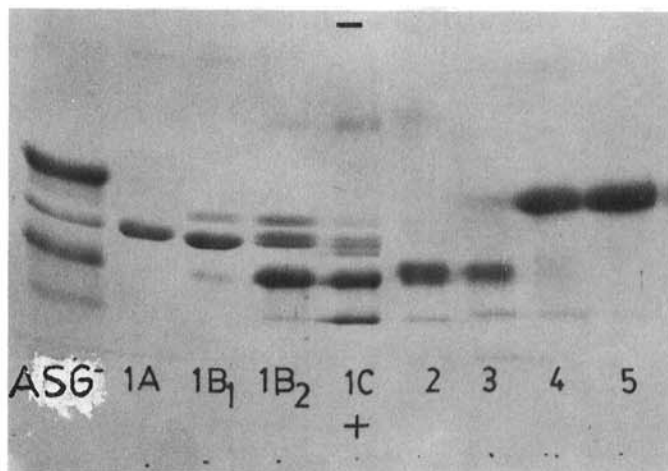


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10–15% pore-gradient) patterns of whole alcohol-soluble reduced glutelin (ASG) and phosphocellulose fractions 1A, 1B<sub>1</sub>, 1B<sub>2</sub>, 1C, and 2–5.

<sup>2</sup> Unpublished results.

suggests that fractions 1B<sub>1</sub>, 1C, 2, and 3 (Fig. 3) should elute in the reverse of the elution order actually obtained, because earlier-eluting fractions contain components having higher pIs than those fractions eluting later. These anomalous results suggest that, in addition to ionic interactions between protein and phosphocellulose, nonionic interactions may occur, or ionic character may be partially masked. Nonuniform distribution of charged residues in these proteins, combined with different conformational states in the two systems, could result in differing surface charge densities and apparently inconsistent elution from phosphocellulose as compared to IEF. Thus, secondary structures of these proteins may

partially resist the attack of 60% IPA containing ME, partially masking charged groups of the proteins and leading to apparent differences in their ionic character as compared to that predicted from IEF. Interactions between ASG fractions and the IEF gel may also lead to anomalous results; this phenomenon is of considerable interest and is currently under investigation.

TABLE I

Amino Acid Composition (moles, in percent) of Fractions 4 and 5 Isolated from Alcohol-Soluble Reduced Glutelin (ASG) by Phosphocellulose Chromatography and of Water-Soluble ASG and Zein<sup>a</sup>

Amino Acid	Fraction 4	Fraction 5	Water-Soluble ASG <sup>a</sup>	Zein <sup>a</sup>
Aspartic acid	0.5	0.2	0.7	5.0
Threonine	4.1	4.1	4.2	3.0
Serine	3.8	3.6	4.6	6.3
Glutamic acid <sup>b</sup>	16.4	15.9	12.8	15.9
Proline	25.6	26.0	25.4	10.5
Glycine	6.9	6.6	7.0	2.4
Alanine	5.6	5.1	6.4	14.2
Valine	7.0	7.2	6.9	4.0
Cysteine <sup>c</sup>	6.5	6.2	5.1 <sup>d,e</sup>	1.6 <sup>d</sup>
Methionine	0.7	0.9	1.4	1.5
Isoleucine	1.9	2.0	1.9	3.6
Leucine	9.4	9.4	10.3	19.0
Tyrosine	2.2	2.2	2.5	5.1
Phenylalanine	1.1	1.5	1.5	5.5
Lysine	0.1	0.1	0.2	0.1
Histidine	5.5	6.0	6.5	1.1
Arginine	2.6	3.2	2.5	1.2

<sup>a</sup>Based on data of Paulis and Wall 1977.

<sup>b</sup>The extent of amidation of aspartic acid and glutamic acid was not determined.

<sup>c</sup>Determined as pyridylethylcysteine.

<sup>d</sup>Based on data of Paulis and Wall 1971.

<sup>e</sup>Value for whole ASG.

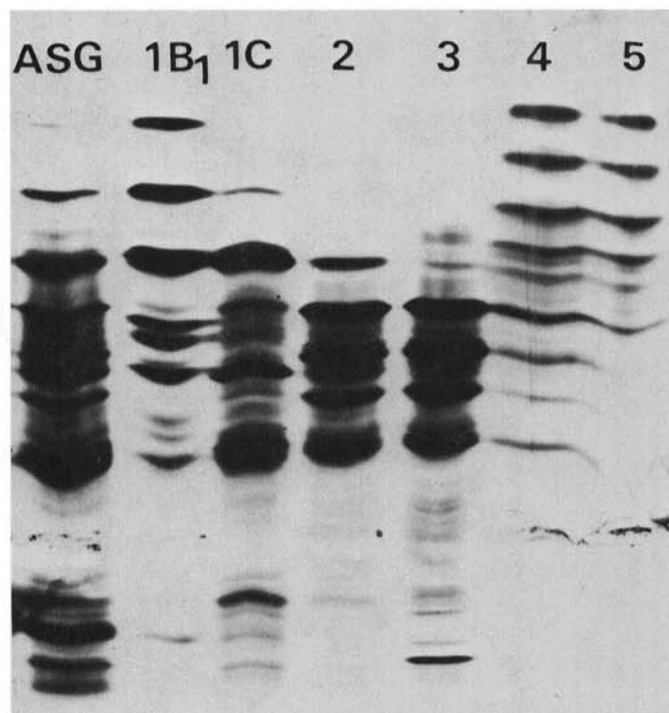


Fig. 3. Isoelectric focusing patterns of whole alcohol-soluble reduced glutelin (ASG) and phosphocellulose fractions 1B<sub>1</sub>, 1C, and 2-5 (cathode at top).

#### Size and Charge Heterogeneity in Fractions 4 and 5

Because fractions 4 and 5 were unique to ASG and were obtained pure (by the criterion of size) in appreciable quantities, they were chosen for further characterization. When fractions 4 and 5 were subjected to SDS-PAGE in homogeneous and pore-gradient gels, each contained one band; the mobility of this band was identical in both fractions (Figs. 2 and 4). The molecular weight estimated from measurements on 10, 12, and 15% gels was 27,500. No size heterogeneity was evident between and within the two fractions when 1:1 mixtures were electrophoresed together or when sample loads were increased or decreased. However, both fractions displayed extensive charge heterogeneity; each had 14-16 components, of which about five were predominant (Fig. 3). These 14-16 components appeared to have pIs between 7.5 and 9.5; some were more basic than the most basic zein components. IEF profiles

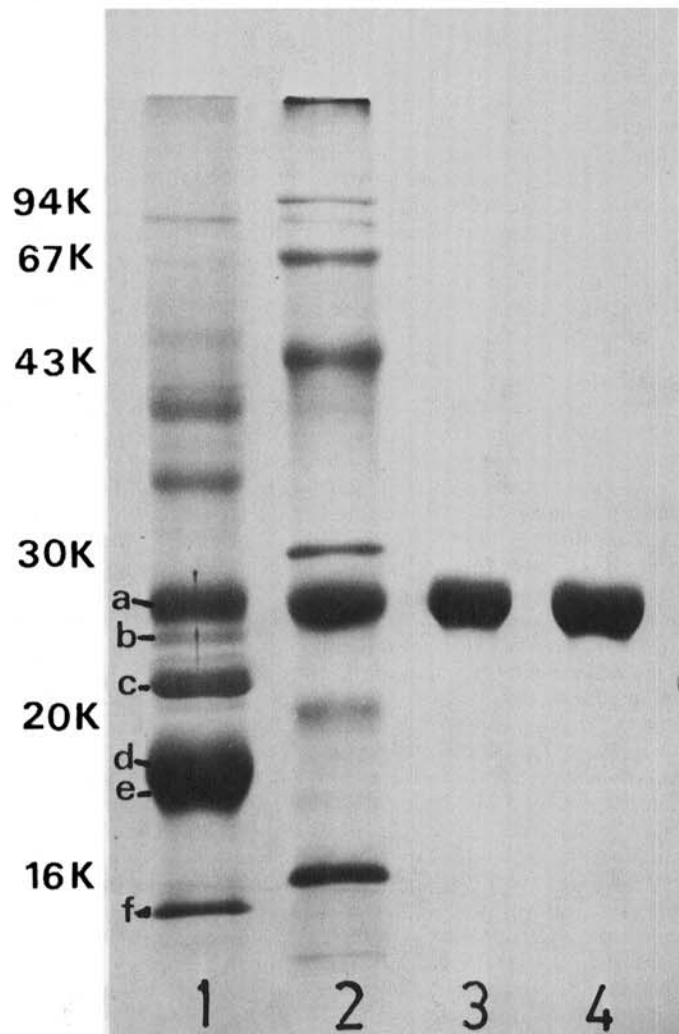


Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% gel) patterns of whole alcohol-soluble reduced glutelin (ASG) (Track 1), molecular weight calibration standards combined with fractions 4 and 5 (Track 2), fraction 4 (Track 3), and fraction 5 (Track 4). Molecular weight standards: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and hemoglobin subunits (16,000). Major size components in whole ASG profile: a, proline-rich component corresponding to fractions 4 and 5 (mol wt 27,500); b, large zein component (mol wt 24,000); c, small zein component (mol wt 22,000); d-f, unnamed low molecular weight components (mol wt 18,000, 17,000, and 13,400, respectively).

of fractions 4 and 5 were qualitatively similar with respect to major components but differed both qualitatively and quantitatively with respect to minor components. These results indicated that fractions 4 and 5 were incompletely resolved and, therefore, include mainly different proportions of 14–16 distinct components having the same molecular weight.

### Solubility Behavior of Fractions 4 and 5

Lyophilized samples of fractions 4 and 5 were soluble in water, as is the water-soluble ASG described by Paulis and Wall (1977). Aqueous solutions having a protein concentration of 2–4 mg/ml were slightly cloudy or opaque, but no visible precipitation occurred upon standing for several days or upon centrifugation at  $27,000 \times g$  for 10 min. The addition of ME (0.35 M) did not abolish the cloudiness, suggesting that intermolecular disulfide bonds were not responsible for it. In contrast, fractions 4 and 5 dissociated to yield clear solutions in 20–80% IPA. The solubility of fraction 4 and 5 proteins in alcohol solutions may derive from their high contents of proline and hydrophobic amino acids, whereas their high content of basic amino acids, combined with a lower nonpolar amino acid content than that of zein, may explain their solubility in water.

### Amino Acid Compositions

Fractions 4 and 5 have nearly identical amino acid compositions (Table I); these fractions are extremely rich in proline (about 26 residues per 100 residues) and, to our knowledge, are exceeded in proline content only by certain fractions of salivary proteins (Isemura et al 1980),  $\omega$ -gliadin (Charbonnier 1974), and C-hordein (Shewry et al 1980). The amino acid compositional data, combined with the electrophoresis and  $\text{NH}_2$ -terminal determinations, suggest substantial homology among proteins in fractions 4 and 5. The amino acid compositions of fractions 4 and 5 are very similar to that of whole water-soluble ASG (Paulis and Wall 1977) (Table I). Slight compositional differences undoubtedly are due to the presence of additional proteins besides fractions 4 and 5 in whole water-soluble ASG (Paulis and Wall 1977).

The amino acid composition data (Table I) showed that proteins of fractions 4 and 5 are not related to zein; these fractions differ significantly from zein in their content of 15 out of 17 amino acid residues compared. For example, they contain more histidine (five times) and arginine but less aspartic acid. This was consistent with the phosphocellulose chromatographic data, in which fractions 4 and 5 eluted much later than any water-insoluble ASG fraction containing subunits similar to zein in molecular weight. Also, fractions 4 and 5 contain 6–7 moles of cysteine, on a percent basis, as opposed to 1–2 moles in zein. This suggests approximately 14 cysteine residues per polypeptide chain, based on an average molecular weight of 27,500. Most of these cysteine residues must form intermolecular disulfide bonds with each other, with zeinlike molecules, and with other ASG components *in vivo*, yielding large oligomers, insoluble in alcohol but soluble after reduction of disulfide bonds.

### N-Terminal Residue

The major  $\text{NH}_2$ -terminal amino acid in both fractions 4 and 5 was found to be threonine. This, in addition to the similar amino acid compositions of fractions 4 and 5, suggests that the 14–16 polypeptides in both fractions are homologous and are products of genes that arose by duplication from a common ancestral gene. Preliminary  $\text{NH}_2$ -terminal sequence data (Bietz et al 1981) also indicate a high degree of homology among polypeptides within and between these two fractions; each fraction shows primarily a single  $\text{NH}_2$ -terminal amino acid sequence, characterized by an initial 11-residue sequence followed by the repeating hexapeptide sequence Pro-Pro-Pro-Val-His-Leu. Thus, although threonine is also the  $\text{NH}_2$ -terminal residue of some zein components, the

polypeptides in fractions 4 and 5 apparently are not homologous to any major zein polypeptide (Bietz et al 1979). Fractionation of ASG by ion exchange chromatography, when combined with other chromatographic and electrophoretic procedures, should lead to purification of individual polypeptides in these fractions and to their further characterization.

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