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# AMINO ACID COMPOSITION OF ZEIN MOLECULAR COMPONENTS

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**Key Word Index**—*Zea mays L.*; Gramineae; maize; protein bodies; zein; amino acid composition; isoelectric focusing.

**Abstract**—Zein extracted from maize endosperm has been fractionated into four polypeptide chains, having the following MWs 23 000, 21 000, 13 500 and 9600. By amino acid analysis the two smaller MW chains (representing 30% of total zeins) have been found to be zein-type molecules. These two chains are thought to be responsible for zein granule formation via –S–S– bridges. Zein is also highly heterogeneous in charge, and is resolved into at least 15 components, with pI's in the pH range 5–9. As demonstrated by amino acid analysis, part of this heterogeneity is due to spot mutations in some of the genes responsible for zein synthesis.

## INTRODUCTION

Zein is the major storage protein from maize and accounts for 50–60% of the total endosperm proteins. It is particularly rich in glutamic acid (20%), leucine (19%), proline (10%) and alanine (12%) and strongly deficient in the essential amino acids lysine and tryptophan [1]. Zein is usually extracted from corn endosperm by alcoholic solvents. However, after alcohol extraction variable amounts of zein remain in the glutelin fraction. This residual zein (named $Z_2$ or $G_1$ by different authors [2, 3]) can be solubilized only by alcoholic solvents on previous reduction of –S–S– bridges with 2-mercapthoethanol.

Recently, it has been demonstrated that zein is a class of macromolecules, heterogeneous in size and charge [4–7]. From the point of view of mass, zeins are resolved by SDS electrophoresis essentially into four polypeptide chains: two having rather similar MW's: 23 000 (Z23) and 21 000 (Z21), one of 13 500 (Z13.5) and a last one of 9600 (Z9.6). These last two components are present together with the higher MW chains only in the $Z_2$ fraction [6].

From the point of view of charge, zeins appear quite heterogeneous by isoelectric focusing (IEF) and are resolved into at least 15 components, isoelectric between pH 5 and 9. Furthermore, $Z_2$ exhibits an IEF pattern very similar to that to $Z_1$ [5, 6]. On the basis of these pI values and of the known content in basic and acidic amino acid residues, we have also demonstrated that at least 90% of the aspartic and glutamic acid residues in zeins are amidated [7].

In this paper we report the amino acid composition of the major classes of zein polypeptide chains: Z23 + Z21, Z13.5 and Z9.6, isolated from normal (+) as well as from mutant *opaque*-2 (*o2*) maizes. We also give the amino acid composition of the major classes of zeins exhibiting differing pI's, as isolated from preparative IEF.

## RESULTS AND DISCUSSION

Table 1 gives the amino acid composition of $Z_1$ and $Z_2$ fractions and of the three major zein chains isolated in SDS: *Z23 + Z21*, *Z13.5* and *Z9.6*. It can be seen that there is a good agreement at the level of most of the amino acid residues between $Z_1$ and $Z_2$. However, some constant and quite strong variations exist between the two fractions, especially for Leu and Met and, to a minor extent, for Gly, Ile, Asp, Pro and Ala. This is consistent with published data [2, 3, 8, 9]. As for the SDS-chains, the levels of 10 amino acids (Lys, His, Arg, Thr, Ser, Glu, Pro, Ala, Val and Tyr) are practically unchanged among the three classes of macromolecules, while the levels of the other six amino acids are significantly modified. In *Z23 + Z21* chains these amino acids are present at *ca* the following levels: Asp (5%), Gly (4%), Met (traces), Ile (3%), Phe (4.5%) and Leu (19%), while in *Z13.5* and *Z9.6* chains are: Asp (2.1%), Gly (8.8%), Met (4–5%), Ile (2%), Phe (2.8%) and Leu (10%).

From these data we can draw the following conclusions: 1. Judging from the paucity of basic amino acids and from the relative abundance of Glu, Pro and Ala, Z13.5 and Z9.6 can indeed be classified as zein chains. This is also confirmed by the fact that these two chains are present in zein protein bodies [4] and that in IEF they give a distribution pattern similar to that of higher MW chains [6]. 2. Z13.5 and Z9.6 chains seem to have partly reduced the overall strong hydrophobicity typical of zein molecules. In fact, there are marked decreases in the levels of Leu, Ile and Phe. 3. Z13.5 and Z9.6 chains are very rich in S-containing amino acids, as compared to Z23 and Z21 chains. This is particularly evident with Met, which barely represents one out of 200 residues in Z23 and Z21 chains, while it is present at the level of 4–5% in Z13.5 and Z9.6 chains. It must be emphasized that these high levels of Met are quite unusual in proteins, which usually contain *ca* 1% Met, as found experimentally in most proteins and theoretically predicted by the theory of molecular evolution [10]. Z9.6 chains are also richer in Cys; however the exact amount of this amino acid can only be determined by –SH group titration. 4. We had previously hypothesized [6] that Z13.5 and Z9.6 could be

fragments of the higher MW chains (Z23 and Z21) as, for instance, in the case of concanavalin A [11]. It is now clear from their amino acid composition that, although they are zein-type molecules, these chains have an independent genetic origin. 5. As for Z23 and Z21 chains, Lee *et al.* [4] have reported that their amino acid compositions are very similar to each other. This in fact can also be deduced from the effect of the *o2* gene, which strongly represses the Z23 chains [4, 6, 12] while leaving unaltered the total amino acid composition of the $Z_1$ fraction [2, 8]. It can also be seen that the *o2* gene does not have any effect on the amino acid composition of the different MW classes of zeins. This is consistent with the hypothesis that *o2* acts mainly as a repressor, by strongly inhibiting the accumulation of zein molecules, and not by synthesizing new types of proteins.

At present, we do not know what is the physiological role of Z13.5 and Z9.6 chains (which generally represent 20–30 % of the total (*i.e.* $Z_1 + Z_2$) zein molecules) in the cell. Due to their very high content in S-containing amino acids, it is tempting to speculate that these chains could play a role in the formation of zein granules in the cell, by stabilizing macromolecular aggregates via formation of –S–S– bridges.

Table 1. Amino acid composition of $Z_1$ and $Z_2$ fractions and of zein chains from SDS-electrophoresis*

| Amino acid | $Z_1(+)$ | $Z_2(+)$ | Z23 + Z21 chains | | Z13.5 chains | | Z9.6 chains |
|---|---|---|---|---|---|---|---|
| | | | + | *o2* | + | *o2* | + |
| Lys | 0.3 | 0.3 | 0.5 | 1.1 | 0.3 | 0.7 | 0.4 |
| His | 0.8 | 2.4 | 3.2 | 1.3 | 1.2 | 1.6 | 1.2 |
| Arg | 1.2 | 1.6 | 1.9 | 1.5 | 2.4 | 2.1 | 2.0 |
| Asp | 5.5 | 3.7 | 5.6 | 4.6 | 2.5 | 1.7 | 2.2 |
| Thr | 3.0 | 4.0 | 3.2 | 2.9 | 3 9 | 3.4 | 4.1 |
| Ser | 6.4 | 6.1 | 7.1 | 6.8 | 6.0 | 6.3 | 5.8 |
| Glu | 23.6 | 20.7 | 17.3 | 18.5 | 17.9 | 20.5 | 22.9 |
| Pro | 10.6 | 15.4 | 11.3 | 11.8 | 11.9 | 11.9 | 11.6 |
| Gly | 2.2 | 5.6 | 4.8 | 3.8 | 9 0 | 8.9 | 8.6 |
| Ala | 12.7 | 11.8 | 11.0 | 11.3 | 10.4 | 10.2 | 10.7 |
| Cys | traces | traces | traces | traces | traces | traces | 1.4 |
| Val | 2.7 | 3.9 | 3.8 | 3.8 | 3.4 | 4.1 | 3.6 |
| Met | 0.1 | 1.0 | traces | 0.3 | 5 3 | 4.9 | 3.9 |
| Ile | 3.8 | 3.0 | 2.5 | 3.4 | 1.3 | 1.2 | 1.1 |
| Leu | 19.8 | 14.5 | 19.4 | 18.0 | 11.6 | 9.1 | 9.6 |
| Tyr | 3.5 | 3.7 | 3.4 | 2.2 | 4.7 | 3.9 | 3.4 |
| Phe | 5.8 | 4.7 | 4.3 | 4.8 | 2 8 | 3.2 | 2.5 |

* Samples were from W64A normal (+) and *o2* inbred lines. Values in μM %. Each entry is the average of duplicate runs.

Table 2 reports the amino acid composition of fractions *A* (the most acidic, pI *ca* 5) through *H* (the most basic, pI *ca* 8.5), isolated from preparative IEF. Here, changes in amino acid composition are more difficult to detect. Most amino acids are identical or show random fluctuations. Only those amino acids which would either progressively increase or decrease in the series of peaks *A*–*H* were considered to show significant changes. On this basis,

Table 2. Amino acid composition of zein fractions from preparative IEF

| Amino acid | Total zein | Relative amounts (μM %) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *A* | *B* | *C* | *D* | *E* | *F* | *H* |
| Lys | 0.3 | 0.2 | 0 8 | 0.3 | 0.2 | 0.2 | 0.3 | 0.2 |
| His | 1.2 | 1.1 | 1.1 | 1.2 | 0.8 | 1.2 | 1.0 | 0.9 |
| Arg | 1.3 | 1.6 | 1.4 | 1.4 | 1.2 | 0 9 | 1 0 | 1.2 |
| Asp | 4.7 | 5.1 | 4.9 | 4.9 | 4.7 | 4.8 | 4.7 | 4.7 |
| Thr | 3.0 | 3.1 | 3.0 | 3.1 | 2 4 | 2.3 | 2.4 | 2.4 |
| Ser | 5.2 | 4.4 | 4.9 | 4.9 | 5.3 | 5.3 | 6.6 | 6 6 |
| Glu | 21.9 | 19.0 | 21.0 | 20.9 | 22.4 | 21.4 | 22.3 | 21.2 |
| Pro | 9.8 | 8.9 | 10.5 | 8 2 | 9 4 | 10.3 | 9.7 | 9.7 |
| Gly | 2.9 | 2.9 | 2.3 | 2.3 | 2.2 | 3.4 | 2.4 | 2.1 |
| Ala | 11.7 | 11.5 | 12.5 | 12.5 | 12.6 | 13.4 | 13.5 | 13.7 |
| Cys | traces | traces | traces | traces | traces | traces | traces | traces |
| Val | 4.2 | 5.8 | 5.0 | 4.9 | 3.7 | 4.2 | 3.6 | 3.6 |
| Met | 0.6 | 1.3 | traces | 0.5 | 0.4 | 0.3 | traces | traces |
| Ile | 3.9 | 4.2 | 4.1 | 3.8 | 3.8 | 3.4 | 4.9 | 4.1 |
| Leu | 17.5 | 18.4 | 19.1 | 18.4 | 20.6 | 19.1 | 19.7 | 20.1 |
| Tyr | 4.1 | 6.2 | 5.4 | 5.4 | 3.8 | 2.7 | 3.1 | 3.1 |
| Phe | 6.4 | 7.6 | 6.8 | 7.5 | 6.2 | 7.1 | 6.4 | 6.3 |

Data from H56 inbred line; fraction *A* through *H* refer to the most acidic until the most basic zein components. Each entry is the average of duplicate runs.

and considering that H56 zeins are constituted essentially of Z23 and Z21 chains (which correspond to about 200 amino acid residues per mole), we have calculated a variation in at least 18 residues in going from peak *A–H*. In particular, there are positive changes in Ala (+4 residues), Ser (+4 residues) and negative changes in Val (−4 residues) and Tyr (−6 residues). This suggests that the zein heterogeneity demonstrated by IEF can be due to mutations in some genes responsible for zein synthesis [7]. However, these changes, which mostly affect neutral or hydrophobic amino acids, are insufficient to explain the broad spectrum of pI's observed in IEF. Possibly, the high charge heterogeneity could be due to the concomitant action of a partial deamidation *in vivo* of some glutamine and asparagine residues.

## EXPERIMENTAL

*Protein extraction.* Endosperms from the inbred lines W64A and H56 were used throughout the work. Zein extraction was done according to ref. [3] with minor modifications [5].

*Preparative SDS-electrophoresis.* This was performed in 6 mm thick gel slabs in a E–C Apparatus cell, by the disc procedure of ref. [12]. A total of 10–20 mg zein from the W64A line, denatured in SDS, was applied to a 15% acrylamide gel. After a 12 hr run, at 50 V, the zein fractions in the gel were revealed by a brief exposure to 10% TCA. The 3 major bands, Z23 + Z21, Z13.5 and Z9.6, were then extracted from their respective zones by four washings with 0.1% SDS in 50 mM Pi buffer pH 6.8. These eluates were extensively dialyzed against 55% (w/v) *iso* PrOH. Complete SDS removal was tested with rosaniline hydrochloride [13]. The N content of each fraction was tested by the method of ref. [14]. Known amounts of protein were then hydrolyzed for amino acid analysis.

*Preparative IEF.* This was done as described in the LKB Instruction Manual I-8100-E04, using the LKB 8100-2 column, with a total sample load of 220 mg zein from the H56 line. The column contained a 5–50% sorbitol gradient, 6 M urea, 1% Ampholine pH 6–9 and 0.1% Ampholine pH 3.5–10. 8 major fractions (peaks A-H) were recovered. Their purity was checked by analytical IEF, as previously described [7, 15]. Fractions A-H were extensively dialyzed against $H_2O$, lyophilized and then weighed for hydrolysis and subsequent amino acid analysis.

*Hydrolysis conditions.* About 0.5 mg of dialyzed and lyophilized protein, either from SDS- or IEF-separations, was suspended in 2 ml of 5.7 N HCl in a hydrolysis vial. Constant boiling HCl was obtained by repeated distillation on quartz, Norleucine (30 nmol) was added as internal standard to each sample. Prior to sealing, the vial was repeatedly evacuated and flushed with $N_2$. The tubes were then sealed under vacuum and hydrolyzed in an oven at 110° for 24 hr. To ensure reproducible conditions all samples from the SDS- or the IEF- runs were treated simultaneously. Hydrolysates were then dryed by rotary evaporation and appropriate sample amounts were loaded in a Beckman Multichrom 4255 analyzer dissolved in the recommended pH 2.2 buffer. About 98% sample recovery was obtained by this procedure, as judged from norleucine recovery. Automatic peak integration was obtained with a Digital PDP8/e computer.

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## REFERENCES

1. Wall, J. S. (1964) in *Proteins and Their Reactions*, Symposium on Foods (Schultz, H. W. and Anglemier, A. F., eds.) pp. 315–341, AVI, West Port.
2. Sodek, L. and Wilson, C. M. (1972) *J. Agric. Food Chem.* **19**, 1144.
3. Landry, J. and Moureaux, T. (1970) *Bull. Soc. Chim. Biol.* **52**, 1021.
4. Lee, K. H., Johns, R. A., Dalby, A. and Tsai, C. Y. (1976) *Biochem. Genet.* **14**, 641.
5. Soave, C., Pioli, F., Viotti, A., Salamini, F. and Righetti, P. G. (1975) *Maydica* **20**, 83.
6. Gianazza, E., Righetti, P. G., Pioli, F., Galante, E. and Soave, C. (1976) *Maydica* **21**, 1.
7. Righetti, P. G., Gianazza, E., Viotti, A. and Soave, C.(1977) *Biochim. Biophys. Acta*, in press.
8. Misra, P. S., Mertz, E. T. and Glover, D. V. (1975) in *High Quality Protein Maize*, CYMMYT-Purdue International Symposium on Protein Quality in Maize, El Batan, Mexico (1972), Dowden, Hutchinson and Ross, Eds.) pp. 291–305. Stroudsburg.
9. Paulis, J. W. and Wall, J. S. (1972) *Biochim. Biophys. Acta* **251**, 57.
10. Ohta, T. and Kimura, M. (1971) *Science* **174**, 150.
11. Wang, J. L., Bruce, A., Cunningham, B. A. and Edelman, G. M. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1130.
12. Laemmli, U. K. (1970) *Nature* **227**, 680.
13. Pitt-Rivers, R. and Impiombato, A. F. S. (1968) *Biochem. J.* **109**, 825.
14. Jaenicke, L. (1974) *Anal. Biochem.* **61**, 623.
15. Righetti, P. G. and Righetti, A. B. B. (1975) in *Isoelectric Focusing* (Arbuthnott, J. P. and Beeley, J. A., eds.) pp. 114–131. Butterworth, London.