STRUCTURAL STUDIES OF THE ZEIN PROTEINS FROM CORN AND STUDIES OF THE NUCLEIC ACIDS RESPONSIBLE FOR ZEIN SYNTHESIS

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LIST OF SYMBOLS AND ABBREVIATIONS

EDTA - Ethylenediamine tetraacetic acid

SDS - Sodium dodecylsulfate

MW - Molecular weight

Tris - Trishydroxymethyl amino methane

TCA - Trichloroacetic acid

IEF - Isoelectric focusing

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

NP-40 - Nonidet P-40

Z_I - Zein I

Z_{II} - Zein II

ASG - Alcohol-soluble reduced glutelin

DAP - Days after pollination

CNBr - Cyanogen bromide

BPB - Bromo phenol blue

2-ME - 2-Mercaptoethanol

O_2 - Opaque-2

CHAPTER I

INTRODUCTION

The proteins of cereal seeds have been customarily divided into three classes, based on their solubilities. (1) Water and saline soluble proteins which during fractionation are generally accompanied by the free amino acids. (2) Alcohol soluble prolamines. (3) Glutelins, generally soluble only under the more extreme conditions of higher pH and/or denaturing conditions.

In terms of absolute amount, the first group — the saline and the water soluble proteins — is of limited significance, contributing less than 10% to the total endosperm nitrogen. Functionally, it is of major importance because of its enzyme content. The converse is true of the other two protein groups, where the bulk of the protein is found in the prolamine and glutelin fractions. Their primary functional role apparently is to act as a repository of nitrogen for the subsequent benefit of the embryo at the time of germination. The proportion of protein in the prolamine fraction to that in the glutelin fraction is a characteristic of each species. In maize, wheat and sorghum, the prolamine fraction accounts for more than half the storage protein (Paulis et al., 1975; Mosse, 1966). In rice, however, most of the storage protein occurs in the glutelin fraction with 3-12% in the prolamine fraction (Cagampang et al., 1966).

The glutelin and prolamine fractions so far examined are all heterogeneous. Glutelin fractions from wheat and maize have generally been extracted by alkali. Glutelin is highly insoluble in the most potent protein-dissociating solvents, but upon reduction of its disulfide bonds, it yields polypeptides which are soluble in 8 M urea, 6 M guanidine hydrochloride, or SDS solutions (Moureaux et al., 1968; Paulis et al., 1971; Paulis et al., 1969). Using these solvents it has been possible to demonstrate heterogeneity of these reduced glutelin polypeptides by starch gel urea electrophoresis (SGUE) (Mita et al., 1971; Dimler, 1974), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bietz et al., 1973; Wasik et al., 1974). Where 10-20 bands are routinely observed in glutelin fractions from wheat and maize, only three components could be detected in rice glutelin (Sawai et al., 1968).

Mature corn endosperm contains protein bodies in a protein matrix (Wolf et al., 1967). The protein body contents are soluble in 70% ethanol, hence are zein; whereas the matrix is not ethanol-soluble. The protein matrix was isolated from immature corn endosperm by means of density gradient sedimentation (Christianson et al., 1974) and has electrophoretic properties, and an amino acid composition quite suggestive of glutelin.

The prolamines are proteins with characteristics similar to zein in all cereal grains. Their name derives from the fact that they are all rich in the amino acid proline and in amide nitrogen (McKinney, 1958). Zein is generally restricted to designate the prolamine of the genus Zea (Paulis et al., 1977a). Prolamines are dissolved by aqueous solutions of urea, detergents, and many polar organic solvents

including alcohols, ketones, and ether (Mosse, 1961). Zein is unique among proteins, since it can be dissolved in high concentration in many purely organic solvents, primarily glycols or amines (Evans et al., 1941a) or in mixtures of organic solvents (Evans et al., 1941b). Zein from corn cannot be dissolved in either dilute acetic acid or 3 M urea as can wheat gliadin, probably because of zein's lower level of basic amino acids and polar amino acids (Wall, 1964). Zein hydrates to a lesser degree, and is more compatible with organic solvents than gliadin as a result of its greater content of hydrophobic amino acids, such as leucine. Numerous workers have concluded that prolamines consist of a group of heterogeneous proteins as evidenced by moving boundary electrophoresis for gliadin (Laws et al., 1948), zein (Foster, 1949), and hordein (Waldschmidt-Leitz, 1959). However, lack of symmetry of the ascending and descending patterns indicated that extensive interactions of the proteins occurred in some buffer systems which also frequently included alcohols or detergents to promote solution (Jones et al., 1959). Aluminum lactate-lactic acid buffers when used in electrophoresis gave several peaks with symmetry between ascending and descending patterns, from which it was clearly demonstrated that gliadin consisted of several components.

The prolamines of wheat, rye, and oats are highly heterogeneous. At least four zones were detected on moving boundary electrophoresis (Waldschmidt-Leitz et al., 1963). Similar fractionation of wheat prolamines has been obtained on sulfoethyl cellulose columns (Heubner et al., 1968). Some of these fractions can be further separated into subfractions, possibly reflecting the hexaploid genetic constitution of wheat (Wrigley et al., 1972). The data suggest that within a fraction of

prolamine, subfractions contain very closely related proteins. About two-thirds of the chymotryptic peptides of three subfractions of SP-2 gliadin are shared by all three subfractions (Booth et al., 1969). The first 10 amino acids in alpha, and alpha 2 gliadin are identical in sequence (Kasarda et al., 1974). Similarity among omega gliadins has also been noted (Charbonnier, 1974). SDS-PAGE of gliadin fractions (Bietz et al., 1973) show the presence of main bands of 44 and 36 kilodaltons. Bands of omega gliadins are found at 69 and 78 kilodaltons. Different sulfoethyl cellulose fractions have slightly different mobilities.

The prolamines of maize, millet and sorghum are much less heterogeneous. Only one fraction was seen in moving boundary electrophoresis (Waldschmidt-Leitz et al., 1962; Scallet, 1947). By SDS-PAGE on 10% gels, zein was resolved into two bands having mol. wt. of 22,000 and 24,000 (Paulis et al., 1977a). It has been demonstrated that zein contains greater number of components in the (PAGE) polyacrylamide gel electrophoresis, by using aluminum lactate-lactic acid buffer (Paulis et al., 1977b). The number of bands could reach 7-8 bands. Each sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) band has been suggested to consist of several proteins varying in charge and amino acid content. Zein has been reported to be very heterogeneous with respect to molecular weight (Turner et al., 1965), and net charge (Scallet et al., 1947; Foster et al., 1950; Sodek et al., 1971). However, on SDS-PAGE gels there are only two bands. These two components do not correspond to α and β zein (McKinney, 1958) which are separated on basis of solubility, probably reflecting changes in secondary or tertiary structure and possibly covalent modifications

(Burr et al., 1979). Heterogeneity of zein was also demonstrated by the finding of two N-terminal and three C-terminal amino acids (Waldschmidt-Leitz et al., 1962). Recently, SDS-PAGE revealed that zein prepared from normal maize inbred contains six separable components (Lee et al., 1976). The two major polypeptides, Zein A and B, have molecular weights estimated by various laboratories as between 21,000 and 25,000 and between 19,000 and 22,000, respectively (Difonzo et al., 1977; Melcher, 1979). Amino acid analysis of these two components show they are similar. This was deduced from SDS-PAGE gel analysis, or from the effect of the opaque-2 gene, which strongly represses the Zein A chains, while leaving unaltered the total amino composition of the Zein I fraction (Sodek et al., 1971; Misra et al., 1975).

Zeins are very rich in glutamic acid (≃ 20%), leucine (≃ 18%), proline (≃ 11%), alanine (≃ 11%), and extremely deficient in the essential amino acids lysine and tryptophane (Wall, 1964). The minor polypeptides which although present in classical zein extracts are more prominent in extracts of the residue remaining after the extraction of zein with ethanol containing a reducing agent (Landry et al., 1970). From the amino acid analysis, the minor bands appear to be zein chains (Gianazza et al., 1977). This is confirmed by the fact that such chains are present in zein protein bodies and that in IEF they give a distribution pattern which is a subset of that of higher MW chains (Gianazza et al., 1976). It has been speculated that such low MW chains have partly reduced the overall strong hydrophobicity typical of zein molecules, with a marked decrease in the levels of leucine, isoleucine and phenylalanine. Also they are very rich in S-containing amino acids as compared to the higher MW chains, particu-

larly in methionine present at the level of 4-5%.

Extraction of the defatted corn endosperm meal with 70% ethanol and 0.5% sodium acetate removes Zein I (Z_I). However variable amounts of zein remain in the residue which contains the glutelin fraction. A fraction has been isolated which is soluble in alcohol containing 0.1 M β-mercaptoethanol, this fraction has been referred to as glutelin-1 (Moureaux et al., 1968), Zein II (Z_{II}) (Sodek et al., 1971), or alcohol-soluble reduced glutelin (ASG) (Paulis et al., 1971). It is very different from the alcohol-insoluble fraction of reduced glutelin which has a much higher lysine content and lower leucine level. It has zein-like properties, being soluble in alcohol, but its amino acid composition and gel filtration pattern show that it is composed of unique proteins. Because of its deficiency in lysine, its quantitative extraction has been used in fractionation schemes for studying proteins of high-lysine corns (Misra et al., 1972; Misra et al., 1975a). By dialyzing the ASG preparation against water, it can be separated into two fractions, distinctly different from each other by amino acid analysis, SDS-PAGE, and PAGE.

The ASG contain 20% of the total nitrogen. As reported by the authors (Paulis et al., 1977b) and others (Landry et al., 1970), ASG is different from zein in amino acid composition. It contains much more histidine, proline, glycine, and methionine and less aspartic acid, glutamic acid, isoleucine, leucine, and phenylalanine than zein.

Fractionation of ASG by dialysis against water produces a soluble protein that represented 33.0% of the total ASG. Except for the amount of lysine, this protein had an amino acid composition different from total ASG, zein, or water insoluble ASG. The biggest differences

were in its high histidine, proline, and valine contents and low aspartic acid, alanine, tyrosine, phenylalanine, and methionine (Misra et al., 1976). Although water-insoluble ASG is closer in amino acid composition to zein than to that of soluble ASG, it exhibits large differences from zein in content of proline, glycine, methionine, isoleucine, leucine, and phenylalanine. There is 4 times as much methionine in this fraction as in zein. The water-soluble ASG may be soluble in both water and alcohol since its amino acid composition contains less nonpolar-amino acids than zein. The high methionine portion of ASG precipitates readily upon dialysis against water and thereby separates from the rest of ASG. This fraction contains mainly low MW components. In earlier studies of the fractionation of glutelin by gel filtration (Paulis et al., 1971) and SDS-PAGE (Paulis et al., 1969), the high methionine protein was also shown to be low MW. The same studies show that zein appears to be cross linked to alcohol soluble glutelin through disulfide bonds.

Isoelectric focusing (IEF) in polyacrylamide gels gives resolution of zein into 8 to 15 bands according to the genotype. An extensive analysis of various maize lines, shows that at least 28 positions appear to be occupied by specific zein bands in the pH range 6 to 9 (Soave et al., 1976).

The possible band positions were codified by progressive numbers from the more alkaline to the more acidic components. The charge heterogeneity was found to be independent of the developmental stage of the endosperm or of environment interaction and to be due essentially to amino acid substitutions among the IEF zein components (Righetti et al., 1977a). The study of several inbred lines of maize proved

that the IEF pattern of zein was characteristic of the genotype and most of the strains gave different patterns (Gentinetta et al., 1975). In crosses between inbreds with different zein patterns, the two reciprocal F_1 generations showed additive patterns and the amount of each components correlated with the gene dose present (Righettiet al., 1977b). When seeds from the F_2 generation were analyzed, simple Mendelian segregation of IEF bands was observed. In recent work, Soave et al. (1978) showed that three zein bands (n^o 1, 2 and 3) appeared to be always associated together and with the zein regulatory element opaque-2 (chr. 7, pos. 16); two other components (n^o 6, 12) segregated independently of themselves and from the three bands linked with the opaque-2 locus. These data indicated that factors responsible for the synthesis of some zein components were located in at least three different regions of the genome and suggested an evolutionary pattern of opaque-2 (02), the overall zein regulatory element, and of the genetic factors specific for each band.

Pernollet (1978) reviewed protein bodies and mentioned that cellular organelles containing storage proteins are found in storage tissue, within the animal as well as the vegetable kingdom, particularly in reproductive organs. They are found in haploid, diploid and triploid tissues not only in angiosperm seeds, but also among gymnosperms and in animal oocytes. The main storage tissue of monocots is a triploid tissue (the endosperm) which is constituted of the starchy endosperm and the aleurone layers (outer layers). Both these tissues contain protein bodies which present some differences which can be correlated with the difference in function. The starchy endosperm is merely a storage tissue while the aleurone layer has an

important role in enzyme secretion during germination processes.

According to Pernollet (1978), protein bodies were named 'aleurone grains' after the Greek word for flour. He confirmed the protein nature of these organelles and the presence of internal inclusions within protein bodies, the globoid (phytin) and the crystalloid (proteins).

As mentioned by Pernollet (1978) protein bodies are small, more or less spherical organelles. They vary in size: diameters have been reported from 0.1 to 25 μm, the average value being a few microns. Protein bodies are bound by a membrane which seems to be single in most cases.

Small electron dense particles containing protein bodies have been identified in the endosperm tissue from corn (Khoo et al., 1970; Wolf et al., 1967), developing wheat kernels (Graham et al., 1962; 1963a; Jennings et al., 1963), rice (Mitsuda et al., 1967), sorghum (Seckinger et al., 1973; Hoseney et al., 1974) and setaria (Rost, 1972). Various internal structures of protein bodies can be distinguished according to Pernollet (1978). Some (e.g. pea cotyledon, Pisum sativum) have a homogeneous or granular structure without inclusion embedded within a homogeneous proteinaceous matrix, while others (e.g. rice endosperm, Oryza sativa) show a granular concentric pattern of electron density with minute granules 150 Å in diameter. Other protein bodies exhibit internal inclusions which can be of two types; the globoid and the crystalloid as in castor bean (Ricinus communis) endosperm. The globoid is a phytic acid storage inclusion, while the crystalloid is proteinaceous. The proteinaceous inclusion is only present when a globoid is seen within a protein body. In case

of Graminea seeds, it is now clear that two different kinds of protein body exist: In the aleurone cells these organelles have inclusions, while in the starchy endosperm no such inclusions have been found. According to Pernollet (1978) a granular concentric structure has been found in the starchy endosperm, in case of barley, wheat, rice, sorghum and various millets. Only maize exhibits a homogeneous protein matrix. It has been possible to purify protein bodies after their separation from other cell organelles. Purified protein body fractions have been isolated from maize, rice, wheat and sorghum. There are numerous differences in biochemical composition between protein bodies from various species. On the average, however, protein bodies are composed of storage proteins (ca 70-80% of dry wt), salt of phytic acid (ca 10% of dry wt), hydrolytic enzymes, cations and ribonucleic acid. Less important and more variable is the presence of such compounds as carbohydrates, oxalic acid salts, lipids and tocopherol as reported by Pernollet (1978).

The protein body contains primarily storage protein. In rice, only 11% of the protein body protein is extractable with NaCl whereas 83% can be recovered with dilute acid. In maize, the protein bodies are isolated in both a free and a matrixed form. The free protein bodies are composed entirely of zein proteins. This can be extracted from the matrixed protein by aqueous alcohol. The protein bodies are almost completely dissolved, leaving behind an insoluble matrix containing components of the glutelin fraction. Glutelin components are found unassociated with the protein bodies, although this glutelin has a different amino acid composition than total glutelin. A similar distribution of prolamine and glutelin has been noted in sorghum

(Seckinger et al., 1973).

Protein bodies can be fragile (Mitsuda et al., 1967). Wheat protein bodies do not survive the dehydration associated with maturation, since they can only be identified in immature kernels (Seckinger et al., 1970). Protein bodies in the soft endosperm of sorghum may rupture on drying (Hoseney et al., 1974). Two sizes of protein bodies have been identified in wheat (Jennings et al., 1963) and there is evidence of a different protein composition in the two fractions (Jennings, 1968; Jennings et al., 1963a). Small protein bodies replace the large ones in the o2 mutant of maize (Wolf et al., 1967). It is thus possible that glutelin is formed in small protein bodies which readily disrupt soon after formation.

Are storage proteins synthesized in the cytoplasm and then transported into protein bodies which would be then considered as vacuoles or are they synthesized within the storage organelles which would then be considered as plastids? The general view of a single membrane binding the protein body would support the first hypothesis, while the presence of functional ribonucleic acids, associated with them would support the second.

Using electron microscopy, as reported by Pernollet (1978), evidence was obtained that proteins were synthesized in the endoplasmic reticulum or the Golgi apparatus. On the other hand, it was found that wheat aleurone layer protein bodies develop close to the Golgi apparatus. In cotton a relationship was found between protein bodies and cytoplasmic proteosynthesizing structures and evidence of a relationship between the lamellar endoplasmic reticulum and protein bodies of lima bean cotyledons was established. Bailey et al. (1970) showed in broad

bean, by autoradiographic studies, that proteosynthesis was associated with endoplasmic reticulum and that the storage proteins were quickly transported into protein bodies. Bain and Mercer (1966) have also shown in the pea that storage proteins are synthesized by the endoplasmic reticulum surrounding vacuoles, in which they accumulate along the external single membrane. Righetti et al. (1977b) described an analogous scheme for maize endosperm protein body ontogeny. In protein bodies with globoids, the early storage protein deposits are located around this inclusion which is formed first. Morton et al. (1964a) showed that isolated protein bodies are able to synthesize storage protein in vitro and that there are two different proteosynthetic systems, one for storage proteins and one for cytoplasmic proteins. The same authors (in 1964b) showed that there are specific ribosomes strongly bound to protein bodies.

This new concept has been criticized. Wilson et al. (1967) could not reproduce Morton's experiments with maize endosperm and claimed that the results were due to bacterial contamination. By contrast, some authors succeeded in reproducing Morton's results. Larkins and Dalby (1975) obtained in vitro zein synthesis with isolated protein bodies from maize endosperm. Burr and Burr (1976) showed clearly by electron microscopy that in maize endosperm, ribosomes are bound externally to the single membranes of protein bodies. As they are isolated with the protein bodies, storage proteins can be synthesized in vitro using protein body isolates. These ideas have been supported by Khoo and Wolf (1970) and Righetti et al. (1977b). Isolation and characterization of ribonucleic acids from protein bodies are in fairly good agreement with this concept as reported by Pernollet (1978).

It is not clear if protein bodies are plastids or if they are formed from the endoplasmic reticulum. As reviewed by Pernollet (1978), results with rice endosperm make the second hypothesis more probable. It has been shown that the protein body double membrane might be an artifact. It was discussed that protein bodies develop within an endoplasmic reticulum lumen and so are surrounded by ER membranes. If this hypothesis is true, the membrane of protein bodies would be initially an ER membrane which would explain the ribosomes surrounding the protein body. Recent work on protein body development has indicated an important role for the Golgi apparatus in protein synthesis. As mentioned by Pernollet, there could be three kinds of protein bodies which are quite different from proteoplasts. In orchid root or in bean root tips, these organelles exhibit a typical structure with double membrane, starchy inclusions and internal ribosomes. Seed protein bodies can no longer be compared with oocyte protein bodies, which are not synthesized within the cell where they are stored, since they are transported into the oocyte by a process akin to pinocytocis.

The processes in protein body development fit in with the hypothesis of an origin from endoplasmic reticulum by accretion of protein synthesized on the rough endoplasmic reticulum ribosomes and vectorially transported to the lumen (Khoo et al., 1970).

Synthesis of the protein of the developing seed is regulated: two phases of protein accumulation occur (Graham et al., 1963b). The salt soluble proteins accumulate during the first phase. The storage proteins are made during the early stage, but the primary accumulation occurs later in development (Jennings et al., 1963b; Palmiano et

al., 1968).

In maize, the saline soluble nitrogen which contains albumins, globulins and non-protein nitrogen increases in the initial and middle stages of maturation and decreases in later stages (DiFonzo et al., 1977; Soave et al., 1975). Zein synthesis begins around the 14th day and then proceeds linearly until the 35th day after pollination (Righetti et al., 1977b; Soave et al., 1975). Glutelin-proteins are synthesized linearly until the late stages. In wheat, the accumulation of alkali soluble storage proteins begins before the accumulation of acetic acid soluble proteins occurs (Jennings, 1968).

Some mutants of maize, Opaque-2, produce a nutritionally favorable effect by lowering the level of lysine-poor protein, zein, and permitting a secondary increase in non-zein proteins, notably the glutelin fraction (Mertz et al., 1964; Jimenez, 1966). Subsequently, several other mutant genes in maize, i.e., floury-2 (fl_2) (Nelson et al., 1965), opaque-7 (O-7) (McWhirter, 1971), opaque-6 (O_6) and floury-3 (fl_3) (Ma and Nelson, 1975) have been found to reduce zein content and to increase lysine and tryptophane levels. Similar genes have been found in barley (Munck et al., 1970; Ingversen et al., 1973) and in sorghum (Singh and Axtell, 1973). These genes reduced the level of prolamine and increased the amount of albumin and globulin.

In a high lysine maize double mutant, sugary-1/opaque-2, 12 out of 16 amino acids showed higher concentration per endosperm in all three stages of development (17, 24, 47 days after pollution (DAP))(Arruda et al., 1978). Significant differences were found for alanine, glutamic acid, aspartic acid, glutamine and asparagine with each showing higher concentration in the high lysine double mutant. As these amino acids are important

components of zein (Sodek et al., 1971; Christianson et al., 1969; Paulis et al., 1969), their data suggest that sugary-1/opaque-2 genes depress zein synthesis with consequent accumulation of alanine, glutamic and aspartic acid, glutamine and asparagine.

The O_2 mutant severely suppresses the synthesis of ZA as well as the total zein. The non-allelic mutant, fl_2, appears to reduce the synthesis of the six zein polypeptides in the same proportion (Lee et al., 1976). In addition, several starch-forming mutants were reported to reduce total zein content (Dalby et al., 1975; Glover et al., 1975; Misra et al., 1975b). Starch-forming mutants may be separated into two groups: starch modified and starch deficient. When O_2 was combined with each of the starch-modified mutants, zein content was further reduced, whereas non-zein protein was not affected (Tsai et al., 1978). These results suggested that there was accumulative effect between O_2 and the starch-modified mutants gene in altering zein synthesis. On the other hand, the combination of O_2 with each of the starch-deficient mutants produced a synergistic effect, and the double mutants accumulated very little zein. The interaction of a starch-deficient-type mutant gene with O_2 appeared to differ from the combination of starch-deficient-mutants with another zein-deficient mutant, fl_2.

The double mutants bt_2O_2 were almost completely deficient in Z_I and Z_{II} at maturity. The biochemical lesion of this double mutant was examined by preparing membrane-bound polyribosomes from 22-day old kernels and <u>in vitro</u>-translation (Tsai et al., 1978). The results showed that less 10% of the ethanol-soluble protein was synthesized and the total RNA directed the synthesis of such protein was only a

trace amount. These observations suggest that the inability of the double mutant to synthesize Z_I and Z_{II} is the consequence of lacking functional Z_I and Z_{II} mRNAs. It is not clear whether the lack of zein mRNAs is the result of a specific postranscriptional modification of these RNA molecules or a transcriptional block of zein genes resulting from the interaction of O_2 with starch-deficient mutant genes.

It was shown that the O_2 mutant had much higher RNase activity than the normal counterpart (Dalby et al., 1967; Wilson et al., 1967). On this basis it was speculated that RNase was degrading zein mRNA more rapidly in the mutant than in normal. However, it was demonstrated later that the maize inbred B37 showed little difference between normal and O_2 RNase activities until much later (25 days after pollination) in development (Cagampang et al., 1972). These observations suggested that an elevated RNase activity might not be the key to the reduction of zein synthesis in maize endosperm. The double mutant bt_2O_2 contained high RNase activity, about 7.5 times greater than normal. Also, at this stage of kernel development (22 days), this double mutant had a high sucrose content. It is speculated that high sucrose concentration may alter the interaction between polysomes and endoplasmic reticulum. The double mutant contained less membrane-bound polyribosomal material than the normal (Tsai et al., 1978). The inability of zein mRNAs to associate with endoplasmic reticulum (membrane-bound polyribosomes are the principle site of zein synthesis) may make them more susceptible to hydrolysis by soluble RNase activity. The mRNAs associated with free polyribosomes are presumably more stable because of their active engagement in translation. Amplification of RNase activity in the bt_2O_2 double-mutant may

completely prevent the synthesis of Z_I and Z_{II} by preferentially degrading zein mRNAs (Tsai et al., 1978).

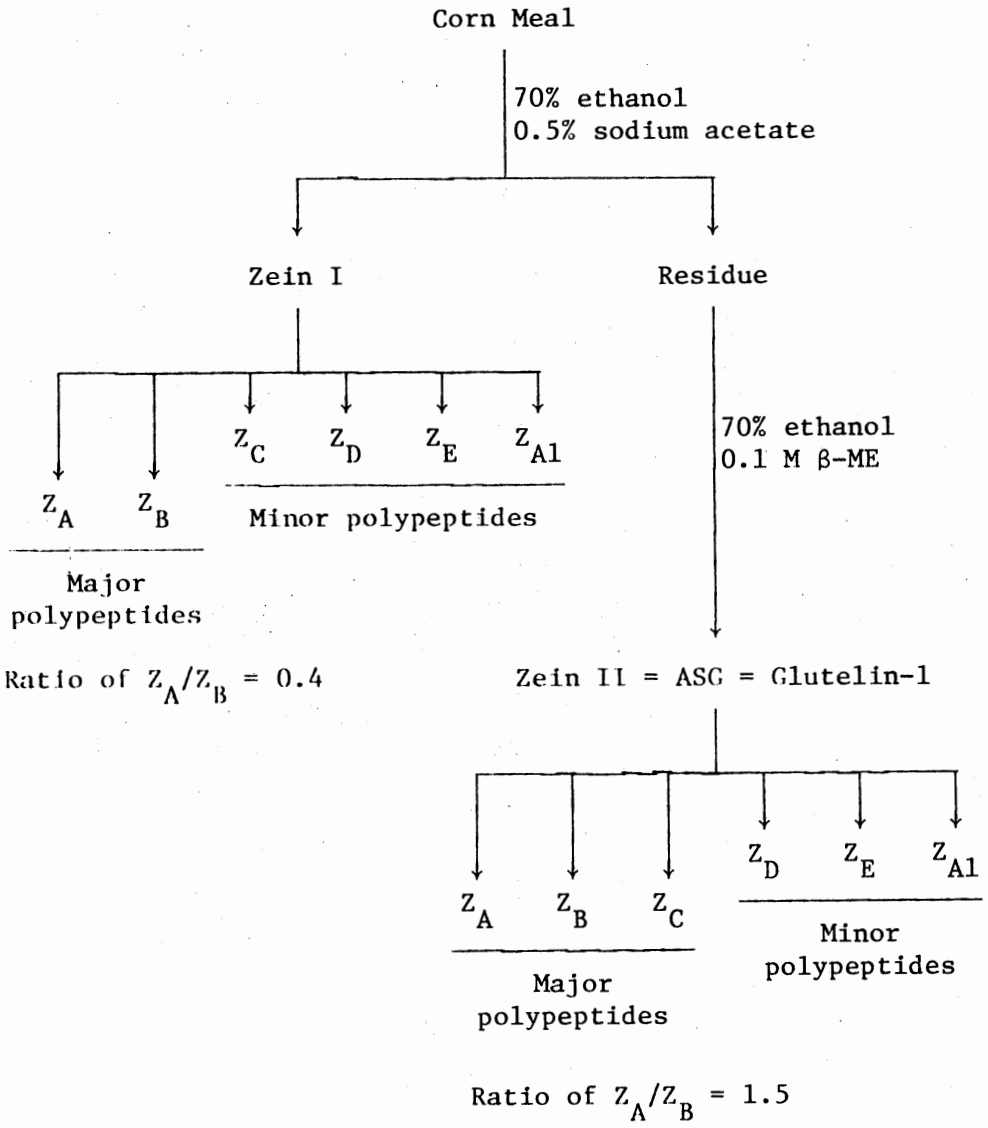
Cell division (Buttrose, 1963; Jennings et al., 1963) and increase in DNA content (Graham et al., 1962; Ingle et al., 1965) are complete early during development. The synthesis of storage proteins is therefore not dependent on concomittant cell division. The level of soluble amino acids reaches a maximum shortly after the cessation of cell division (Ingle et al., 1965). The decrease in level beyond this time is probably due to the more rapid utilization for the synthesis of protein which begins during this time (Cruz et al., 1970; Murphy et al., 1971). The level of most amino acid activating enzymes reaches a peak at the fifth week after flowering of wheat and then declines (Norris et al., 1973). The level of these enzymes could play a role in the regulation of the bulk of reserve protein synthesis since deviations from the general pattern were noted with some synthetases. RNA undergoes its most rapid accumulation before or close to the time that reserve protein synthesis becomes maximum (Cruz et al., 1970; Graham et al., 1962; Ingle et al., 1965; Jennings et al., 1963c). The level of RNA declines during the maturation of maize so that only 10% of the maximum level is left at maturity.

The interest of this laboratory in this study is to find out how the zein proteins are different in heterogeneity. It is important to know whether such heterogeneity is due to differences in primary amino acid sequences or post-synthetic modifications. Such knowledge is required to understand the possible multiplicity of mRNAs coding for zein. Estimation of the number of genes responsible for zein synthesis is necessary towards an understanding of the protein synthesis in corn

endosperm, and may lead to a site of regulation of the storage protein synthesis.

The previous description of zein proteins and its extraction can be summarized as shown in Figure 1.

Figure 1. Classification Scheme of Zein.



Molecular Weights

Z_A = Zein A = 21,600	Z_D = Zein D = 10,000
Z_B = Zein B = 19,600	Z_E = Zein E = 9,000
Z_C = Zein C = 14,400	Z_{A1} = Zein A_1 = 24,000

CHAPTER II

MATERIALS AND METHODS

Materials

Hydroxylapatite (Bio-Gel HT) was obtained from Bio-Rad Laboratories, alpha-chymotrypsin and trypsin were obtained from Worthington. Dansyl chloride and iodoacetamide were obtained from Pierce Chemical Company. Sodium dodecyl sulfate was a Serva Product, while dithiothreitol was obtained from Sigma. Corn meal was obtained locally through a retail grocer. Ampholine carrier ampholytes for the pH range 3.5 to 10 were purchased from LKB Producter. Coomassie Brilliant Blue R-250 was a Serva product, Ultra pure urea was purchased from Schwarz/Mann. Agarose and EcoRI were purchased from Sigma. Pancreatic RNase was a Worthington product. Nitrocellulose filters BA 85 were obtained from Schleicher and Schuell. Self adhesive plastic (Seal-N-Save) was obtained from Sears.

Methods

Zein Extraction

Corn meal was defatted by stirring overnight in n-hexane. Zein fractions were obtained by extracting the defatted meal with 70% ethanol, 0.5% sodium acetate (zein I) (Paulis et al., 1975). After two further extractions with the same solvent the zein II fraction

was obtained by extraction with 70% ethanol, 0.5% sodium acetate, 0.6% 2-mercaptoethanol (Sodek et al., 1971; Paulis et al., 1975). All extractions were with 10 ml solvent per g of meal at room temperature for 60 min. Albumins and globulins were not pre-extracted from the meal. The extracts were dialysed extensively against water and the precipitate which formed was dried in a vacuum dessicator.

SDS-Polyacrylamide Gel Electrophoresis (Tubes)

Polyacrylamide gel electrophoresis in SDS was carried out according to Weber and Osborn (1969). Generally 25-50 µg protein in 0.1 g/100 ml SDS, 0.01 M sodium phosphate, pH 7.0 were placed onto a 12.5 g/100 ml polyacrylamide (acrylamide:bisacrylamide::30:0.8) gel cylinder. Electrophoresis was for 12 hr at 7 mamp per cylinder. Gels were stained with coomassie brilliant blue R-250 and destained in 10% acetic acid.

Hydroxylapatite Chromatography

Zein was complexed with dodecyl sulfate by a 2 min incubation at 100^{o} of 4 mg zein in 1.0 ml 0.01 M sodium phosphate, pH 6.4, 2 g/100 ml SDS, 2 g/100 ml 2-mercaptoethanol. Samples were then dialyzed overnight against 0.01 M sodium phosphate, pH 6.4, 0.1 g/100 ml SDS, 1 mM dithiothreitol (starting buffer) (Moss et al., 1972). A slurry of hydroxylapatite was poured into a 1 cm x 50 cm column over a 0.3-0.7 cm layer of Sephadex G-25 medium. The column was equilibrated with starting buffer. Protein samples of 3 to 5 ml in volume were applied and the column was then washed with 2 column volumes of starting buffer. Linear gradients (0.25 M to 0.42 M sodium phosphate) were

formed with a simple two chambered device and flow rates of 5-10 ml per hour were obtained by gravity. Ninety drop fractions of approximately 1.5 ml were collected and their absorbance at 280 nm determined.

Amino Acid Analysis

Fractions from the hydroxylapatite chromatography were dialyzed against water and lyophilized. The protein was hydrolyzed in evacuated sealed tubes at 110°C for 22 hr in 0.20 ml 6 N HCl. Amino acid analysis was performed on a modified analyzer of the Spackman et al. (1958) type in a 7.8 mm diameter column (Liao et al., 1973).

N-Terminal Analysis

Fractions from hydroxyapatite chromatography were dialyzed against water and lyophilized and dissolved in 0.1 g/100 ml SDS, 0.2 M $NaHCO_3$, pH 8. Dansylation and hydrolysis was performed according to Gray (1972). The hydrolyzate was spotted on 15 cm x 15 cm polyamide sheets and fluorescent spots were identified under ultraviolet light after chromatograpy as described by Woods and Wang (1967).

Cyanogen Bromide Cleavage

Prior to treatment with CNBr, some zein fractions were treated with 40% mercaptoacetic acid (Alfagame et al., 1974) at room temperature for 16 hours, then dialyzed against water. About 0.10 mg of zein from selected column fractions were cleaved with a 40-fold weight excess of CNBr in 0.25 ml of 70 g/100 ml formic acid. The cleavage reaction was allowed to proceed for 25 hours at 25°C. The mixture was lyophilized at the end of the reaction.

Digestion with Proteolytic Enzymes and Peptide Mapping

Fractions from hydroxylapatite chromatography containing zein-dodecyl sulfate complexes were dialyzed against water overnight and then lyophilized. Samples were redissolved in 1 g/100 ml NH_4HCO_3, pH 8 and trypsin and chymotrypsin were added at an enzyme to protein ratio of 1:40. Digestion proceeded for 5 hr at 37°C (Salnikow et al., 1973).

Approximately 20 nmoles of protein were transferred to a spot no larger than 0.5 cm in diameter on Whatman 3MM chromatography paper. Descending chromatography in 1-butanol:acetic acid:water:(200:30:75) was used in the first dimension for 9-12 hours. After drying, electrophoresis in the second dimension was carried out at 44 volts/cm for 45 min on a flat surface or in tanks (Savant Insturments) containing Varsol as coolant and formic acid:acetic acid:water, pH 1.9 (25:100:875) as solvent. Papers were dried and stained with ninhydrin-cadmium reagent (Dreger et al., 1967). Photography was by transillumination.

Isoelectric Focusing

Zein extraction and chromatography of zein-SDS complexes on hydroxylapatite were as previously described. Isoelectric focusing was done in vertical gel tubes and gel slabs. The slabs were of 16 x 9 x 0.75 cm in a home made apparatus. Tube gels were of 11.5 x 0.6 cm. The gels (either the tubes or the slabs) contained 5% acrylamide and bis (25:1 ratio), 2% ampholines (pH 3.5-10) and 8 M urea.

Samples containing lyophilized protein-SDS complexes either from

column fractions or from whole zein complexed with SDS were dissolved in 2.5 g urea in 2.5 ml deionized water and 80 µl NP-40. Zein-samples containing SDS were dialysed against 0.01 M sodium phosphate overnight and then against distilled water. This procedure was necessary to remove most of the SDS. The samples was then lyophilized. The addition of NP-40 was necessary to avoid precipitation of the protein at the top of the gels, at the anodic side.

Other samples, not containing SDS were dissolved in 8 M urea and NP-40, and still others in 6 M urea, 10 mM Tris-glycine, pH 8.5 and 2% 2-mercaptoethanol. The gels were pre-run by applying 100V for 15 min and then 250V for 1 hr before application of sample. The slots were dried with strips of filter paper and the samples were applied. Focusing proceded for 1 hr at 100V and then at 220V for 20 hr (20V/cm). Cathode solution (bottom) and anode solution (top) were prepared according to Righetti et al. (1977b) except that sorbitol was omitted from the solutions. For tube gels cathode solution (top) contained 5 mM NaOH pH > 10 and anode solution (bottom) contained 5 mM H_3P_4 or Hc pH < 3). After focusing, the gel was washed with distilled water and then stained with $CuSO_4$:Coomassie Brilliant Blue:ethanol for 3 hrs then destained in ethanol:acetic acid:water overnight according to Ostavsky and Drysdale (1975). Bands were photographed by transillumination.

Staining for carbohydrate was with periodic acid-Schiff reagent (Fairbanks et al., 1971).

Alkylation with Iodoacetamide and Acrylonitrile

Protein was reduced overnight in 0.1 ml of 1% v/v 2-mercaptoethanol

at room temperature (Paulis et al., 1975) in deionized 8 M urea. The protein was alkylated by the addition of 20 ml of 8.1% w/v acrylonitrile-8 M urea. Incubation was for 1 hour at room temperature. For alkylation with iodoacetamide, samples were dissolved in 0.01 M sodium phosphate, pH 6.4, 2% SDS, 2% mercaptoethanol by placing in a boiling water bath for 2 min. An excess of iodoacetamide over sulfhydryl was added and the mixture left for 1 hr. 1 M NaOH was added as needed to maintain the pH greater than 8. It occasionally dropped below pH 6. After alkylation with either acrylonitrile or iodacetamide, the samples were dialyzed against water and the samples then lyophilized.

Alkylation with ^{14}C-iodoacetate

0.4 mg of Z11 was dissolved in 0.2 ml of 8 M urea, 0.1 M Tris-HCl (pH 8.0) and 120 mM 2-mercaptoethanol. After 30 min 25 µCi of [^{14}C]iodacetate (50 mCi/mmole, Amersham) was added. After a further 30 min of reaction the mixture was dialyzed against 250 ml of 10 mM Na-phosphate (pH 7), 0.1% SDS overnight.

In Vitro Product - Isoelectric Focusing

Poly A (+) RNA was extracted from developing corn endosperm as previously described (Melcher, 1979). The RNA was translated in wheat germ extracts (Marcu and Dudock, 1974) containing 100 mM KCl, 2.5 mM $MgCl_2$ by incubation for 60 min at 25°C. The total *in vitro* products were prepared for IEF by precipitation with 10% cold trichloroacetic acid. After heating to 90° for 15 min, the samples were centrifuged and the pellets washed with ether. Ethanol soluble *in vitro* products

were prepared for IEF by addition of 150-200 µg of native zein to the in vitro incubation mixture. The sample was then adjusted to 70% ethanol (total volume:1.0 ml). The tubes were heated in a 65°C water bath for 90 min with occasional shaking (Dalby, 1974). The mixture was then centrifuged at 1,000 x g for 10 min and the supernatant dialyzed against distilled water for 5 hrs. The sample was then lyophilized. A similar extraction was also performed including 1% 2-mercaptoethanol in the ethanolic extraction solution. All dried samples were dissolved in 10 mM Tris-glycine pH 8.5, 6 M urea.

mRNA Isolation

Corn (Zea mays cv. Weathermaster Ex 2) was grown in fields in Goodwell, Oklahoma in 1975 and in Perkins, Oklahoma in 1976 and 1977. At the early milk stage of development, approximately 20 to 25 days after silk emergence, the ears were removed and quick frozen in liquid N_2 and transported to the laboratory on dry ice, then stored at -70°C. Kernels were removed while frozen. Using the procedure of Larkins et al. (1976) frozen kernels were homogenized in 3 volumes of buffer A (0.2 M Tris-HCl (pH 8.5), 0.2 M sucrose, 60 mM KCl, 50 µM $MgCl_2$, 1 mm DTT), the extract was strained through cheesecloth and centrifuged in Beckman rotor JA 20 at 500 x g for 5 min. Total membrane-bound polyribosomes in the 500 g supernatant (which contained a mixture of protein bodies and rough endoplasmic reticulum (RER) were obtained by centrifugation at 37,000 x g for 10 min according to Larkins et al. (1976). The membranous pellet was suspended in buffer A containing 1% Triton X-100, and detergent-insoluble material was removed by centrifugation at 37,000 x g for 10 min. The supernatant fraction

was layered over 4 ml of 2 M sucrose in buffer B (40 mM Tris-HCl (pH 8.5), 20 mM KCl, 10 mM $MgCl_2$) and polyribosomes were pelleted by centrifugation at 50,000 rpm in 75 Ti rotor of a Sorvall OTD-50 ultracentrifuge. The polyribosomal pellet was suspended in 0.5% SDS, 10 mM Tris, 1 mM Na_2EDTA, 0.5 M NaCl (pH 7.5) and adjusted to a concentration of 20 to 25 A_{260}. A volume not exceeding 10.0 ml was applied to 0.5 g Oligo(dT)-cellulose at room temperature. Poly (A)-containing molecules were eluted with 10 mM Tris pH 7 after washing by the procedure of Aviv and Leder (1972). The sample was heated to 60°C for 3 min and rapidly chilled to 4°C, the sample was then brought to 0.3 M NaCl and the RNA was rehybridized to oligo(dT)-cellulose according to Melcher (1979). This procedure was repeated three times, and the RNA was precipitated with 2-3 volumes of ethanol after adjusting to 0.3 M NaCl. Yield was generally between 20 and 50 mg poly (A) RNA/100 g kernels. The mRNA was dissolved in 200 µl sterile distilled water and stored at -70°C.

In Vitro Protein Synthesis

A standard cell-free protein synthesis system according to Marcu and Dudock (1974) was prepared from wheat germ. The complete system in a volume of 50 µL contained: 15 µl of wheat germ supernatant (1 A_{260} unit), 20 mM HEPES (pH 7.4), 2 mM DTT, 1 mM ATP, 20 mM GTP, 40 mg/ml creatine phosphokinase, 8 mM creatine phosphate, 2.5 mM Mg-Acetate, 100 mM KCl and addition of 0.1 mM spermine according to Melcher (1979). At this point 0.25 µCi of [^{14}C]Leucine (Amersham) or 0.25 µCi of [4,5-^{3}H]-Leucine (Amersham and ICN) and 25 µM 19 unlabeled amino acids were added. Samples were incubated at room temperature after addition

of 2-3 mL of poly (A) RNA in H_2O or 2-3 ml of Tobacco Mosiac Virus (TMV). A third sample was left without RNA addition and was as a blank for radioactivity incorporation background. RNA extract from TMV (obtained from E. Fowlkes, Bishops College, Dallas, Texas) according to the procedure of Marcus and Dudock (1974), was used as standard for the activity of wheat germ. The samples were incubated for 1 hr. Protein synthesis was terminated by the addition of 1 ml 10% trichloroacetic acid to 5 mL aliquots of the incubation mixture for 10 min. Then the samples were heated at 95°C for 15 min; then chilled in ice for 10 min. The precipitated protein was collected on glass fiber filters for counting in toluene ppo in a Beckman L3150 T Liquid Scintillation Counter. The rest of the samples were stored at -20°C.

SDS-Polyacrylamide Gel Electrophoresis (Slabs) and Fluorography

Radioactive samples were analyzed on slab gels with a SDS-polyacrylamide gel electrophoresis system similar to that described by Laemmli (1970). Gels were 1.5 mm thick and consisted of 10 cm running gel of 12.5% acrylamide (acrylamide/bis acrylamide = 30:0.8) in 3.75 mM Tris-HCl (pH 8.9), 0.058 mM TEMED, and 0.075% SDS. The running gel was overlaid with a 2.5 cm stacking gel of 5% acrylamide. Freshly prepared ammonium persulfate solution was added immediately before each gel layer was poured. Samples were applied in sample buffer (0.024 M Tris-HCl (pH 8.3), 1% SDS, 1% 2-mercaptoethanol, 0.002% bromophenol blue and 10% glycerol). Electrophoresis was carried out at room temperature at 15 mamp constant current through the stacking gel and 30 mamp constant current through the running gel until the stacking

dye reached the bottom of the gel. Gels were stained in a solution of 0.1% Coomassie blue, 25% isopropanol, 10% acetic acid for 3 hrs and destained in 10% acetic acid for 5 hrs. Zein I isolated by ethanol extraction from the corn meal was used as a marker of the native protein. Some zein I was labeled with ^{14}C iodoacetate and used as radioactive marker for the in vitro zein products. Slab gels which contained radioactive samples were impregnated with ppo, dried, and exposed to preflashed (Saskey et al., 1975) Royal X-omat film according to the procedures of Bonner and Laskey (1974) and Laskey and Mills (1975).

DNA Extraction and Purification

DNA was prepared from sweet corn seeds. Homogenization was carried out in 1 volume /gm corn of 1 M NaCl, 0.1 M EDTA (pH 8). Some acid-washed sand was used to improve homogenization. 0.1 volume of 20% SDS was added to make 2% SDS final concentration.

The homogenate in some experiments was made 1 M $NaClO_4$ by adding solid or solution of 5 M stock sodium perchlorate (this step improved the extraction of pigments into the $CHCl_3$ layer, but interferred with spooling and thus lowered the yield). The mixture was heated to 70-72°C for 5 min and then cooled in ice. One volume chloroform:octanol (8:1) was added to the homogenate and the mixture stirred for 1 hr at room temperature. The aqueous phase was recovered by centrifugation at (12,000 x g) for 20 min and re-extracted with chloroform:octanol for 1 hr at room temperature. The aqueous phase was again recovered as described above and the nucleic acids precipitated by the addition of 2-3 volumes of cold ethanol.

The precipitate was collected by spooling and resuspended in 10

ml of 0.01 M NaCl, 0.01 Tris-HCl (pH 8.0) and incubated with 1 mg pancreatic RNase was heated to 80°C for 20 min prior to use. 1 mg of proteinase K was added and the mixture incubated for 1 hr at 40°C. An equal vol. of chloroform:octanol was added and stirred for 30 min. The aqueous phase was removed by centrifugation at 12,000 x g for 10 min. 2 volumes of cold ethanol was added and the DNA fibers were respooled and dissolved in 0.01 M NaCl, 0.01 M Tris-HCl (pH 8). Solid NaCl was added to make 0.3 M total. After ethanol precipitation, the DNA was recovered by spooling. The dissolution and spooling step was done twice before the DNA was dissolved in the Tris-saline (0.01 M NaCl, 0.01 M Tris-HCl (pH 8)) and stored at 4°C over a few drops of chloroform.

Iodination of RNA

Iodination of 75-100 µg of rRNA or 20-40 µg of mRNA was performed in 185 µL of 0.05 M sodium acetate (pH 4.5), 7×10^{-4} M thallium trichloride (dissolved immediately before use), 0.5% SDS and 1.4×10^{-5} NaI (including 100 µCi ^{125}I, Amersham, England) by a modification of the procedure of Commerford (1971). Since traces of free ^{125}I are easily released during the labeling procedure, incorporation was performed in the hood. The tube was capped and sealed with parafilm and incubated at 60°C for 20 min, then chilled in ice and opened in the hood, 2 volumes 0.1 M Tris-HCl (pH 9) were added and the mixture was incubated at 40°C. After 10 min the mixture was chilled and made 40% ethanol, 0.3 M NaCl and then loaded onto a small column containing 0.5 g cellulose (Whatman CF11) equilibrated with 150 mM NaCl, 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5% SDS, 35% ethanol and washed with

the equilibrating buffer until no further radioactivity was eluted. Small RNA fragments (≤ 5 S) were not efficiently bound by this column and were discarded. ^{125}I RNA fragments bound to the cellulose was eluted quantitatively with 0.01 M Tris-HCl (pH 7.9), 100-150 µg yeast tRNA was added, then solid NaCl was added to 0.3 M and 2-3 volumes of ethanol was added and stored at -70°C for 24 hrs. The ^{125}I RNA was recovered by centrifugation at 12,000 x g for 20 min, dried in a vacuum dessicator and dissolved in water before storage at -70°C.

DNA Restriction and Gel Electrophoresis

Restriction endonuclease with $EcoR_1$:Digestion reaction contained 10-15 µg corn DNA and 1 unit per µg DNA in 0.05 ml reaction mixture containing 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM NaCl, 100 µg/ml Bovine Serum Albumin. After incubation for 3-4 hr or overnight incubation at 37°C. 5 µl of stop solution (equal volumes of 20% SDS, 0.1 M EDTA) and 15 µl of sucrose-BpB were added and incubated at 65°C for 5 min. The sample was then applied to a vertical slab gel of dimensions 17 x 11 x 0.3 cm of 1% agarose in (EC apparatus). Electrophoresis was for 4 hrs at 100V.

Molecular weight of the restriction fragments was estimated by comparison to $EcoR_1$ restriction fragments of cabbage mosaic virus (CaMV). Buffer for electrophoresis was 30 mM Tris-base (pH 7.8), 36 mM sodium phosphate and 2 mM Na_2EDTA.

Transfer of DNA from Agarose Gels to Nitrocellulose Filters

Corn DNA digested with $EcoR_1$ and fractionated on agarose gels was

transferred directly to 17 cm strips of nitrocellulose filters by the method of Southern (1975).

RNA-DNA Hybridization

Radioactive RNAs was hybridized to DNA on nitrocellulose filters in two ways: RNA in 2 x SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) was used to wet DNA-containing filter strips. The filters were wrapped in self-adhesive plastic and incubated at 65°C for 24 hr. Alternatively RNA in 1.2-1.5 ml of 2 x SSC along with filter strips were placed in a vessel made from perspex (internal dimension of 0.8 mm deep by 2 cm high and about 1 cm longer than the strip of nitrocellulose) according to Southern (1975). The vessel was filled with 2 x SSC and incubated at 65°C for 10 min and the strip was fed in through the narrow opening in the top. The solvent was then drained off and the RNA solution 2 x SSC was introduced and incubated at 65°C for 24 hr. After the incubation period, strips were washed exhaustively in 1 SSC for 30 min at 65°C and treated with a solution of RNase A (20 µg/ml in 2 x SSC) at room temperature for 1 hr. After a final rinse in 2 x SSC the filter was dried in air. Autoradiography was performed for 15-20 hr at -70°C or -20°C with X-ray film and a fluorescent intensifier screen according to Swanstrom and Shank (1978).

CHAPTER III

RESULTS

Unfractionated Extracts

The predominant polypeptide in zein I extracts is zein B (Lee et al., 1976; Burr et al., 1978; Misra et al., 1975a), whose molecular weight as examined by SDS-polyacrylamide gel electrophoresis in several concentrations of gels and percentage crosslinks was 19,600. Zein A, molecular weight of 21,600, although present in zein I extracts, was more prominent in zein II extracts (Figure 2). Another band with 24,000 MW appeared only by discontinuous SDS-PAGE and this zein was called ZA_1. By densitometry it was estimated that the ratio of zein A to zein B was 0.4 in zein I extracts and 1.5 in zein II extracts. Zein C, although present in zein I extracts is only detectable when substantially more protein is applied to the gels. It is readily detectable in zein II extracts. When zein II was reduced and alkylated with iodoacetamide and acrylonitrile, zein A and zein B were shown clearly. In addition zein C and zein C_1 were seen staining in good amount (Figure 3). If the samples were not alkylated zein C and C_1 appeared as streaks (Figure 2 - tube 3); thus the alkylation resulted in better resolution and separation. Zein C_1 is probably composed of smaller polypeptides (a dimer of zein E), since when fractions from Sepharose CL-4B column containing zein C_1 were reduced again and then ran on SDS-PAGE,

Figure 2. SDS-Polyacrylamide Gel Analysis of Total Zein I Extracts. Samples in both slots contained equal amounts of protein (100 mg). A_1: is zein A, with 24,000 daltons; A: is zein A with 21,600 daltons; B: is zein B with 19,600 daltons and C: is zein C with 14,400 daltons.

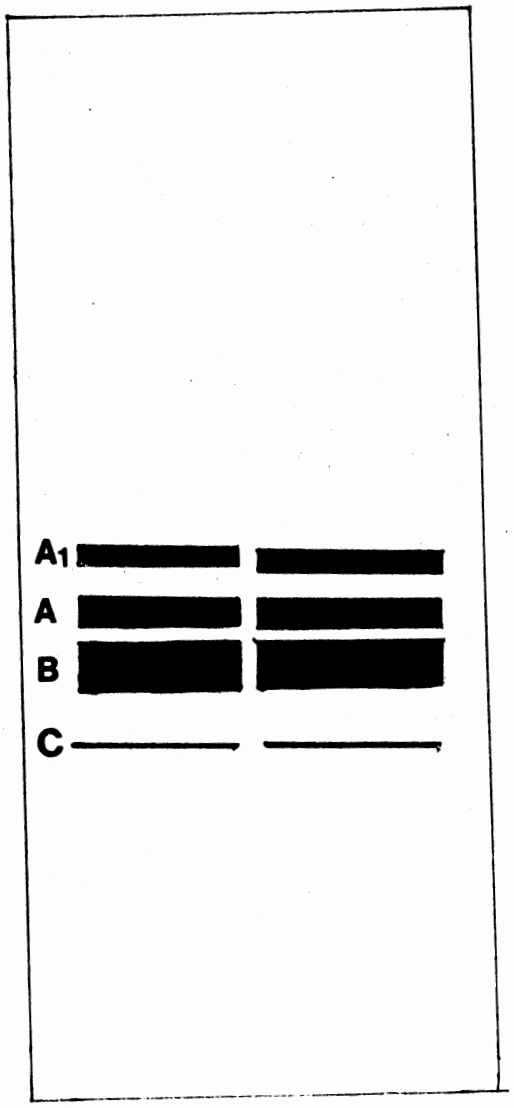
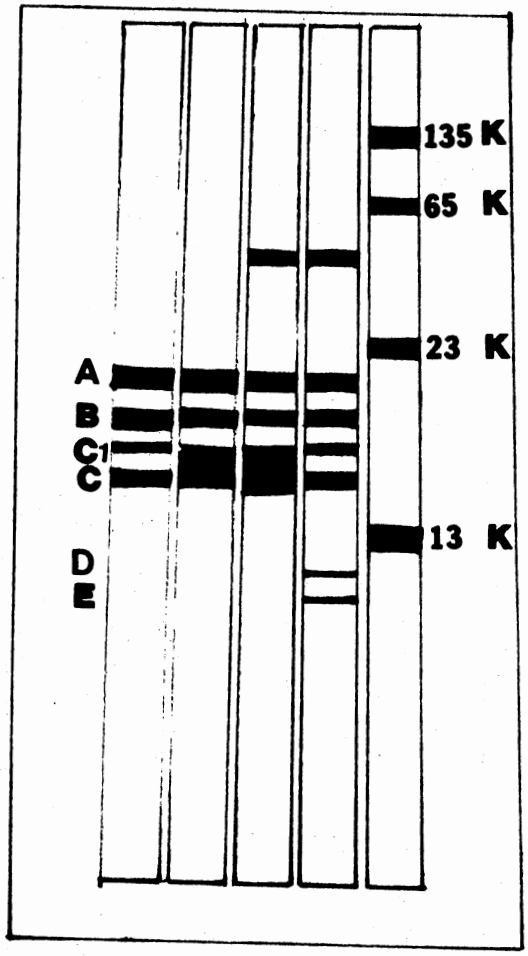

A1
A
B
C

Figure 3. SDS-Polyacrylamide Gel Electrophoresis in 12.5% Tube Gel. 1: Zein II reduced with 2-mercaptoethanol (2-ME) and alkylated with iodoacetamine. 2: Zein I reduced and alkylated as in 1. 3: Zein II reduced with (2-ME) but not alkylated. 4: Zein II reduced with (2-ME) and alkylated with acrylonitrile. 5: Markers contains from bottom to top: cytochrome c, 13,370 daltson, α-chymotrypsinogen, 23,140 daltons; bovine serum albumin, 65,400 daltons; β-galactosidase, 135,000 daltons. A: is zein A; B: is zein B; C_1: is uncompletely reduced zein polypeptides containing dimers of zein E. C: is zein C and D, and E belongs to the faint color bands of zein D and E.


135 K
65 K
23 K
13 K
A
B
C1
C
D
E

zein C_1 was converted to a low molecular weight band (zein E) (unpublished observation by Melcher). Another unreduced band can be seen occasionally on the top of the gels. This is due to the difficulty of reducing zein C. However, another two bands could be seen on the gel in faint color and were called zein D and E with estimated MW of 10 and 9 kilodaltons. These low molecular weight zeins were reported to be rich in sulfur containing amino acids and it was speculated by Gianazza et al. (1977) that these chains could play a role in the formation of zein granules in the cell, by stabilizing macromolecular aggregates via formation of disulfide bridges.

Generally, zein A and B are the dominant polypeptides in zein I as appeared in SDS-PAGE; while zein A_1 and C are less dominant. In zein II, in addition to zein A and B, zeins C, D and E are present in considerable amounts.

Hydroxylapatite Chromatography

In view of the insolubility of zeins in the aqueous and salt solutions commonly used for chromatographic separations of proteins and the similar size and amino acid composition of zeins A and B, it was anticipated that separation would be difficult to obtain. A successful partial separation of zein A from zein B was achieved by chromatography of SDS complexes on hydroxylapatite (HT) according to Moss and Rosenblum (1972). A successful partial separation of zein B from zein A was also obtained by gel filtration of SDS-complexes on Sephadex G-200. This latter approach was abandoned because it was too tedious. For hydroxylapatite chromatography, zein I either was reduced or reduced and alkylated with iodoacetamide then complexed

with SDS in 0.01 M sodium phosphate and applied to a column of hydroxylapatite. Zein-dodecyl sulfate complexes bound to the column under these conditions and were then recovered by elution with a gradient of sodium phosphate. Figure 4 shows the elution profile of zein I reduced and Figure 5 shows the elution profile for zein I reduced and alkylated. The profiles shows the broad and irregular protein elution; this is suggestive of the elution of several different molecules. The reduction and alkylation of zein spread out the elution profile and gave better separation. Patterns obtained by electrophoresis of aliquots of selected fractions in polyacrylamide gels containing SDS are shown in Figure 6. The zein B polypeptides eluted first and are the sole components of the first peak. As the salt concentration increased, zein A polypeptides also eluted resulting in fractions containing both bands. These fractions were pooled and treated again with 5% 2-mercaptoethanol in order to reduce the disulfide bridges, and then applied to a new column. Despite such treatment, it was not possible to separate this fraction of zein A and zein B polypeptides further.

Finally, the proportion of zein B polypeptides in the elutes decreased drastically so that the last fractions contained only the larger zein A polypeptides.

Amino Acid Analysis

Purified zein A and zein B obtained by hydroxylapatite chromatography were hydrolysed and submitted to amino acid analysis. The results (Table I) are expressed as residues per mole, ignoring the contribution of tryptophan and cysteine to the molecular weight. The amino acid compositions resemble one another and also the composition

Figure 4. Elution Profile of Reduced Zein I Polypeptides. 7 mg of zein I was reduced with ME and complexed with SDS then applied to hydroxylapatite (HT) 1 x 50 cm column. The protein was eluted with a gradient of sodium phosphate. Fractions were assayed for absorbance (left ordinate) and for conductivity (right ordinate).

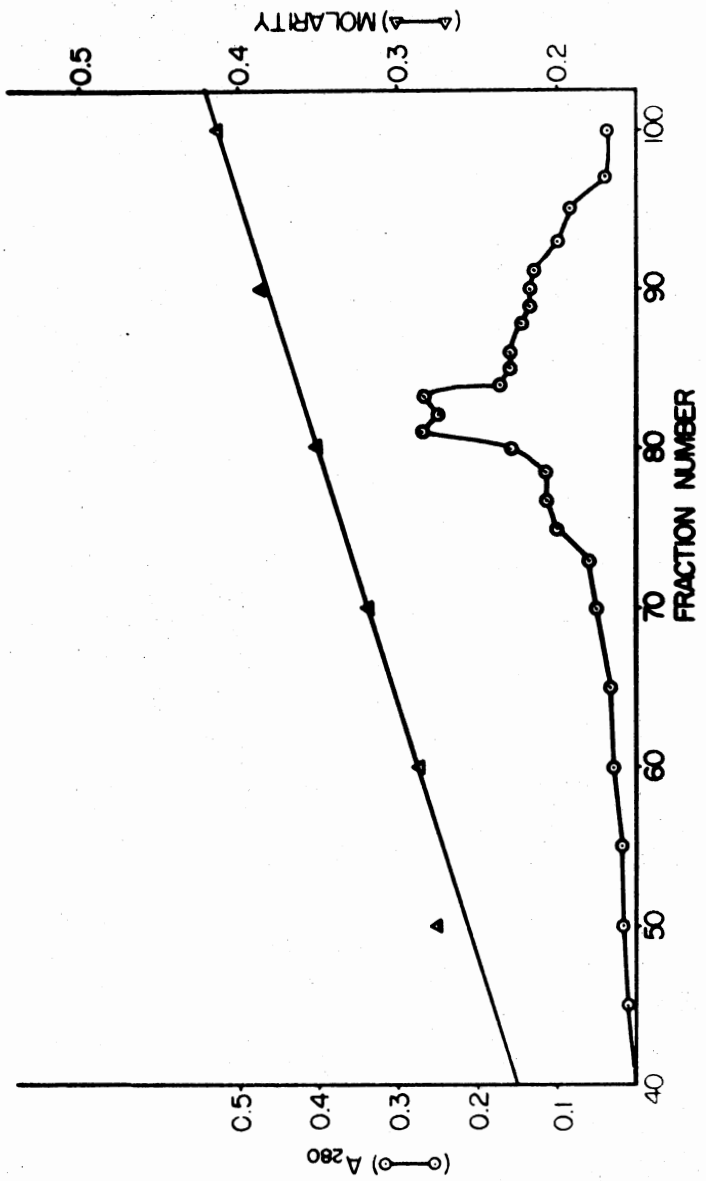

(▲—▲) MOLARITY
0.5
0.4
0.3
0.2
100
90
80
70
60
50
40
FRACTION NUMBER
0.5
0.4
0.3
0.2
0.1
(o—o) A280

Figure 5. Elution Profile of Reduced or Alkylated Zein I Polypeptides. 7 mg of zein I was reduced with (2-ME) and alkylated with iodoacetamine then complexed with SDS and applied to the hydroxyapatite column (as in Figure 4).

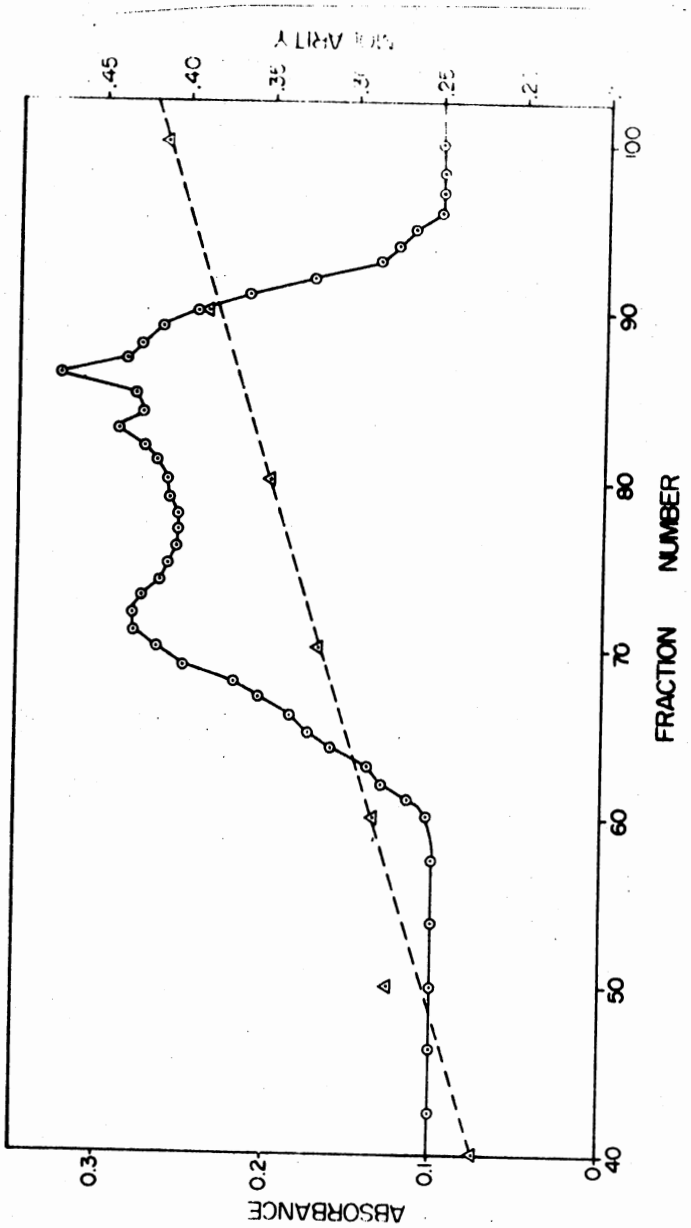

ACTIVITY
.45
.40
.35
.30
.25
.20
ABSORBANCE
0.3
0.2
0.1
0
40
50
60
70
80
90
100
FRACTION NUMBER

Figure 6. 12.5% Polyacrylamide Tube Gel Electrophoresis in SDS. Sample numbers correspond to the gradient fractions and were aliquots of selected fractions in Figure 5 used for the analysis. The photographs show the distribution of the two major polypeptides. A: is zein A; B: is zein B, in these fractions.

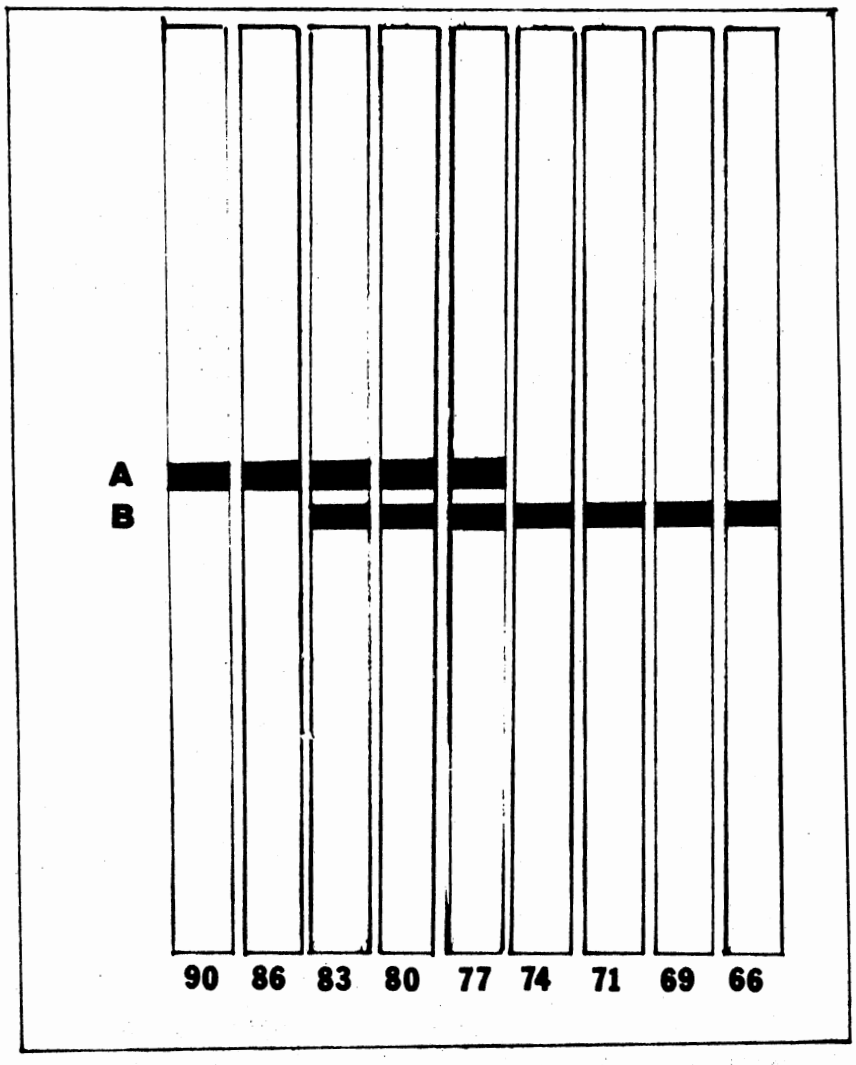

A
B
90
86
83
80
77
74
71
69
66

TABLE I

AMINO ACID COMPOSITION OF SEPARATED ZEINS*

	Zein A	Zein B
Asp	11.6	9.4
Thr	6.5	5.4
Ser	16.1	13.7
Glu	39.3	35.8
Pro	18.1	17.4
Gly	8.9	7.1
Ala	25.3	24.4
Val	8.7	6.4
Met	1.9	0.9
Ile	7.0	6.5
Leu	32.8	31.7
Tyr	6.2	6.3
Phe	8.5	9.8
His	2.5	1.8
Lys	1.5	0.8
Arg	3.4	2.2

*Expressed as moles of amino acid residue per mole of polypeptide assuming mol. wt. of 21,600 for Zein A and 19,600 for Zein B.

of total zein as reported by Sodek et al., 1971 and Waldschmidt-Leitz et al., 1962. The compositions are characterized by a low lysine content, and high content of glutamic acid (most likely as its amide, glutamine) leucine, and alanine. For both zeins, the content of the hydrophobic amino acids, leucine, alanine, proline, tyrosine, phenylalanine, valine, isoleucine and methionine is approximately 60%, consistent with the hydrophobic properties of these proteins. Zein A has one to three additional residues of each of the analyzed amino acids except tyrosine, phenylalanine, histidine, and lysine. Only in the case of phenylalanine are there fewer residues in zein B than in zein A. The difference in arginine content (two for zein B and three for zein A) could account for the more basic pI's on isoelectric focusing of zein A bands (see below). The presence of two methionines in zein A as opposed to one in zein B is consistent with the results of in vitro incorporation of radioactive methionine and leucine into zein bands in response to zein mRNA.

Lee et al. (1976) have also determined the amino acid compositions of zein A and zein B by hydrolysis of slices of stained bands from polyacrylamide gels. Their composition for zein B agrees closely with that reported here except for a higher content of proline (23 residues as opposed to 17). Larger differences are seen in zein A, particularly in glycine and proline where the estimates of Lee et al. (1976) are about twice the present estimates. It is unclear whether these differences are due to the method of protein separation, the genetic source of the zein or the method of analysis. Due to the presence of SDS in the samples, formation of sulphate from SDS during acid hydrolysis is possible. The content of serine and threonine may be changed

due to modification which occurred by the sulfate ions which were formed.

N-Terminal Analyses of Zein Fractions

The results of N-terminal analyses of zein extracts and column fractions, as shown in Table II, are consistent with the above interpretations. Zein I extracts contain two predominant N-terminal amino acids, phenylalanine and threonine, while the zein II extract is very heterogeneous containing phenylalanine, threonine, alanine, isoleucine, leucine, methionine, serine, glycine and valine as distinguishable N-terminal amino acids. The threonine N-terminal is associated with all fractions of the hydroxylapatite column that contain zein B, both the early fractions of pure zein B and the later fractions where a mixture of zein A and zein B is present. Phenylalanine is not present as an N-terminal amino acid in fractions containing only zein B but is present when zein A begins to appear in these fractions. The very last fractions of the column did not produce any identifiable N-terminal in three separate attempts. Enough protein was present in these reactions as judged by the intensity of O-dansyltyrosine spots to give an identifiable N-terminal dansyl amino acid, thus raising the possibility that polypeptides in this region of the column had blocked N-termini. Our results differ from those of Waldschmidt-Leitz et al. (1962) in that they detected serine instead of phenylalanine as the second major amino acid in addition to threonine.

Cyanogen Bromide Cleavage

It was observed by Melcher that when zein I and zein II protein

TABLE II

N-TERMINAL AMINO ACIDS OF ZEIN FRACTIONS

Fraction	N-terminal Residue
Zein I	Phe, Thr
Zein A_1*	Phe
Zein A_2**	None
Zein B	Thr
Zein II	Phe, Thr, Ala, Ile, Leu, Met, Ser, Gly, Val

*Zein A_1: The early fractions of the 21,600 mol. wt. band.

**Zein A_2: The late fractions of the 21,600 mol. wt. band.

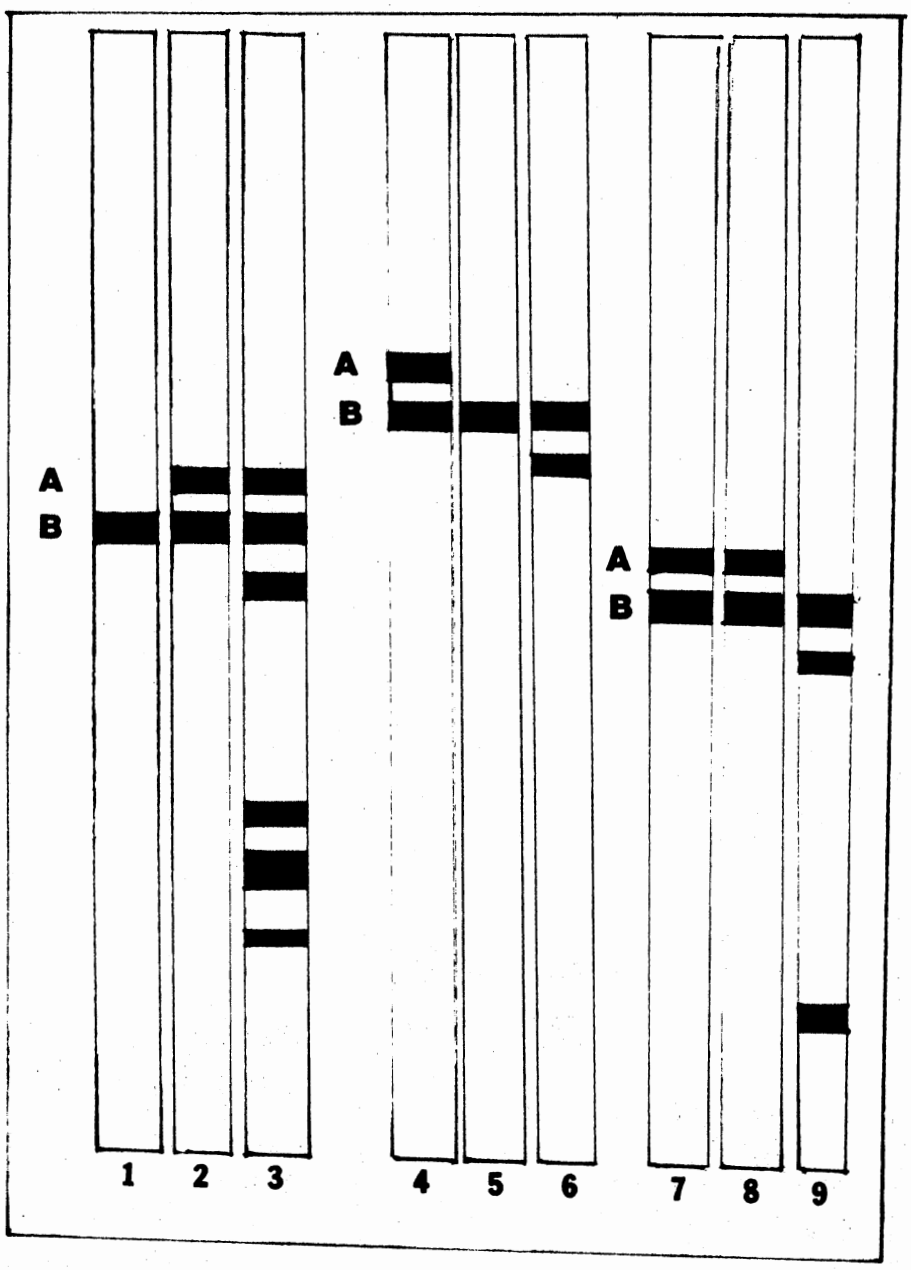
extract were treated with cyanogen bromide and the products electrophoresed on SDS-PAGE, the zein A band almost completely disappeared (Fralj and Melcher, 1979). Despite repeated attempts to obtain complete cyanogen bromide cleavage of zein A, a small amount was left unaltered in molecular weight. The zein B band remained.

Burr et al. (1978) reported that cyanogen bromide fragmented the zein A into peptides of 17,000, 4,000 and 1,000 daltons. They suggested that zein A was cleaved at two methionines. On the other hand, Melcher (1979) has reported that cyanogen bromide treatment of a mixture of *in vitro* products results in the replacement of the zein A *in vitro* product with a band at 19,600 daltons. No 17,000 dalton product was found.

To clarify these results and to investigate further the structure of the fractionated zein polypeptides, fractions from the hydroxylapatite chromatography containing both bands in approximately equal amounts (as described by densitometry) were submitted to cyanogen bromide cleavage after reaction with mercaptoacetic acid (Figure 7, tube 3). The slower zein A band disappeared on cyanogen bromide treatment. A densely staining band at the position of zein B was found as well as new bands at about 17,000 molecular weight and three bands in the molecular weight region below 5,000. Of these latter three, the middle one (suspected to be of 2,000 daltons) was the most intense. A small amount of zein A was left unaltered.

Other fractions which contained both bands (Figure 7), were treated with CNBr. Tube number 8 showed that zein A remained uncleaved while with later fractions from the same column run also containing both bands (tube 7), zein A was totally cleaved and a

Figure 7. SDS-Polyacrylamide Tube Gel Electrophoresis (12.5%) of Zein Fractions From Hydroxylapatite Column Chromatography and Cyanogen Bromide Cleavage Fragments.
Tubes 2, 4, 7: are intermediate fractions containing zein A and B mixture used as standard for the cleavage with CNBr. Tubes 2, 5: zein B treated with CNBr, was not changed. Tube 3: equivalent amount of zein A and zein B from the immediate fraction cleaved with CNBr. In addition to faint remnant zein A and dense, heavy bands of zein B, another band at $\sim$ 17,000 daltons and three bands below 5,000 daltons. Tube 6: pure zein A treated with CNBr, the cleavage product is zein B. The other cleavage product was undetected. Tube 8: different intermediate fractions contain zein A and zein B treated with CNBr. Zein A was not cleaved as well as zein B. Tube 9: zein A pure was treated and the cleaving product is the 19,000 daltons and another band around 5,000 daltons. The three sets of tubes are from different runs.


A
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9

clearly stained band at the position of zein B was found and faint bands at about 17,000, and below 5,000 daltons were seen.

When fractions containing exclusively zein B (Figure 7, tubes 1 and 5) were treated with cyanogen bromide, no cleavage products were detected, thus confirming that zein B is resistant to cyanogen bromide treatment even though it contains one methionine. This methionine might be so close to a terminus of the molecule that cleavage would not result in a change in size or it might be present in a met-ser or met-thr sequence, sequences which have been shown to be resistant to cyanogen bromide cleavage as reported by Shome et al. (1971). When protein from the fractions which contained only zein A polypeptides was cleaved with cyanogen bromide the zein A band disappeared and was replaced by a major band in the position of zein B (tube 6), thus confirming the hypothesis that zein A was cleaved at a methionine approximately 2,000 from one end. In addition to this major band a minor band at about 17,000 mol. wt. was detected, corresponding to the cleavage observed by Burr et al. (1978). The low molecular weight products formed three bands, the middle one of which was most stained.

The result suggests that the slow band (zein A) consists of at least three polypeptides. The first polypeptide has one cyanogen bromide sensitive site generating fragments of 19,600 and 2,000 daltons. The second polypeptide has two cyanogen bromide sensitive sites generating fragments of 17,000, 4,000 and 1,000 mol. wt. as identified by Burr et al. (1978). And the third polypeptide has no cleavable methionine.

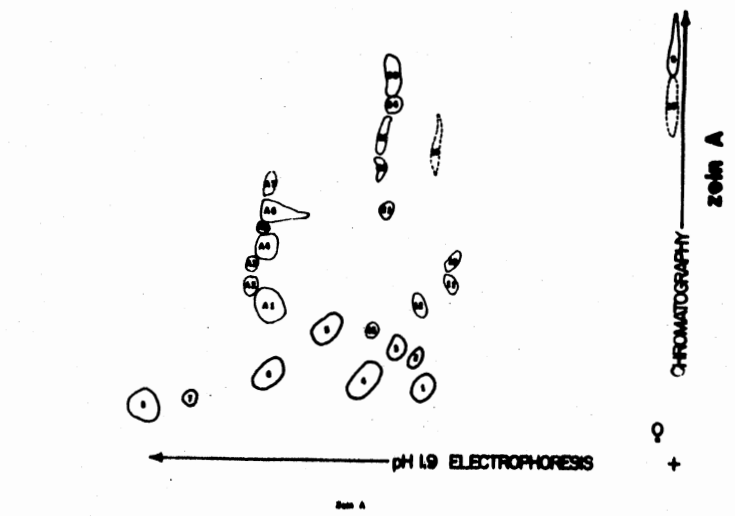
Peptide Mapping

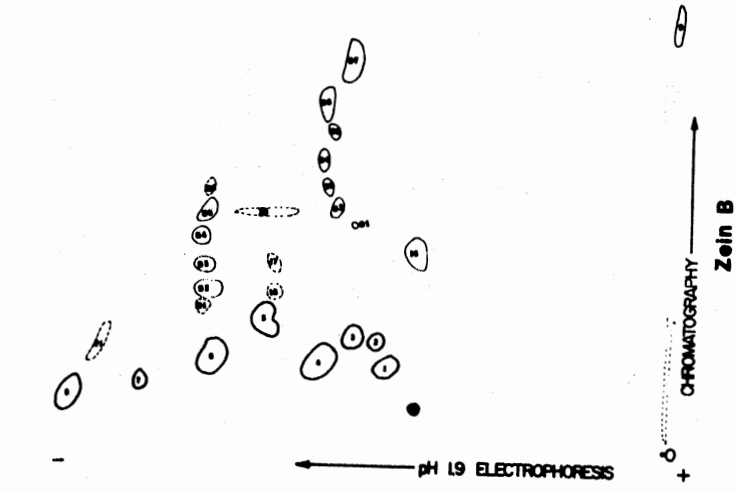
In order to examine whether zein A and zein B polypeptides were similar in structure as suggested by the amino acid analysis, fractions from Sephadex G-200 chromatography which contained only zein A, and those that contained zein B and some total zein I was complexed in the same manner with SDS. The samples were dialyzed against distilled water, lyophilized and digested with trypsin and chymotrypsin as described in the methods. The peptides produced were separated by two dimensional chromatography and electrophoresis on paper. Initial experiments were done using pH 3.5 and 6.5 for electrophoresis. At these pH's only few peptides moved and these moved toward the cathode. The absence of anodically migrating peptides despite the high glutamic acid values in amino acid analysis (Table I) may indicate that most, if not all, of the glutamic and aspartic acid residues detected are present in the protein as their amides. Righetti et al. (1977) concluded that at least 90% of the glutamic and aspartic acids were present as glutamine and asparagine residues. Wall (1964) reported also such a high value for asparagine and glutamine.

Preliminary peptide maps for zein A and zein B (Figure 8) were from Sephadex G-200 column fractions. Flat surface high voltage electrophoresis was used in the second dimension. 20-30 spots were detected by staining with ninhydrin. About 25 spots are expected from the known amino acid composition assuming a monomer molecular weight of 20,000.

Zein A and zein B peptides have three similar electrophoretic mobility patterns:

Figure 8. Peptide Maps of Zein Fractions.
Top is zein A and bottom is zein B. Purified zein A and B were from Sephadex G-200 column. The proteins were digested with trypsin and chymotrypsin and spotted on 3 mm paper. Chromatography is butanol:acetic acid:water preceded electrophoresis at pH 1.9 in flat surface high voltage apparatus.


CHROMATOGRAPHY
zein A
pH 1.9 ELECTROPHORESIS
+


CHROMATOGRAPHY
Zein B
pH 1.9 ELECTROPHORESIS
+

a - spots (1-9) with dark intensity staining, moved in similar pattern for both zein A and zein B.

b - spots A-A_7 and B_1-B_6 had a lesser staining intensity, but moved in another pattern. In addition there were 4 spots unique to zein A (10, 11, 12, 13), and 4 spots unique to zein B (14, 15, 16, 17).

Spots in the first series (1-9) are shared between zein A and zein B judging from the identical staining intensity and mobility patterns. For the other two series of spots it was hard to distinguish which are shared between zein A and zein B. However, it is likely that most of them are shared between the two zeins. In Figure 8 a total zein I digest contains clear spots of the first series (spots 1-8), except spot 9 was missing. The other two series in this digest appeared as long streaks and diffuse spots, indicating larger peptides which resulted probably from incomplete digestion of zein I polypeptides, or a different separation mobility either in first or second dimension. This could be as a result of disulfide bonds connecting the resultant peptides or presence of some pigments and/or lipids components with whole zein I extracts. Righettiet al. (1977) found 3-5% of the total zein proteins contained lipids. Such lipoprotein contains a carotenoid covalently bound to the polypeptide backbone and was found to be a component of the membrane that envelopes the zein protein bodies in the endosperm. They speculated that such lipid moiety is bounded to the polypeptide chain, since it was not released by alcohol, Triton X-100, or urea treatment. Presence of such components could hinder the enzymatic digestion and/or the separation in the peptide mapping for zein I extracts, while in the fractionated zein I components (zein A and zein B), it is very likely that these components were separated in some

other fractions.

In Figure 9, zein I was reacted with cyanogen bromide, dialyzed and lyophilized and then digested in the same manner. There are two differences from zein I peptide maps:

a - Spots (2, 3) in zein I disappeared and two spots (M_1, M_2) appeared. Spot M_1 could be the same as spot 3 in location, and spot M_2 could result from a new fragment peptide of the diffuse spots or the streaks.

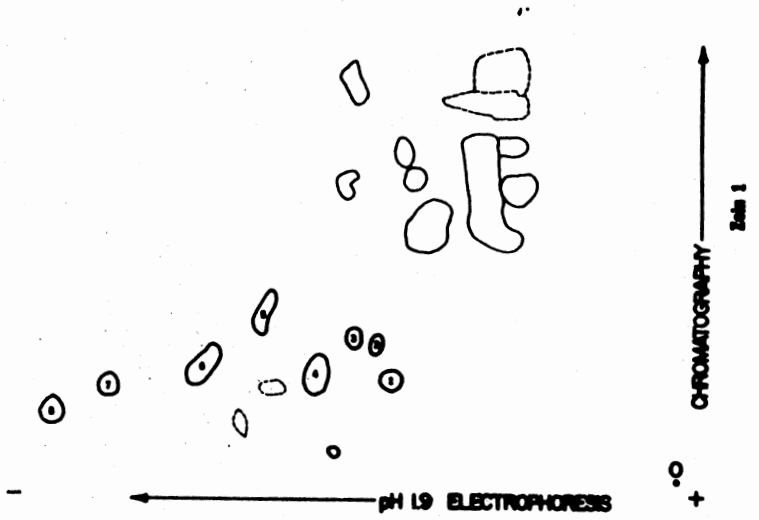
b - Most of the streaks and the diffuse spots did not show clearly and it is possible they were faint so it was not possible to distinguish them from the background stain. Another possibility is they were lost because of their small size after CNBr cleavage followed by dialysis.

For the main spots (1-8) which are shared in zein I, zein A, and zein B, only two spots have methionines reactive to CNBr cleavage, these are indicated by spots (2, 3). The other major spots remained untouched. This agrees with the amino acid analysis for zein A and zein B and with the results which will be discussed later, which show that only zein A contains cleavable methionine (one or two).

Another peptide mapping was done on fractions from hydroxylapatite chromatography. Some fractions contained zein A; those that contained a mixture of zein A and B together, and those that contained only zein B and some total zein 1 were complexed with SDS in the same manner and treated as mentioned before, were digested and separated as above, except the second dimension was in an electrophoresis tank. Total spots detected by staining with ninhydrin numbered 26, 32, and 28 for zein B, the mixture, and zein A, respectively.

Spots were numbered on the map of the intermediate fraction polypeptides (Figure 10). With a few exceptions (spots 32, 33, 34, and

Figure 9. Peptide Maps of Total Zein I in the Top and Total Zein I Cleaved with Cyanogen Bromide Dialyzed Then Digested and Separated as in Figure 8.
Unnumbered spots belong to diffuse spots or long streaks and does not match with the peptide mapping of zein A and zein B.


CHROMATOGRAPHY
pH L9 ELECTROPHORESIS
-
+

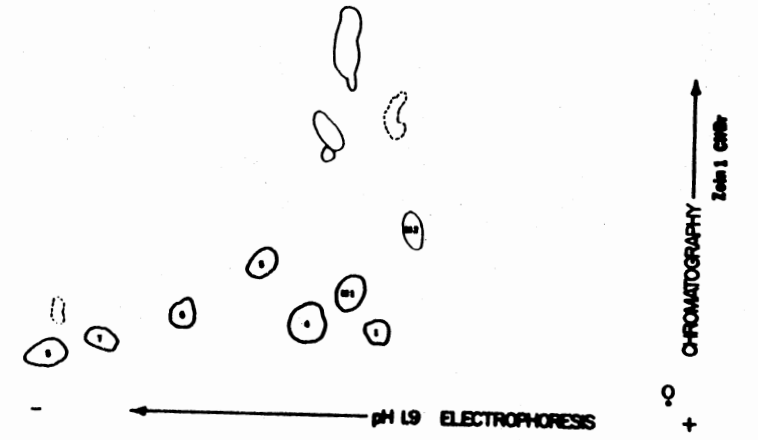
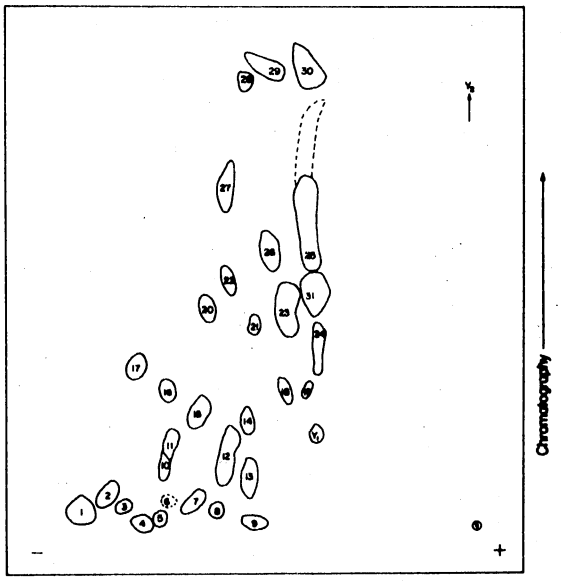

CHROMATOGRAPHY
pH L9 ELECTROPHORESIS
-
+

Figure 10. Peptide Maps of Zein I From the Intermediate Fractions of Hydroxylapatite Column Containing Both Zein A and Zein B. Proteins were digested with trypsin and chymotrypsin and spotted on 3 mm paper. Chromatography in butanol:acetic acid:water preceded electrophoresis at pH 1.9 in tanks electrophoresis apparatus. The top of the figure is a photograph of the chromatogram and the bottom is illustrating drawing.



Both Bands

Y1) all peptides of digests of zein A and of zein B corresponded to peptides in the zein A-zein B mixture. Peptides 1 through 10 of Figure 13 have an artefactually higher mobility than the other peptides probably due to uneven voltage distribution during electrophoresis. Not all spots were of equal intensity, suggesting that some peptides are present in less than molar amounts per mole of polypeptide. From 20 to 21 zein B peptides and 17 to 21 zein A peptides were judged to be major peptides. Based on the amino acid analyses reported above 18 to 21 spots would be expected for polypeptides of 20,000 and 22,000 daltons. The number of major spots observed is thus entirely consistent with the presence of single polypeptides of the size and composition of zeins A and B. The minor spots could be due to the presence of additional unrelated polypeptides or to heterogeneity within limited regions of the peptide sequences of zeins A and B.

Of the major peptides, 13 to 16 are shared between zein A (Figure 11) and zein B (Figure 12). Four peptides (28, 29, Y1, Y2) are unique to zein B and two peptides (7, 31) although major in zein B are only present in minor amounts in zein A. Conversely, four major peptides (16, 17, 20, 27) are found only in zein A and not in zein B. Most of the minor peptides are not shared between zein A and zein B. The comparison of peptide maps of zein A and zein B is consistent with the idea that zein A and zein B polypeptide chains share identical sequences for over half of their polypeptide structures.

Comparison of peptide maps for zeins A and B with those of digests of the mixture derived from intermediate column fractions yields indications of heterogeneity within zein A and zein B. Zein B peptides 32 and Y2 are not found in the map of fractions containing both bands

Figure 11. Peptide Maps of Zein I From the Late Fractions of the Hydroxylapatite Column Which Contains Pure Zein A. Protein was digested and separated as in Figure 10. The top of the figure is a photograph of the chromatogram and the bottom is illustrating drawing.

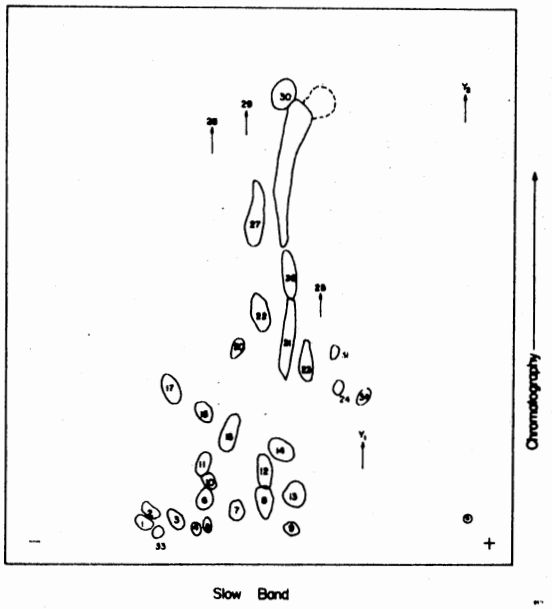
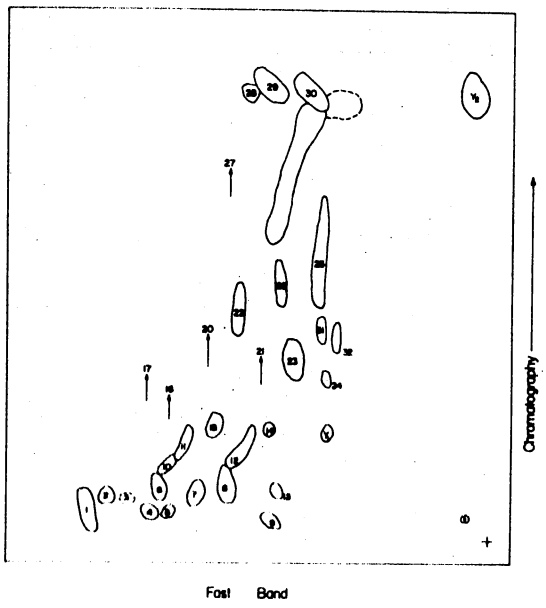

Chromatography
Slow Band
–
+

Figure 12. Peptide Maps of Zein I From the Early Fractions of the Hydroxylapatite Column Which Contain Pure Zein B. Protein was digested and separated as in Figure 10. The top of the figure is a photograph of the chromatogram and the bottom is illustrating drawing.


Chromatography
Fast Band

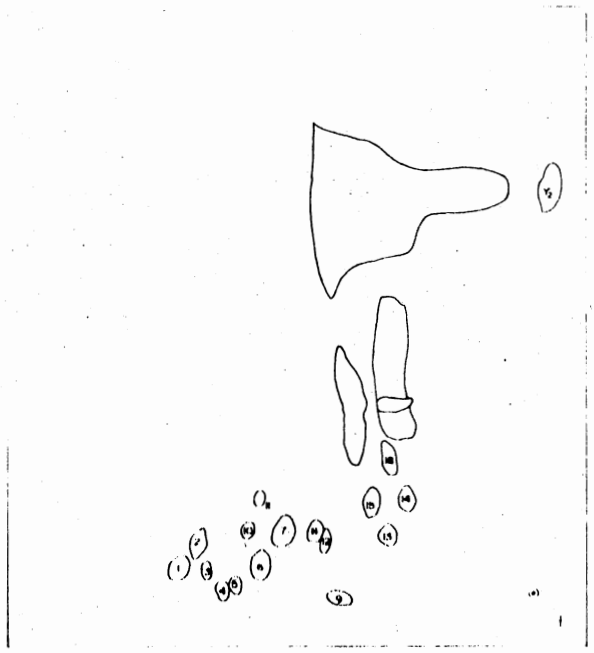
and peptide 6 is only faintly detected in this map. Two zein A peptides (33 and 34) are not found in digests of intermediate fractions. Although the minor spots (32, 33, 34) may have gone undetected because of staining sensitivity, spot Y2 was a densely yellow staining peptide definitely absent from the zein A-B mixture. This implies the existence of at least two zein B polypeptides, one containing Y2 and 6, eluting early, and the other lacking Y2, eluting later. Two minor spots (18 and 19) were only detected in the zein A-B mixture.

Figure 13 shows peptides in the total zein I digest which were separated in tank electrophoresis. The pattern looks different from the separated zeins and the same explanation mentioned before (see above) is applicable here again.

Isoelectric Focusing of Native Zeins

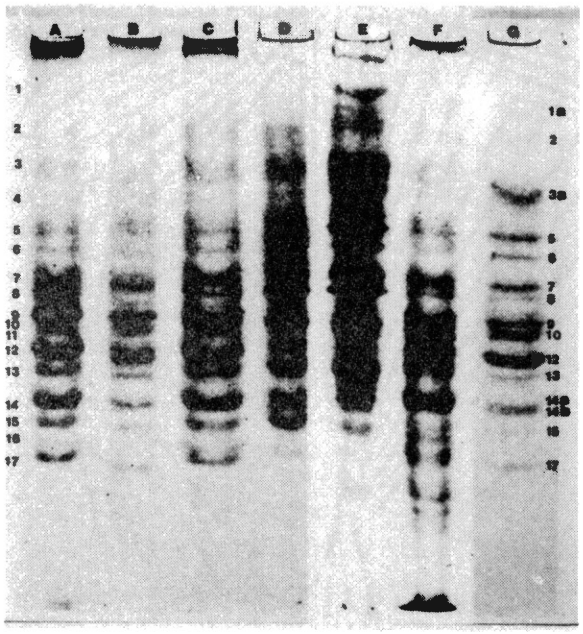
Preliminary isoelectric focusing of zeins using tube gels and no urea in the buffer gave a few bands (5 to 8) with poor resolution. This was most likely due to precipitation of the protein in the top of the gels due to insufficient detergent to keep the protein soluble. In spite of that, zein fractions from the hydroxylapatite column gave separations which showed that the zein A components focused in the basic region. This is consistent with the higher arginine in zein A as shown by amino acid analysis (see p. 44). Also, different fractions contained different bands indicative of elution of several polypeptides which are different in their pI's. When urea was used in the buffer system (as in Materials and Methods) using slab gels and pH from 3-10 in order to focus all zein polypeptides on the same gel, better separation and resolution was obtained. Figure 14A and 14G show the IEF pattern

Figure 13. Peptide Maps of Total Zein I Extract Which Was Complexed with SDS and Dialyzed, Lyophilized Then Digested and Separated as in Figure 10.
The top of the figure is a photograph of the chromatogram and the bottom is illustrating drawing. The unnumbered spots are either diffused spots or streaks.



Total Z_1

Figure 14. Isoelectric Focusing Profiles of Modified and Unmodified Zeins.
A) zein I, B) zein I in the presence of SDS, C) zein I alkylated with iodoacetamide, D) zein I alkylated with acrylonitrile, E) zein II alkylated with acrylonitrile, F) zein II alkylated with iodoacetamide, G) zein II unmodified.


A
B
C
D
E
F
G

obtained with zein I and zein II extracts, respectively. In accord with the results of others (Righetti et al., 1977) approximately 17 bands could be distinguished and these are numbered beginning with the most acidic in the zein I profile. Within the resolution of the method, most of the zein I bands can also be found in zein II. In the acidic region there are six diffuse faint bands (1-6) in zein I. Two of these (5 and 6) are more prominent in zein II. Zein II also contains two acidic bands (1a and 3a) not found in the zein I profile. In the neutral to basic region, there are three further differences between zein I and II profiles, excluding differences in relative staining intensity. The faint zein I band 16 could not be detected in zein II. Zein I band 14 appears in zein II as a closely spaced set of two bands (14a and 14b). There are two bands (10 and 11) between the dark zein I bands 9 and 12 in zein I, but only one in zein II. Some bands appear bowed while others are straight. The cause of this bowing is unknown, but it was reproducibly observed and thus used to distinguish these bands.

Covalent Modification of Zeins

The low molecular weight zeins are known to be rich in methionine (Gianazza et al., 1977) while zeins A and B have an average of only 2 or 1 methionine residues per chain, respectively (Fraij and Melcher, 1978). In an effort to test whether bands in the acidic region are components of the low molecular weight zeins we have examined the IEF pattern of zeins after modification with iodoacetamide. Alkylation of cysteine residues with iodoacetamide should not alter the isoelectric points of polypeptides (as reported by Scott et al., 1973),

unless these are very basic, since a neutral group is replaced by a neutral group. Alkylation of methionine residues, however, creates a non-titratable positive charge out of a neutral moiety and should thus cause molecules to focus at a more basic point.

Alkylation of zein I and zein II with iodoacetamide was performed under neutral to slightly acidic conditions. Figure 14C shows that the IEF pattern of zein I polypeptides was not altered by alkylation. On the other hand, the zein II pattern was altered considerably (Figure 14F). Bands 7-17, those also present in zein I, have not changed, but bands 1a and 3a have disappeared. There is also a diminished relative intensity of bands 5 and 6. The pattern of acidic bands in zein II after iodoacetamide alkylation is indistinguishable from the pattern of zein I bands in this region. There are a series of new bands focusing in the region on the basic side. Bands 15 and 17 appeared as two closely new bands and below band 17 there are at least 5 bands. An interpretation of this is to mean that bands 1a, 3a, 5 and 6 contain residues that are susceptible to alkylation under these conditions while bands 7 through 16 do not contain susceptible residues. The large shift observed could be due to the presence of several methionine residues per chain in the acidic zeins (Gianazza et al., 1977).

When zein I was modified with acrylonitrile (Figure 14D), bands in the acidic region were not affected. The pattern of the bands in the neutral to basic region was, however, altered considerably. These appeared to shift to more acidic isoelectric points. Since the mobility changes of isolated bands were not examined definite conclusions as to which bands were modified are not possible. The simplest

interpretation, however, is illustrated in Figure 15 where the modified and unmodified zein I lanes have been cut from a photograph and repositioned to align bands 9 and 12 of the unmodified zein with the two darkest modified bands. This alignment resulted also in an alignment of bands 13 and 15. The shift in mobility of bands 14, 16 and 17 was greater than that of 9 and 12. Whether bands 10 and 11 shifted could not be determined since the modified band 13 obscures this area of the gel.

The result implies that most of the zein I bands contain acrylonitrile modifiable residues and that some of the more alkaline bands (14, 16 and 17) may contain more such residues than others (13 and 15). The results of alkylation of zein II are shown in Figure 14E and realigned as the zein I photographs were in Figure 15. Bands 5 and 6 have not shifted while bands 1a, 2 and 3a have undergone a shift to more acidic pH. For the most part the positions of the modified zein II bands in the alkaline half of the gel correspond to those of modified zein I bands. It was unable to explain the higher relative intensity of the modified bands 5, 6 and 8.

Zein I and zein II were also alkylated with radioactive iodoacetate under conditions where alkylation of cysteine is favored. Such alkylation should introduce negative charges into cysteine containing molecules and thus shift their IEF positions to the acidic side. If alkylation of methionine residues occurred it should not affect the pI since both a positive and a negative charge will be introduced. The pattern of [^{14}C]-iodoacetate modified zein I bands (Figure 16c) is similar to that of acrylonitrile modified bands (Figure 16d). Acidic bands corresponding to modified bands 1a and 3a are visible in zein I

Figure 15. Comparison of IEF Profiles of Unmodified Zeins with Zeins Modified with Acrylonitrile.
Lanes were cut from a photograph of the gel shown in Figure 2 and the acrylonitrile modified zeins repositioned to show the correspondence between modified and unmodified bands. Identification of lanes as in Figure 2.

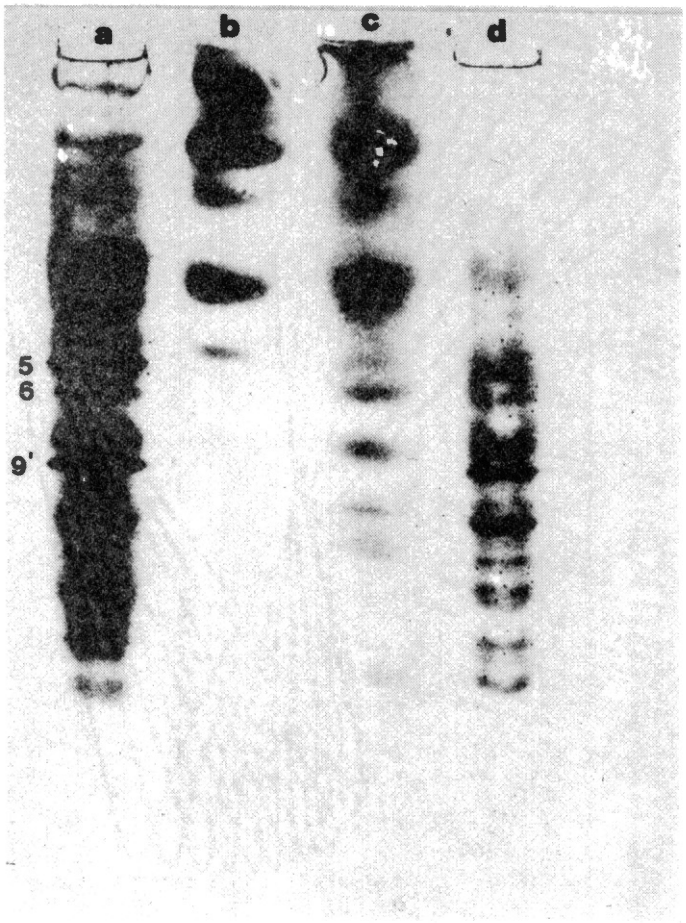
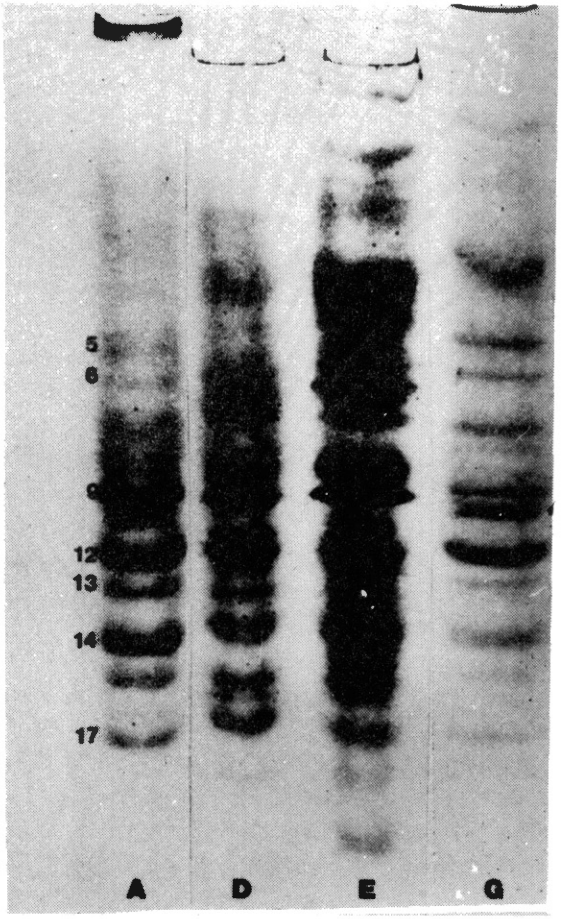

a
b
c
d
5
6
9'

Figure 16. Comparison of IEF Profile of Zeins Modified with ^{14}C - iodoacetate, (b and c) and with Acrylonitrile (a and d). A and b) zein II; C and D) zein I. ^{14}C bands detected by fluorography.


5
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12
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14
17
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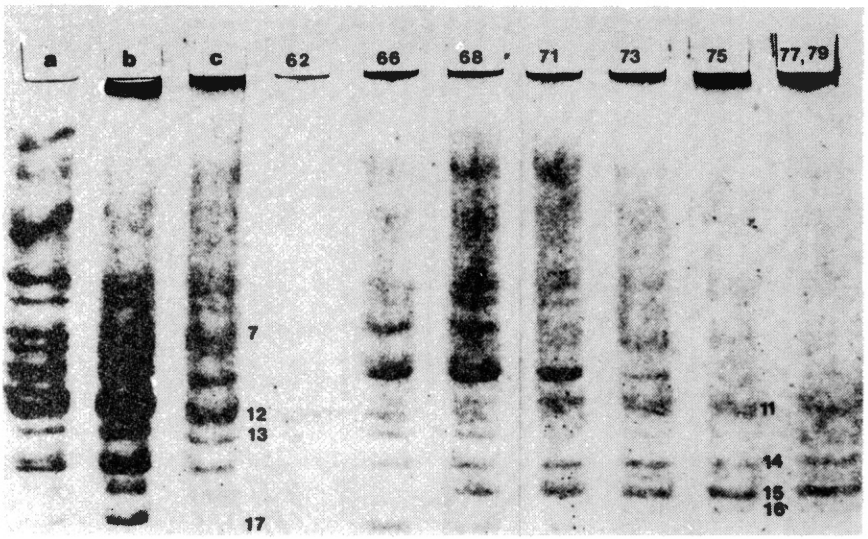
whereas they could not be detected in that extract by Coomassie blue staining (Figure 16a). The greater fluorographic intensity relative to Coomassie blue staining of these acidic bands in both zein I and II is consistent with a higher content of cysteine of low molecular zeins since the specific radioactivity of these bands should be higher than that of bands containing low amounts of cysteine. Bands corresponding to stained bands 5, 6 and 9 are noticeably absent among the radioactive zeins (Figure 16b and 16c). The absence of labeled bands 5 and 6 is consistent with the inability to detect a shift in IEF position after acrylonitrile treatment. Band 9, however, was shifted after the latter treatment (Figure 15) suggesting that a residue other than cysteine had reacted with acrylonitrile.

Hydroxylapatite Chromatography

It has been shown that chromatography of zein dodecyl sulfate complexes on hydroxylapatite produces some fractions that contain only zein A and others that contain only zein B when judged by SDS-PAGE (see above). Hydroxylapatite chromatography followed by IEF should thus permit the assignment of some of the IEF bands to zein A and others to zein B. There is, however, concern that IEF of zein dodecyl sulfate complexes may lead to artifactual results due to the negative charge of dodecyl sulfate. Figure 17C shows the IEF profile of zein-dodecyl sulfate complexes. The pattern is basically similar to that of zein in the absence of dodecyl sulfate. It is, however, less distinct and less intense. A precipitate, probably containing protein, formed in the slots of lanes to which SDS-protein complexes were applied. The addition of NP-40 to the zein-SDS complexes prevented this precipi-

Figure 17. IEF Profile of Fractionated Zein.
Zein fractionated by hydroxylapatite chromatography was submitted to IEF. Numbers refer to chromatographic fractions. a) unfractionated zein II; b) unfractionated zein I; c) unfractionated zein I with SDS and NP-40.


a
b
c
62
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68
71
73
75
77,79
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13
17
11
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15
16

tation (Figure 17b). The pattern of zein peptides was the same with or without SDS (Figure 17c and 17b, respectively) except that the presence of SDS caused the whole pattern to shift by about 2 mm to the more basic side. Similar reports that the use of NP-40 makes possible the IEF separation of SDS protein complexes have recently been published (Tuzynski et al., 1979).

Figure 5 also shows the IEF pattern of selected fractions from hydroxylapatite chromatography. By SDS-PAGE it was determined that fraction 62 contained only zein B and that fractions 77 and 79 contained only zein A. Intermediate fractions contain mixtures of zein A and B. Several of the IEF bands could thus be assigned to zein B: bands 7, 12, 13 and 17 (some of these are not visible on the photograph but were clearly seen in the gel). Similarly four bands (11, 14, 15 and 16) can be identified as zein A polypeptides. Peptides 5, 6, 8 and 9 are found predominantly in intermediate fractions and can thus not be assigned, although it is probable they belong to the zein B group since members of this group are predominant in zein I. It is of interest that although on the average zein A bands are more basic than zein B bands, the most basic band (17) is associated with zein B and that a zein A band (1) is found among the central bands in the IEF pattern. The more basic pI values for zein A are consistent with their higher content of arginine (as shown in the amino acid analysis).

Carbohydrate

It has been reported that zein preparations contain carbohydrate, an observation that has been confirmed by Melcher (Fraij and Melcher, 1979). However, Burr et al. (1978) speculated that a covalent bond may

exist between the sugar moiety and the polypeptides. To further investigate the possible presence of sugar, an IEF gel was stained with periodic acid-Schiff reagent, which reportedly stains zein peptides on SDS-PAGE (Burr and Burr, 1979). None of the zein bands in Figure 15 were stainable, however, one band that stained with periodic acid-Schiff reagent in the acidic region of the gel about 1 cm from the origin when NP-40 had been added to the sample was detected. It is thus possible that the carbohydrate observed in zein preparations remains associated with zein during SDS-PAGE but is dissociated from it under the conditions of IEF. A similar absence of carbohydrate staining of IEF zein bands has been reported by others (Righetti et al., 1977b).

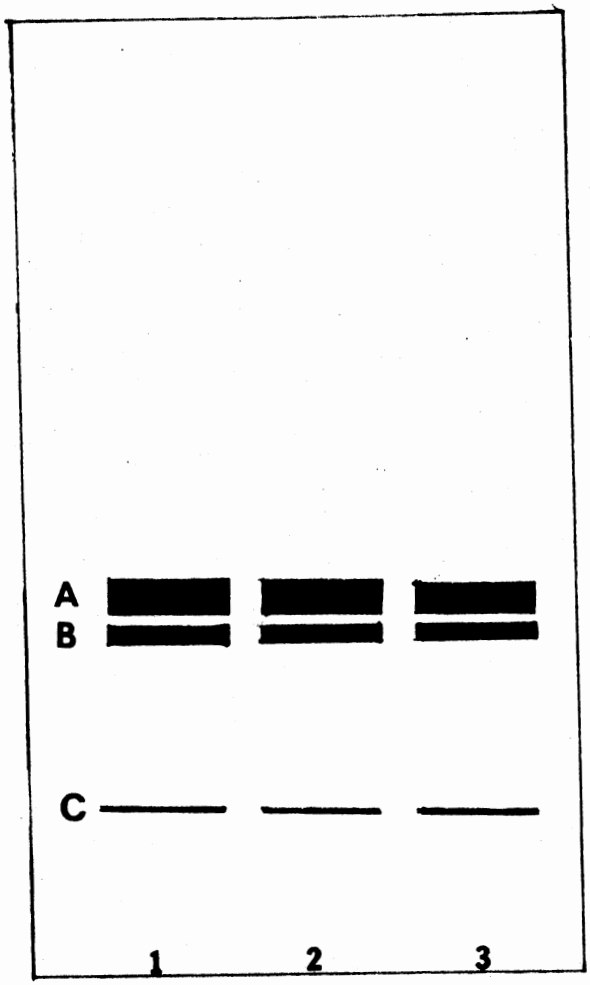
In vitro Products

When mRNA extracted from polysomes of a protein body enriched fraction of developing corn endosperm is translated in a cell free extract of wheat germ, the products detected can be identified as precursors to zein polypeptides (Burr et al., 1978; Melcher, 1979; Wienand and Feix, 1978; Larkins and Hurkman, 1978).

Zein mRNA was isolated and translated in vitro (as described in Materials and Methods). The translation products were applied to SDS-PAGE. The radioautograph of the product is shown in Figure 18. The polypeptide formed from the isolated message was identified as precursors to zein A, B and C which is in agreement with published results by Melcher (1979).

So far, all the results support that zein is indeed a heterogeneous protein and such heterogeneity is due to primary amino acid

Figure 18. Discontinuous SDS-PAGE of *in vitro* Products of Zein mRNA with a Running Gel of 12.5% Detected by Fluorography. Tube 1 contains 2 µℓ of mRNA and tube 2 contains 3 µℓ mRNA from the same extract. Tube 3 contains 3 µℓ of another extract mRNA. A, B and C are zeins A, B and C.


A
B
C
1
2
3

differences. To gain more results supporting this conclusion and to examine such heterogeneity at the molecular level in an effort to estimate the number of genes responsible for zein biosynthesis, two approaches have been made: First, studying the *in vitro* product by isoelectric focusing to see whether such a product is as heterogeneous as the native zein. The second approach was the hybridization of the isolated zein mRNA to the extracted corn seed DNA cleaved with restriction endonuclease (EcoR).

Isoelectric Focusing of Zein *in vitro* Products

The *in vitro* translation products were submitted to IEF and the radioactive products were detected by fluorography. In the pH range from 3.5 to 10 there were at least 18 distinguishable bands distributed among a wide range of isoelectric points (Figure 19). To ascertain whether all of these *in vitro* products were zein polypeptides, the incubation mixture was extracted with ethanol or ethanol and a reducing agent. Figure 20c and 20f show these extracts and Figure 20d and 20e the residue remaining after these extractions. Identical patterns were obtained in the two kinds of extracts. Approximately 90% of the radioactivity was extractable with ethanol. The polypeptides remaining in the residue for the most part focused to the same positions as those extracted with ethanol. There were, however, two acidic bands present in the residue which were not extracted with ethanol. These two polypeptides may be zein precursors which are not ethanol soluble, other storage proteins, or membrane components coded for by minor mRNA molecules in the preparation. It is unlikely that they are wheat germ polypeptides since this cell free extract has a very low background

Figure 19. Isoelectric Focusing Profiles of *in vitro* Zein Products Detected by Fluorography.
a - To 50 µl incubation mixture 1 ml of 10% cold TCA was added, then suspension was centrifuged and the pellet washed with 1 ml of 0.2 M Na acetate (pH 4.5). The final pellet was resuspended in Tris-glycine buffer and applied to the gel. b - As in a, except the wash step was substituted by ether.

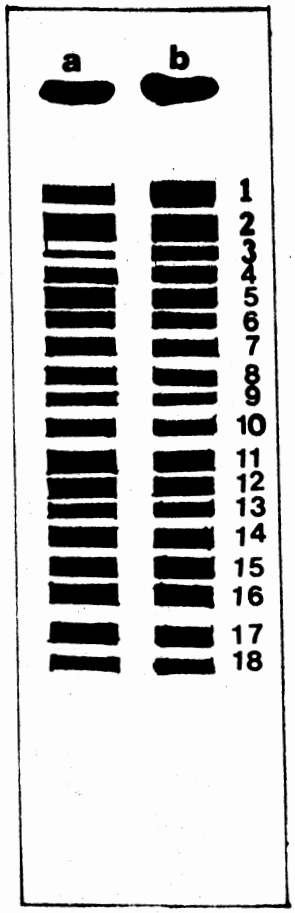
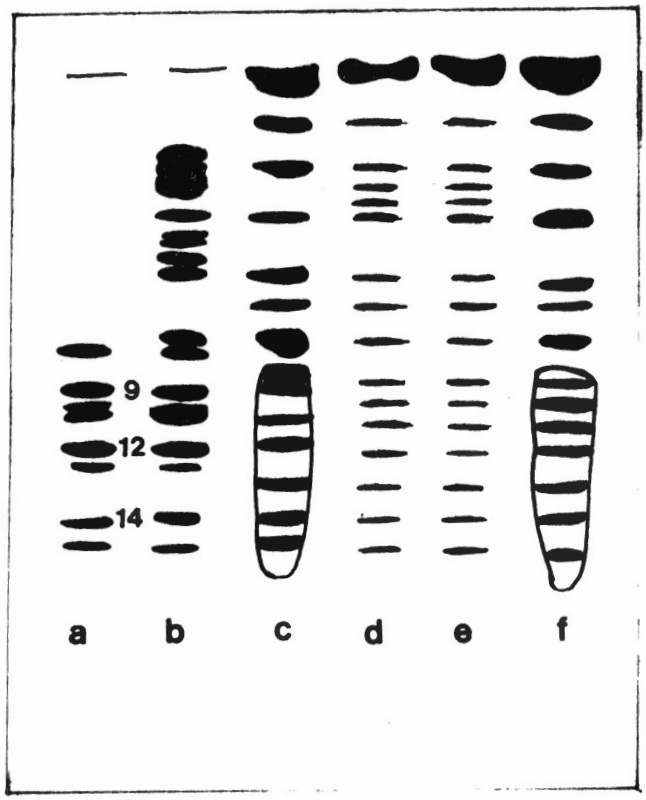

a
b
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Figure 20. Comparison of IEF Profile of Products of *in vitro* Protein Synthesis Detected by Fluorography (c-f) with Coomassie Blue Stained Proteins (a, b).
a) Zein I; b) Zein I + wheat germ extract; polypeptides extracted with c; ethanol, f) ethanol and 2-mercaptoethanol; d) and e) residues of extractions c) and f), respectively.


9
12
14
a
b
c
d
e
f

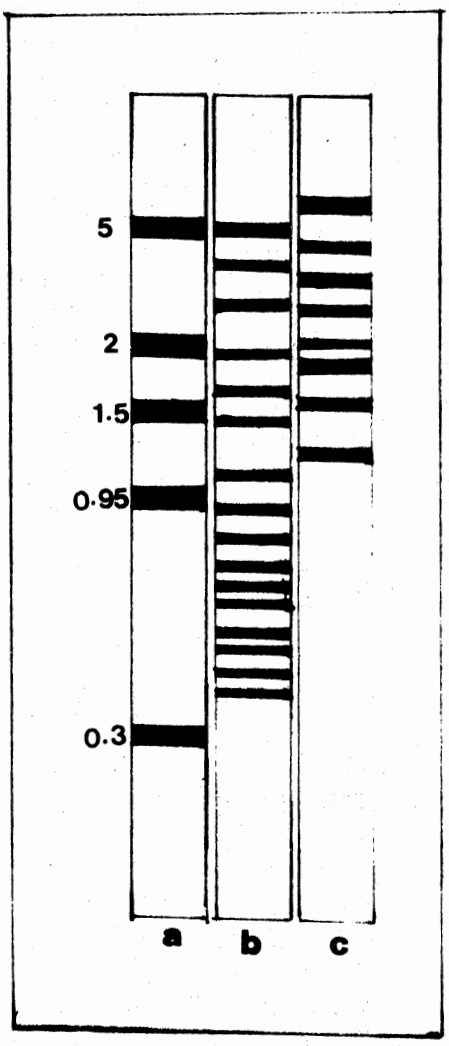
incorporation of amino acids in the absence of added mRNA.

Also displayed in Figure 20 are the Coomassie blue staining patterns of this gel. Figure 20a shows zein without any wheat germ extract while the Figure 20b shows the whole incubation mixture to which unlabeled zein had been added. The multiple bands in the neutral to acidic region can be seen to arise from components of the wheat germ extract. The pattern of the in vitro products were detected by fluorography. Although one or two fluorographic bands may align with stranded bands, the alignment may be fortuitous. The possibility that genetic differences in the source of the RNA and of the standard zein were responsible for this difference was considered. Zein extracted from the same variety of corn as that from which the RNA was obtained showed an IEF profile identical to that shown in Figure 14a except for a diminished intensity of band number 13. The difference in IEF pattern must therefore be due to the lack of post-synthetic modification (removal of the signal peptide) of the zein in this in vitro incubation.

Hybridization of the Isolated Zein mRNA To the Restricted Corn DNA

Zein mRNA was isolated and labeled with ^{125}iodine. Extracted corn seed DNA was cleaved with restriction endonuclease ($EcoR_1$) and the fragments were separated by agarose gel electrophoresis before being transferred to nitrocellulose strips (as mentioned in Materials and Methods). Hybridization of the immobilized DNA fragments with the labeled mRNA gave 15-20 distinct bands (Figure 21). It was reported by Wienand et al. (1978) that the molecular weight for zein

Figure 21. Agarose Gel Electrophoresis of DNA Cleaved with $EcoR_1$. a - CaMV markers: CaMV DNA cleaved with $EcoR_1$: M.W. (megadaltons); b - radioautograph of hybridization strip of corn DNA/mRNA; c - radioautograph of hybridization strip of corn DNA/rRNA.


5
2
1.5
0.95
0.3
a
b
c

A mRNA is 500,000 while zein B mRNA is 330,000. In Figure 21A standard markers range from 5 million MW to 0.3 million of double stranded CaMV DNA. Since zein A mRNA has a MW 500,000 the double standard DNA coding for this mRNA is equivalent to 1 million MW. Over one half of the radioactive bands can be seen with MW higher than 1 million. Thus over one half of zein genes are located in DNA sequences with a MW higher than needed for zein A mRNA. For all bands that were seen on the strip, it is not possible to know whether each band is a complete zein DNA sequence or zein DNA sequence plus some other sequences which were fractionated as one band on the gel.

It is possible that the large number of sequences in corn DNA which hybridized to the zein mRNAs could be considered as single genes which codes for a large number of polypeptides. Alternatively each band could represent a sequence of a split gene, where introns separated extrons (the sequences which codes for the polypeptides). From the radioautograph (Figure 21) some bands are seen more intense in radioactivity than others. This is seen especially in the bands from top to bottom where the top bands are more intense and such intensity becomes fainter. This means that the sequence which gave more intense bands were larger than the fainter bands and this is in agreement with the molecular weight range such bands moved. Zein A and B are the dominant polypeptides in zein proteins as mentioned above. The existence of other corn MW polypeptides like zein C, D and E also were mentioned. Thus it is expected that there are major zein mRNAs for the major zeins, zein A and B. Such mRNAs may give the intense bands, while the fainter bands may come from smaller zein mRNAs belonging to the low MW zein polypeptides.

The results discussed above are in good agreement with the 15-20 bands found in electrofocusing results. Such results would eliminate or make unlikely that a post-synthetic modification which might alter the electrophoretic mobility and such variation in this process would lead to apparent heterogeneity.

The possibility of the existence of other than zein mRNAs in the extract is possible, since two minor bands out of eighteen bands of the *in vitro* products that were shown in the IEF results were not ethanol soluble and hence possibly not zein proteins. Such minor mRNA could have some sequences which gave some of the bands on the hybridization strip, but the numbers must be very limited and should not be a major source of raising the number of the detected bands.

Another possible RNA sequence which could give a radioactive band in this system is contamination of mRNA with rRNA. To be certain labeled rRNA also was hybridized separately on another strip from the same gel run (Figure 21). Comparing rRNA labeled bands with mRNA labeled bands reveals that these bands do not match. This proves that the labeled mRNA was free from any rRNA contamination.

CHAPTER IV

DISCUSSION AND CONCLUSION

Zein I and zein II fractions have some similarities and some differences. The closest similarities are seen in the IEF patterns of zein I and zein II (Figure 14) where most of the zein I bands can also be seen in zein II. In the acidic region there are six different faint bands in both zein I and zein II; two of these (5 and 6) are more prominent in zein II. In addition zein II contains two additional acidic bands (1a and 3a) not found in zein I. Another two bands in the neutral region also differ between zein I and zein II (bands 16 found only in zein I, and band 14 in zein I appears as a closely spaced set of two bands in zein II). However, SDS-PAGE demonstrates in zein II two new polypeptide chains (zein D and E) absent in zein I. Zein C also was most prominent in zein II and very faint in zein I (Figure 3). From the point of view of solubility, zein II behaves differently from zein I since it is only soluble in alcohol containing 2-ME. It could be that zein II is linked into supramolecular complexes by disulfide bridges formed either among zein II chains, or among zein II chains and other non-zein components like glutelin as reported by Paulis et al. (1969). The alcohol soluble polypeptides consist of a few components represented in zein I as zein A_1, zein A and zein B. The residual part of zein (zein II) has indeed polypeptides with mobilities equal to those present in zein I. There are zein A_1, zein A and zein B

chains represented, however, in absolute terms, a minor part of the zein II since this fraction contains a considerable amount of other subunits with lower MW (zein C and zein D). This is in agreement with results reported by Salamini et al. (1977).

Zein A_1, zein A and zein B of zein II possibly represent a part of zein which has lost its typical solubility through linking with other components by disulfide bridges.

The most prominent difference between zein I and zein II as shown in both the SDS-PAGE and IEF is the presence of the low MW polypeptides (zein C, D and E). Such polypeptides have been reported to be typical zein polypeptides containing 4-5% sulfur-containing amino acids particularly methionine (Gianazza et al., 1977). The above mentioned bands in zein II (Figure 14) are reported to belong to the low MW polypeptides (Gianazza et al., 1976).

When zein II was alkylated with acrylonitrile bands 1a and 3a have disappeared and a series of at least five new bands focusing in the basion region appeared. This means that the acidic bands contain residues that are susceptible to alkylation with acrylonitrile. The large shift observed could be due to the presence of several methionine residues per chain in the acidic zeins (Gianazza et al., 1977).

The present results establish that the large heterogeneity in zein I polypeptides can be demonstrated by several methods: by column chromatography on hydroxylapatite, CNBr cleavage, N-terminal analysis, and by isoelectric focusing. By hydroxylapatite column chromatography the profile of zein I elution was much broader than that obtained when a homogeneous protein is chromatographed (Moss et al., 1972) under identical conditions.

This suggests that perhaps the profile observed is a collection of peaks due to a collection of molecules with only very slight differences from one another. Zein A differs from zein B in molecular weight (21,000 vs 19,600). The profile results from the elution at slightly differing salt concentrations of the various zein polypeptides. By size analysis it was demonstrated that, in general, the zein B polypeptides elute at lower salt concentrations than the zein A polypeptides. However, some zein B polypeptides elute at the same salt concentration as some zein A polypeptides resulting in fractions that contain both size classes of zein polypeptides. Such fractions were applied to a new column after reduction for the second time at 5% 2-ME although it was not possible to achieve further separation between those particular zein A and zein B polypeptides. This implies that the two zeins in these fractions have a different ability to bind the hydroxylapatite from the rest of the separated zein A and zein B. This means that there are differences existing in their structure. This was proved by IEF (see below) and by peptide mapping where two spots were unique to such fractions.

The separation on hydroxylapatite column was evidenced by the observation that certain tryptic-chymotryptic peptides are localized to particular regions of the column.

Two different zein B molecules can be distinguished on the basis of peptide mapping. For example, peptide Y2 is found only in early fractions from the hydroxylapatite column where zein B is uncontaminated by zein A. The spot is absent from peptide maps of fractions containing polypeptides of both molecular weights. Two different zein A polypeptides can be distinguished on the basis of N-terminal analysis, one

polypeptide with phenylalanine and the other with blocked N-termini. However, by cyanogen bromide cleavage, three zein A can be distinguished, first polypeptide with no cleavable site, the second with one cleavable site and the third with two cleavable sites. Although only five different zeins A and zeins B could be distinguished structurally by the mentioned techniques, it was likely that these polypeptides were still more complex as we will see later.

Zein A and zein B polypeptides are very similar not only to members of the same class but also to members of the other class. Zein A fractions had only minor differences in amino acid composition when compared with zein B containing fractions. Peptide maps of zein A and zein B fractions show that of the 21 to 24 major peptides, 13-16 occur in both maps. Based on the sizes and amino acid compositions of zein A and B, approximately 20 tryptic-chymotryptic peptides are expected.

Three-fourths of the major spots are shared between zein A and B. This means that the polypeptides of the zein A and B size classes have a common core structure accounting for approximately three-fourths of the length of the polypeptides. The existance of such common core structure is also reflected in the similarity of the amino acid compositions of the separated zeins. Such similarity in amino acid composition is due to selective pressure on the seed to store only certain amino acids (the hydrophobic), almost 60% of the amino acid residues in zein are hydrophobic. Such forces are a functional requirement for zein accumulation to maintain their ability to be packaged into granules. Also certain amino acids to be stored in seeds have to be compatible with the metabolism of the developing and the germinating

seeds. The observation that zein polypeptides have a common core structure is consistent with similar observations on gliadin (Bietz et al., 1970), the analogous protein fraction of wheat. The heterogeneity of the zein polypeptides results from two kinds of variation. A limited variation results in the distinction between zein A and zein B polypeptides. In addition to the common core peptides there is a series of eight major peptides, four of which are found only in zein A containing fractions, and the other four found only in zein B containing fractions. It is very likely that the four zein A and zein B peptides form variable peptides and probably such variable peptides are the source of differences in the four polypeptides for each zein A and zein B. This conclusion is based on the existence of four bands for zein A and another four bands for zein B from the purified hydroxylapatite fractions on the IEF as will be discussed later.

The middle fractions of the column which contains both zein A and B together gave 3-4 more peptides than the pure zein A or B. In addition there are two major spots found only in this fraction which are absent from the pure A or B peptides. From IEF results (see below) there are four bands in this fraction which are different from the zein A or zein B bands. In the peptide maps of the tryptic-chymotryptic digests of all fractions examined there were peptide spots that were not as intense as others. These peptides were different for each fraction. They could originate from polypeptides other than zein A or zein B, such as zein A_1 or the low MW zein, zein C, D and E. Alternatively, the minor spots could come from a hypothetical zein A and zein B variable region. As if there are several zein A polypeptides differing in sequence in only few peptides and it is very likely

this is the case for most of these faint peptides where all the results prove the existence of several polypeptides for each zein A and zein B. Such variable region may be the structural basis for the heterogeneity observed by IEF and hydroxylapatite chromatography.

It can be concluded that some of the variation so far detected is due to sequence differences. The difference between zein A and zein B must be due to differences in sequence since zein A is cleaved by reaction with cyanogen bromide while zein B is unaltered. Furthermore, the observation of three different cyanogen bromide cleavage patterns with zein A must indicate that there are at least three different amino acid sequences in zein A. Common core sequences cannot be at the N-terminal end of zein polypeptides since two different N-terminal amino acids were found for zein A and zein B polypeptides. That only one amino acid could be identified as N-terminal for each zein A and zein B polypeptides suggests that the variable region sequences may be at the N-terminal end. Consistent with this hypothesis is the observation that in vitro synthesized prezein A is cleaved by cyanogen bromide to generate a large fragment of the same size as the 19,600 dalton fragment generated by cyanogen bromide treatment of native zein A as reported by Melcher (1979). Since the precursor bears an extra stretch of polypeptide presumably at the N-terminal end, the methionine at which cleavage occurs must also be near the N-terminal end of the chain. The minor cleavage pattern of zein A to generate a large fragment of 17,000 daltons suggests, also, that the variable sections can be located near the N-terminus, perhaps interspaced with common core sequences.

Additional evidence which establishes that the heterogeneity in zein polypeptides is due to differences in amino acids in the primary sequence came from chemical modification of zein proteins. Protein modification reactions are seldom specific for any one particular kind of residue. Nonetheless, if the same residues are present in the same environment in two polypeptide chains, they should be equally modified. Thus differences in the modification of a population of polypeptides implies a difference in the content of modifiable residues or a difference in the primary amino acid sequence around these residues. Thus the observations presented here that some polypeptides change in isoelectric point while others do not and that some polypeptides are shifted more or less than others can be taken as evidence that the polypeptides differ in amino acid sequence.

Of the modifications attempted here, the modification with iodoacetamide was probably the least specific. Under the conditions used modifications of cysteine, histidine and methionine residues are possible. Alkylation of amino groups are probably not significant because most of the amino groups were in the non-reactive protonated form during the experiment, and because the reaction rate of amino groups is much slower than with the other residues (Means and Feeney, 1971). Although alkylation of cysteine residues probably occurred it cannot be responsible for the basic shifts in isoelectric point that were observed since the modification should not change the charge except at fairly basic pH (Stott and Feinstein, 1973). Since reaction with histidine residues is fairly slow, it is likely that the shifts observed are due to alkylation of methionine residues. Consistent with this interpretation, only the acidic bands shift position to

more basic pH. These polypeptides are known to contain considerable amounts of methionine (Gianazza et al., 1977).

Acrylonitrile modifies sulfhydryl and amino groups creating the cyanoethyl derivatives. Since modified polypeptides focus at more acidic pH values, we believe that the cyanoethyl groups have been converted to carboxyethyl groups by the strong acid present in the electrode solution of the IEF apparatus. The modification of amino groups proceeds much less rapidly and at higher pH than the modification of sulfhydryl groups (Means and Feeney, 1971). It is thus likely that the shifts observed with this reagent are due mainly to modification of cysteine residues. Since in the radioactive labeling of zeins with [^{14}C]-iodoacetate an excess of iodoacetate over sulfhydryl was not used, it is likely that only cysteine residues have become modified with carboxymethyl groups. That the pattern of ^{14}C labeled bands is similar to that of acrylonitrile modified bands argues that acrylonitrile also modifies only cysteine under the conditions employed. Band 9, modified by acrylonitrile but not by [^{14}C]-iodoacetate is the exception to this generalization. It is interesting to speculate that band 9 may contain lysine residues which react with acrylonitrile but not with iodoacetate.

These considerations lead to certain conclusions about zein A, zein B, and low molecular weight zeins. Zein A of 22,600 daltons, consists of at least four polypeptides that generally focus in the basic region of IEF gels, although at least one of them (band 11) is considerably less basic than the others. The basic behavior is consistent with the arginine content of this fraction (Fraij and

Melcher, 1978). The IEF positions of zein A bands are not modified by reaction with iodoacetamide although amino acid analysis suggests the presence of two methionines per chain. The observation of methionine incorporation during *in vitro* synthesis and the susceptibility of zein A chains to cyanogen bromide cleavage supports the presence of methionine in these chains (Melcher, 1978). All zein A bands, with the possible exception of band 11 probably contain cysteine. Mobility shift analysis of modified polypeptides has been shown to be a reliable method of estimating relative contents of cysteine (Stott and Feinstein, 1973). Thus, the larger shift in mobility of bands 16 and 14B when compared with 15 is an indication of a difference in amino acid composition, namely a higher cysteine content. Such a difference must derive from a difference in coding sequences of the mRNA.

Zein B of 19,600 daltons consists of at least four polypeptides that generally focus in the neutral to basic region of IEF gels and include the most basic band detected in unfractionated zein. The IEF positions of zein B polypeptides, like those of zeins A, are not modified by reaction with iodoacetamide. This observation is consistent with the lack of cleavage by cyanogen bromide, but contrasts with the observation of one methionine per chain in amino acid analysis. This methionine may derive from small amounts of low molecular weight zeins which may be present in these fractions. All the identifiable zein B polypeptides appear, from their shift in mobility upon acrylonitrile and iodoacetate reaction to contain cysteine, with band 17 containing more cysteine than the others.

The low molecular weight zein polypeptides are most prominent in the zein II extract, contain methionine and cysteine, and electrofocus

in the acidic region of IEF gels. It should be pointed out, however, that not all polypeptides that focus in this region share these properties. There are a series of faint diffuse bands that do not become modified with iodoacetamide. There are 4 bands (5, 6, 8 and 9) which because of their elution in the middle of the hydroxylapatite elution profile cannot be assigned to a molecular weight. Of these, bands 8 and 9 whose IEF positions are not modified by iodoacetamide reaction but are modified by acrylonitrile. Band 9 may contain lysine since it does not react with iodoacetate. On the other hand, bands 5 and 6 are not modified by acrylonitrile or iodoacetate reaction. Reaction of zein with iodoacetamide reduces the relative intensity of bands 5 and 6 raising the possibility that each consists of both modifiable and unmodifiable polypeptides.

The products of in vitro translation of zein mRNA in wheat germ extracts have previously been shown to be precursors of zein polypeptides. They are longer by 10 to 20 amino acids than the final polypeptides (Burr et al., 1978; Melcher, 1979; Wienand and Feix, 1978; Larkins and Hurkman, 1978). It has also been shown that when membranes are included in an in vitro zein protein synthesis reaction these precursors are trimmed to their native size (Larkins and Hurkman, 1978). It has been suggested that in such incubations glycosylation can also occur (Burr and Burr, 1979). The number of zein precursor products formed in the wheat germ system is similar to the number of zein polypeptides detectable by Coomassie blue staining of IEF gels. However, the isoelectric focusing pattern is quite different. The polypeptides detected can be confidently identified as zein precursors for two reasons. First, on SDS-PAGE zein precursor molecules are

almost exclusively detected. Second, the bands extractable with ethanol are the same as those seen without extraction. Ethanol extraction is the criterion used to define zeins. The possibility that the efficiency of translation of the different mRNA species in the wheat germ extracts differs from that in vivo is ruled out by the observation that IEF patterns identical to mature zein are obtained when in vitro products are made in the presence of membranes (Viotti et al., 1978).

In most animal (Schecter et al., 1975; Devillers-thiery et al., 1975) and some bacterial proteins (Inouye et al., 1977) made on membranes for transport or secretion have mRNAs which, when translated in cell-free systems without membranes, serve as templates for larger-than-authentic sized products. This is also the case for zein which also passes through a membrane during or after synthesis. It is also larger when made in a cell-free system (Burr et al., 1978; Melcher, 1979). The additional length has been called the signal peptide (Blobel and Dobberstein, 1975) and is thought to be cleaved as the amino terminus traverses the membrane on which it is made. Another plant protein which is also proteolytically cleaved is the light chain of ribulose diphosphate carboxylase (Dobberstein et al., 1977; Cashmore et al., 1978). The altered IEF behavior of zein precursors can thus be attributed to the properties of the signal peptides. These must be also heterogeneous in sequence, since a single signal sequence present on all precursors ought to produce a uniform mobility shift for all bands.

The initial products of translation in this processing system are heterogeneous. It is concluded that the primary amino acid sequences, and thus the genes coding for these polypeptides are also heterogeneous.

This conclusion is strengthened by the mRNA/DNA hybridization results, where zein mRNA sequences are found on 15-20 different sizes of $EcoR_1$ restriction fragments of mDNA.

The presence of such a large number of sequence coding for the zein polypeptides raises two possibilities. The first is that the similar polypeptides of zein are coded by diverging genes derived from a common ancestral gene when a mutation (e.g. point mutations lead to a new gene which codes for a new polypeptide; hence, in a few amino acid(s) in the sequence). Polydisperity in storage proteins has been reported in wheat gliadins (Wrigley and Shepherd, 1973) and in potato storage proteins (Stegeman et al., 1973).

The second is a process of splicing different small gene segments to a core gene for the common sequence. Such a process is possible if the structural differences among zein polypeptides were localized to one region of the polypeptide, leaving a region of shared sequence. Although the intensity of the restriction bands are different, some bands are darker than the others, which implies that the dark bands came from longer sequences of the message which exists in a predominant form. Such results cannot favor one of the mentioned possibilities, but indeed explain that zeins are polypeptides and are heterogeneous because the zein genes are also heterogeneous and thus excluding the postmodification effect on the heterogeneity observed. It is not possible to estimate the exact number of zein genes from the present results, but the results demonstrate that the heterogeneity observed in the present study of zein proteins must be due to primary differences in the amino acid sequence. Thus the number of genes coding for such

a large number of polypeptides have to be large in number as well. Supporting such a conclusion is the observation of 18 different polypeptides which are seen by IEF and such number corresponds to the 15-20 bands seen in the restriction fragments of the corn DNA.

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