PROTEOLYSIS OF ZEINS IN THE ENDOSPERM OF GERMINATING MAIZE SEEDS

by

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(ABSTRACT)

The pattern and sequence of zein degradation in the endosperm of germinating maize seeds were investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. The proteases involved in the degradation of various zein components (α, β and γ) were extracted with three buffer systems and partially characterized with respect to their ability to degrade various zein components. They were also investigated *in vivo* by germinating the seeds in the presence of protease inhibitors used singly and in combination.

Of the various zein components, γ-zein (27-kD) was the first to be degraded and its degradation was complete by the third day after germination (DAG). Beta-zeins (17- and 18-kD) began to be degraded on the second DAG, degradation being complete by the seventh day for the 17-kD polypeptide, and the fourth day for the 18-kD polypeptide. The degradation of 10-kD- zein began on the fourth DAG and was complete by the eighth day. The α-zein fraction (22-and 24-kD) was degraded beginning on the fifth day and continued gradually until after the tenth day.

From the results of these studies, the arrangement of various zein fractions within the protein bodies can be deduced and this was consistent with the immunocytochemical data published by others. Gamma-zein is situated in the peripheral region of the protein bodies and could be a structural component of the protein body membrane or it may

be directly anchored in the membrane. Beta-zeins are internal to γ-zein with the 18-kD in the interface between the 17-kD and γ-zein. The 10- kD zein is located between the 17-kD and α-zein or interlacing with α-zein in the protein body core. Finally, α-zeins are in the protein body core. Based on these observations the proteolysis of the protein in protein bodies of maize would start from the periphery and proceed towards their core.

The proteases involved in degradation of various zein components were synthesized *de novo*. The mRNAs pre- existing in dry seeds appeared to direct the synthesis of active proteases required for zein degradation at least during the initial stages of germination. Serine protease was responsible for the degradation of α- and β-zeins while aspartic (acid) protease may play some role in β-zein degradation. Serine and cysteine (thiol) proteases worked synergistically in γ-zein degradation. Enzymes extractable from the endosperm of germinating seeds with 0.2 M acetate buffer (pH 3.8) were able to degrade the α-, β- and γ-zeins in an *in vitro* assay.

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Introduction

Just as wheat and barley are the major cereals of the Old World, maize is the major cereal in the New World. Soon after the discovery of the New World, maize was introduced into the Old World and has since been extremely important in the agricultural economies of all tropical, subtropical, and warm temperate regions of both Worlds. In exceptionally favorable circumstances, a hectare of maize yields more than 20 tons of grain, compared to 10 tons for a hectare of the highest yielding wheat or barley (Baker, 1978).

Maize is very well suited for various scientific investigations and there are several reasons for this. Maize is easy to cultivate and is highly adaptable to a wide range of environmental conditions. Biologically, maize is a monoecious plant in which the staminate and pistillate flowers are separate, but borne on the same plant. This makes the desired pollinations or crosses simpler. Maize is a naturally cross-pollinating plant; therefore, it has an extremely variable genetic base. A single pollination can yield several hundreds of kernels from one ear. Maize is an annual plant, and thus, results of a desired cross can be obtained within a year. Analyses at the cytogenetic and cytological levels are easy because maize has only 20 chromosomes ($2n = 20$). Furthermore, the genetics of maize has been investigated intensively, probably more than any other plant species.

Most of the genetic improvement studies in maize have been done through classical breeding which relies on the transfer of genetic materials from parental plants to offsprings, in the hope that random recombinations would bring out the desired characteristics. However, problems such as restricted parental selection and "unlucky" recombination may bring about a low frequency of success. The process of artificial

selection is time-consuming and the cost of labor is very high (Whitehouse, 1973). With the advent of newer and more sophisticated molecular techniques, most of the above-mentioned problems are expected to be remedied in the future.

Maize seeds are made up of storage proteins (10-12%), starch (80%), and oil (8-10%) (Shollenberger and Jaeger, 1943). Thus, maize could contribute towards meeting the food and feed needs of the world's population. Because of this potential, numerous studies have been directed at the improvement of the nutritional quality of maize. However, most of the physiological and genetical studies on maize have dealt with the development of the seeds: the time of storage protein synthesis, how, when, and where this protein is synthesized and accumulated, and in what amount. Consequently, the other equally important aspect of maize seed, that is germination, has been neglected. Germination represents the period when the various storage proteins are degraded to provide nitrogen to the young sporophyte until it establishes itself and is capable of photosynthesis and nutrient uptake. The study of the germination process deserves emphasis because this stage of maize plant development determines the type of yield obtained from the mature plants later. If the developmental events during germination are impeded, it will have a direct bearing on the goals of all the research aimed at improving the maize with respect to other desirable characteristics such as increased yield and higher contents of essential amino acids.

A major objective of some genetic manipulations in maize is to improve the nutritional quality of its protein because the major storage protein in maize (zein) is known to be essentially devoid of two essential amino acids for human nutrition, lysine and tryptophan. It is conceivable that during the course of evolution of maize, there might have been certain advantages to select against these two amino acids. Before trying to improve the quality of protein in maize, several questions need to be answered. The most important of these questions is: How will the improved protein content affect the

germination of maize seeds and the development of the maize seedlings? In other words, will the improved protein quality have any effect on the ability of maize seedlings to mature into productive plants? It is important to know which of the storage proteins is/are essential for germination and development of maize seedlings before any manipulation is undertaken. This study was thus undertaken to investigate one aspect of maize seed germination, that is, the degradation of various zein fractions and to partially characterize the proteases involved in this process.

The term germination is somewhat more difficult to define than it appears at first sight. Many laboratory workers take the protrusion of the radicle (seed root) through the testa (seed coat) as the beginning of germination. For gardeners and farmers, germination is when the shoot makes its first emergence above the ground. Neither of these definitions is satisfactory because both focus attention specifically on the growing parts of the seed, and overlook other equally important parts, such as mobilization of food reserves during germination. A more general definition should cover all the processes involved in the transformation of a previously dormant plant embryo into an independent and established seedling. Thus, it should also encompass all the processes such as the hydrolysis of food reserves which may begin before as well as persist for a period of time after the root and shoot emerge. The period of germination should, therefore, include the period from the start of imbibition of the seeds to the period at which the starch and storage proteins in the endosperm are completely degraded or the germinating plant ceases to become dependent on them.

An ideal model for the study of seed protein degradation and of the proteolytic enzymes involved would be one in which a few protein substrates, present in high concentrations, are hydrolyzed to free amino acids by a small number of specific proteases in a relatively short period of high metabolic activity. Germinating maize seeds meet most, if not all, of these requirements.

Hypotheses

1. Different classes of zeins are degraded at different times following a definite pattern during germination of maize seeds.

2. The different classes of zeins are degraded by different classes of proteases.

3. Proteases extracted from germinating maize seeds degrade the different classes of zeins *in vitro*.

Research Objectives

The research was carried out with the following objectives in mind:

1. To determine when, and in what sequence the various zein components (α-, β-, γ-, and 10 kD-zeins) are degraded, and when their degradations are completed during germination of maize seeds (Chapter 2).

2. To identify the various classes of proteases that are degrading the various zein components by using specific protease inhibitors *in vivo* (Chapter 3).

3. To isolate the proteases from germinating maize endosperm, and to show that they are able to degrade the various zein components *in vitro* (Chapter 3).

Literature Review

I. History of Maize

Maize first became known to the Old World in 1492, after being discovered by two Spaniards sent by Columbus to explore the hinterland of Cuba, although it was already established as a crop plant in the New World. There is even evidence that it was in Mexico more than 5000 years ago. In fact, all of the principle types of maize recognized today were already in existence in the pre-Columbian times, and each of these types of maize is included in the same species, *Zea mays* L. (Baker, 1978).

Today, maize is known only in cultivation and no wild form has ever been found. Although the time of maize domestication is uncertain, radioactive decay measurements on maize cobs found in archaeological excavations of caves dated them back to around 7000 years ago (Mangelsdorf, 1974). One author mentioned that some 60,000 year old pollen grains taken from a drill core two hundred feet below Mexico City have been thought to belong to this species (Baker, 1978).

The oldest form of maize found appeared to be a "podded" popcorn, which was discovered in 1940 in an abandoned rock cavern in New Mexico (Galinat, 1971). It is believed that maize never occurred in the wild since it was thought to have been selected by humans out of teosinte (presumably the ancestor of present day maize). When the amino acid compositions and electrophoretic profiles for various protein fractions of maize were compared to that of its presumed wild relatives, *Teosinte* and *Tripsacum*, it was concluded that "*Teosinte* is closely related to corn and may be, its direct ancestor" (Paulis and Wall, 1977). However, there is no direct archaeological evidence for the

hypothesis that domestication of teosinte represents the beginning of maize and its evolution under artificial selection.

Maize proteins have been subjected to intensive studies for over 150 years (Gorham, 1821). Selection for high protein maize was initiated by Hopkins (1899), using a variety known as "Burr's white". Several strains have been developed from the original one with the Illinois High Protein line (IHP) and the Illinois High Oil (IHO) line, to name two (Dudley *et al.*, 1974). Most of the high protein lines obtained had been due to an increase in the amounts of a group of low quality storage proteins, that is zein, in the endosperm (Schneider *et al.*, 1952). Zein is known to be deficient in two nutritionally essential amino acids, lysine and tryptophan. One of the few exceptions to this rule was the discovery of a high lysine mutant, the *Opaque-2* (Mertz *et al.*, 1964). The increase in the protein quality in maize is not without a price. Some undesirable traits are decreased resistance to insect pests, storage problems and decreased yield. However, it is expected that in the future, maize with most, if not all of the desired characteristics will be produced with the advent of modern research protocols and facilities, such as greater precision in gene isolation.

II. Biological Importance of Maize

The maize plant has a unique structure (Figure 1 on page 9). Although it is an annual plant, it produces several tillers, and each shoot is supported by prop (adventitious) roots developed from the lowermost stem node. The leaves spread along the stem, each with sheathing bases. An inflorecence (tassel) containing the staminate flowers terminates the stem, while the pistillate flowers are borne upon highly condensed inflorescences (ears) in the axils of leaves. This separation of pollen- and seed- produc-

ing flowers is unusual among grasses (Kiesselbach, 1949) and characterizes the tribe Sorgheae into which *Zea* is placed, all of which are monoecious.

The ear of maize is unique in that it represents a lateral shoot with many internodes, which is subtended by several leaves (up to seven) on a very short, stout stem below the pistillate flowers. The pistillate flowers are crowded in rows along the axis (cob) and each flower produces a single one-seeded fruit, often called a grain (kernel). During flowering, each pistillate flower produces a long silk, which is functionally the stigma and style. The kernel is made up of two major parts (Figure 2 on page 10), the endosperm and the embryo (Shollenberger and Jaeger, 1943). The embryo is a diploid plantlet derived from the fusion of one male gamete and one female gamete. However, the endosperm is a triploid tissue resulting from the fusion of one male gamete and two female polar nuclei (Randolph, 1936).

The major function of the endosperm is to synthesize, accumulate and store nutrient reserves (e.g. starch and proteins) during seed development (sink), and later on to provide the necessary nutrients (source) to the embryo during germination and early seedling growth (Tsai *et al.*, 1978 and 1980). The aleurone layer (fused fruit wall and seed coat) and the embryo contain proteins, oil and vitamins, while the endosperm contains primarily starch and proteins. Thus, maize is important to human diet directly as food and indirectly as feed for domesticated animals.

The leaves and stems, as well as the grain, can be used as livestock feed, and in fact in countries (much of northern Europe) where the summer is too cool for the grain to ripen, silage is prepared from leaves and stems. It is advantageous to include the tassels of male flowers because they are very rich in vitamins. Corn whiskey is made from grains by breaking the starch down to sugar, followed by alcohol fermentation by yeast and distillation to concentrate the alcohol (Baker, 1978).

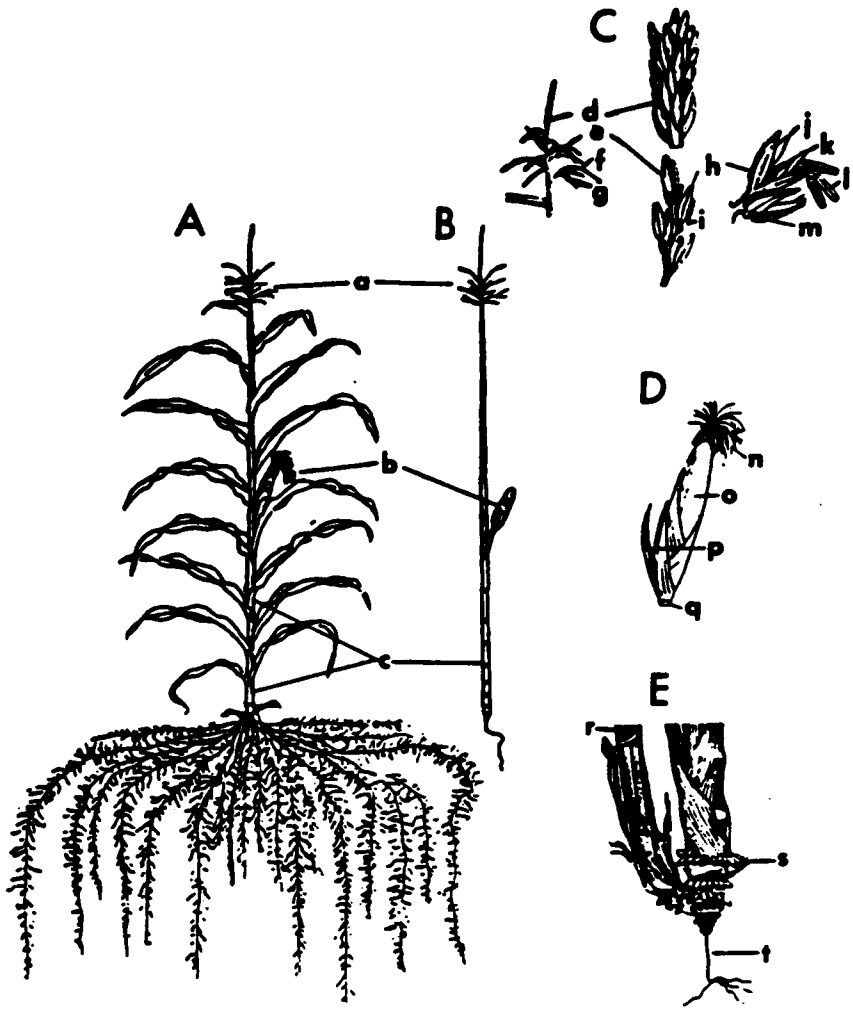

Figure 1. **Mature maize plant and its major parts:** A, mature aerial and root system of a typical maize plant (Kiesselbach, 1949). B, mature maize plant drawn without leaves and adventitious roots. The apical end of the main stem (culm) terminates in the tassel, while the basal end terminates in the primary root (radicle). The ear shoot arises from an internode near the center of the culm. C, the tassel and its spikelets. D, the ear shoot. E, base of main stalk and attached growing tiller and seminal roots with the adventitious (crown) roots been cut off to show their arrangement at the crown. a, tassel; b, ear shoot; c, culm; d, central spike; e, lateral branch; f, first order spike; g, second order spike; h, pedicillate spikelet; i, sessile spikelet; j, secondary male floret; k, primary male spikelet; l, anthers; m, glumes; n, silk; o, husks; p, prophyll; q, shank; r, tiller; s, adventitious root; t, seminal root (Poethig, 1982).

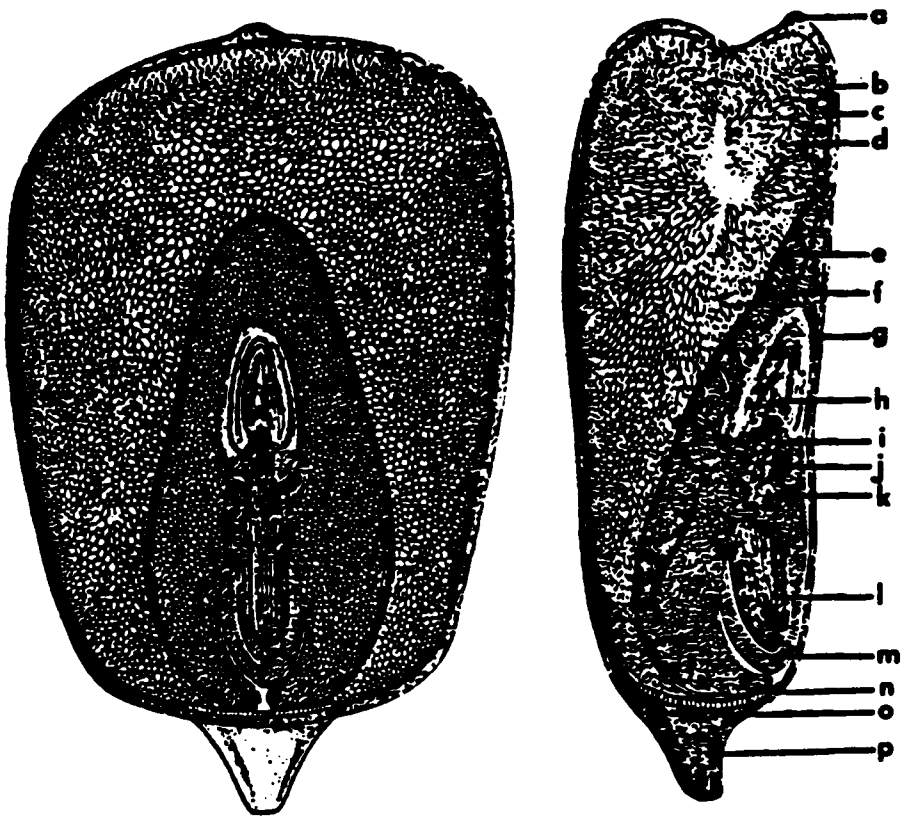

Figure 2. **The mature kernel of Maize:** A and B, vertical sections in two planes of a mature kernel of dent corn, showing arrangement of organs and tissues. a, silk scar; b, pericarp; c, aleurone; d, endosperm; e, scutellum; f, glandular layer of scutellum; g, coleoptile; h, plumule with stem and leaves; i, first internode; j, lateral seminal node; k, scutellar node; l, primary root; m, coleorhiza; n, basal conducting cells of endosperm; o, brown abscission layer; p, pedicel or flower stalk (Keisselbach, 1949).

III. Storage Protein of Maize

During germination the seed rapidly changes from a quiescent state to a dynamic state, with metabolism increasing rapidly. The substrate for this rapid metabolism is known as the storage reserves. The largest amounts of these storage reserves are usually found in the endosperm of monocots and in the cotyledons of dicots; however, other organs may also be involved. These storage reserves consist of lipids, proteins, and carbohydrates. During germination they are hydrolyzed and the products are used by the axis for the synthesis of cellular structural components and subsequent growth. The initial energy required for the metabolic processes and growth is also derived from these storage reserves. Although all three types of storage reserves occur in most seeds, the relative proportions of each varies considerably among species. In reality there is no clear definition as to what constitutes the storage protein. However, the general consensus is that it: (1) has no enzymatic activity, (2) provides nitrogen for germinating seedlings, (3) usually accumulates in aggregates called protein bodies, and (4) is often composed of a related group of polypeptides (Larkins *et al.*, 1984). This review will emphasize only the storage proteins of maize endosperm since they have direct relevance to, and were the subject, of my research.

Storage proteins in maize are primarily located within distinct organelles known as the protein bodies (Duvick, 1961; Wolf *et al.*, 1969; Khoo and Wolf, 1970; Christiansen *et al.*, 1974; Burr and Burr, 1976; Larkins and Hurkman, 1978; Dierks-Ventling and Ventling, 1982). Maize proteins are made up of a heterogeneous mixture of polypeptides which differ in both their physical and chemical properties. Gorsham (1821) coined the word "zeins" for those proteins which are soluble in 70% alcohol. Fractionation of maize proteins into albumins (water and salt soluble) and globulins (soluble in salt and

insoluble in water) was done by Chittenden and Osborne (1891). Osborne and Mendel (1914) were able to extract yet another protein, glutelins, which was soluble in dilute acid or alkali but not in water, salt or alcohol. Osborne and Mendel (1914) also were the first to sequentially extract maize protein using different solvents. They first extracted albumins and some globulins with water. Then globulins were extracted with either sodium or potassium chloride. Zeins were extracted with 70% alcohol. Finally, glutelins were extracted with dilute alkali. The Osborne-Mendel protocols were later modified by Landry and Moureaux (1970). The Landry-Moureaux procedure extracts albumins and globulins in the first extraction step using 0.5 M sodium chloride, then dialyzing the extract in water to precipitate down the globulins. These two procedures were further modified by other investigators (Sodex and Wilson, 1971; Wall *et al.*, 1975; Paulis and Wall, 1977; Paulis, 1981 and 1982). Alternate solvents and procedures to maximize the recovery of various storage proteins in maize have been employed by other researchers. These include defatting, greater fineness of meal and greater solvent to meal ratios (Nagy *et al.*, 1941), and the use of a detergent as solvent (Foster *et al.*, 1950).

Zein is thought to serve as nitrogen sink in developing seeds since zein comprises the major portion of the endosperm protein (Tsai *et al.*, 1978). This hypothesis was based on the observations that nitrogen sink capacity increased with additional available nitrogen fertilizer. Zein and glutelin are also believed to be dynamic nitrogen sinks because especially zein was readily manipulated by nitrogen fertilization and genetic means. Furthermore, increases in nitrogen deposition in the endosperm induced by nitrogen fertilizer were confined to an increase in zein content (Tsai *et al.*, 1980).

Corn meal, in addition to the four protein fractions, was also shown to contain other non-protein nitrogenous (NPN) compounds such as nucleic acids and small peptides. This had been investigated by Zeleny (1935), Baudet *et al.* (1966), and Paulis and Wall (1969).

A. Protein Bodies: Formation and Degradation

The bulk of the storage proteins found in maize is primarily located within distinct subcellular organelles which are often referred to as "protein bodies" in current literature. Other terms such as protein granule, protein vacuole, aleurone body, aleurone vacuole, and aleurone grains are also frequently used (Altschul *et al.*, 1966).

A brief review on the structure and composition of protein bodies was given by Weber and Neumann (1980). Protein bodies are spherical or oval organelles that contain storage protein and other substances bound by a single limiting membrane which is often difficult to be seen in electronmicrographs. They vary in internal structure and size. The simplest protein bodies have a granular or homogeneous matrix without subunits (Saio and Watanabe, 1966; Rost, 1972), while others are somewhat more highly structured with a concentric pattern of light and dark layers (Mitsuda *et al.*, 1969; Khoo and Wolf, 1970; Rost, 1971). The most highly structured protein bodies have one or more subunits embedded within a granular proteinaceous matrix (Rost, 1972). Using the subunit types, Rost (1972) categorized angiosperm protein bodies into three groups: (1) protein bodies without subunits, (2) protein bodies with globoid subunits, and (3) protein bodies with globoid and crystalloid subunits. The crystalloid is a crystalline protein deposit and the globiod consist of storage phosphate compounds.

The formation of protein bodies in the aleurone and the endosperm of maize follows two different courses, that is, by filling of vacuoles in the aleurone layer, and by dilatation of endoplasmic reticulum (ER) cisternae in the endosperm (Kyle and Style, 1978). The enlargement of cisternae of the ER was also observed by Khoo and Wolf (1970). Recently, the measurement of cytochrome oxidase c reductase activity and electron micrograph studies further supported the occurrence of ER derived protein bodies in developing maize endosperm (Larkins and Hurkman, 1978). Also, a similar

polypeptide pattern of storage protein synthesized *in vitro* by membrane-bound polysomes as well as by polysomes attached to protein bodies supported this hypothesis. Furthermore, polysomes attached to protein bodies of maize were successfully used for the *in vitro* synthesis of zein polypeptides (Burr and Burr, 1976; Burr *et al.*, 1978; Viotti *et al.*, 1978). However, Viotti *et al.* (1978) indicated possible contaminations by rough endoplasmic reticulum (RER), which appeared partly attached to protein bodies, besides those organelles lacking attached polysomes detectable in the electron micrographs.

Zein synthesis is initiated in the maize endosperm approximately 12 days after pollination, and continues until maturity. Larkins and Hurkman (1978) reported mRNAs that direct zein synthesis were localized on membrane-bound polysomes. The primary translation products had "signal peptides" which were cleaved as the proteins enter the lumen of the RER (Burr *et al.*, 1978). Once inside the RER, polypeptides associated to form dense insoluble masses called protein bodies. This process appeared to be protein dependent since it also occur in *Xenopus laevis* oocytes injected with zein mRNAs (Hurkman *et al.*, 1981).

Storage protein breakdown during seed germination corresponds to changes in protein body ultrastructure. The contemporary knowledge about the fate of protein bodies in germinating seeds was summarized by Ashton (1976). Protein bodies undergo either internal or peripheral degradation, finally leading to the formation of central vacuole(s) in the storage cells of seeds. The pattern of protein body fate most commonly reported was internal. This was proceeded by swelling of the organelle, followed by a flocculation of its content, and finally by fusion to larger vacuoles or to protein masses (Bagley *et al.*, 1963; Horner and Arnott, 1965; Opik, 1966; Smith and Flinn, 1967; Briarty *et al.*, 1969; Krik and Pyliotis, 1976). Peripheral degradation proceeded from the protein body periphery inwards, and may originate from several locations of the protein body

surface. This proceeded until a large central vacuole remains (Horner and Arnott, 1965; Mollenhauer and Totten, 1971; Rost, 1972).

B. Albumins and Globulins

Albumins and globulins are soluble in salt solution. Albumins on the other hand are also soluble in water in which globulins are not (Chittenden and Osborne 1891). Albumins make up about 4%, while globulins represent about 2% of the total endosperm proteins (Whitehouse, 1973). Most biologically important proteins such as enzymes (proteases, nucleases, etc.), glycoproteins, membrane and nucleoproteins are derived from these two fractions. In their native states, albumins and globulins are tightly folded into compact globular structures held together by intramolecular disulfide linkages and noncovalent interactions.

Albumins and globulins are the less characterized groups of proteins, especially in corn than other cereals. Ultracentrifugation analyses of albumins and globulins, however, revealed two components with molecular weights of 26 kD and 166 kD, respectively (Danielsson, 1949). Paulis *et al.* (1975) compared the migration rates on SDS-PAGE with standard globulin proteins and found that the proteins have molecular weights of 12, 15, 41 and 62 kD, while Misra and Mertz (1976) reported major components of both protein fractions having molecular weights of 13.4, 22, 24.5 and 58 kD. Even though they appeared to be heterogeneous by high resolution electrophoretic and chromatographic techniques, starch gel electrophoresis patterns and amino acid analyses showed some differences between albumins and globulins (Paulis and Wall, 1969). Albumins were shown to be richer in aspartic acid and threonine than globulins (Paulis and Wall, 1969).

Several factors such as ionic strength, pH, and temperature affect the solubility of albumins and globulins (Foster *et al.*, 1950; Paulis and Wall, 1969). In general globulins require higher ionic strength than albumins to remain in solution. Albumin solubility may be reduced when bound by phytic acid (Craine *et al.*, 1961).

Using starch gel electrophoresis, Paulis and Wall (1969) showed that albumins and globulins were both heterogeneous and complex. The heterogeneity of these two protein fractions was further demonstrated by SDS-PAGE especially under high reducing and denaturing condition which resolved more than 20 bands (Paulis et al., 1975; Wilson *et al.*, 1981). Resolution of more bands under reducing and denaturing conditions suggested that these proteins formed intermolecular aggregates in their native states.

C. Prolamins (Zeins)

Gorsham (1821) coined the word "zeins" for maize proteins which are soluble in 70% alcohol. Due to their high content of proline and glutamine, the alcohol-soluble storage proteins in cereals are collectively called "prolamins" (Osborne, 1908). In maize, zeins constitute 50-60% of the total protein in the endosperm (Whitehouse, 1973).

Zein was previously thought to be present in both the endosperm and the embryo (Schneider *et al.*, 1952). They studied the histological location of zein in maize grain by analyzing the nitrogen content of the alcohol soluble fractions of several maize varieties, and found that 43% of zein was located in the endosperm and less than 1% in the embryo. Lee and Tsai (1984) found that zein made up about 50% of the endosperm protein and only 4% was present in 50-day old embryos. These results agreed with those reported earlier (Tsai, 1979 and 1983; Wall and Paulis, 1978). However, Burr *et al.* (1977) and Dierks-Ventling and Ventling (1982) using tissue- specific immunofluorescent procedures found that zein was localized only in the endosperm and

none was detected in the embryo. Dierk-Ventling and Ventling (1982), Ludevid *et al.* (1984), Larkins (1986), and Lending *et al.*, (1988) were able to localize glutelin-2 (γ-zein) within the protein bodies which was distributed around the periphery of the protein bodies. Alpha-zeins were located in the core of the protein bodies, while β-zeins were located around the periphery of the protein bodies (Larkins, 1986; Lending *et al.*, 1988). However, the electron micrographs did not clearly show the arrangement of various zeins within the protein bodies. Furthermore, protein bodies were isolated from kernels 18 days after pollination (a stage in which the protein bodies were still in the process of active development). Sanchez-Martinez *et al.* (1987) confirmed that zein was not synthesized in the embryo by doing hybridization experiments and immunoprecipitation of the *in vitro* translated product of total poly(A^+)- or poly(A^-)-RNA fractions from various stages of development of embryo tissues.

By differential solubility in alcohol, zein can be separated into three distinct fractions (Esen, 1986). They had been designated as α-zein, β-zein, and γ-zein. Alpha- zeins polypeptides are soluble in 90% isopropanol (IPA) but not in 30% IPA/30 mM Sodium acetate (NaAc), making up to about 80-85% of total zein, include at least eight size classes with estimated molecular weights between 22 to 25 kD. Beta-zeins are about 10-15% of the total zein, soluble in 60% IPA but not in 90% IPA or 30% IPA/30 mM NaAc, and have an estimated molecular weights of 17 and 18 kD, respectively. Gamma-zein is about 5-10% of total zein, soluble in 30% IPA/30 mM NaAc (also soluble in water and dilute salt solution), with a molecular weight of 27 kD. However, based on calculated molecular weights from nucleotide sequence data, the sizes of the 22 and 24 kD α-zeins have recently been changed to 23.8 and 26.7 kD, respectively (Esen, 1987).

Of the three zein classes, α-zein is the most heterogeneous, resolvable into up to 25 different charged species by isoelectric focussing (Gianazza *et al.*, 1976). Alpha-zein is

encoded by a multigene family containing up to three subfamilies (Park *et al.*, 1980; Hagen and Rubenstain, 1981). Sequence comparisons indicate 60-70% homology among α-zein polypeptides. To date, complete primary structures of about 15 α-zein polypeptides have been deduced from cloned cDNA and genomic DNA sequences (Geraghty *et al.*, 1981; Argos *et al.*, 1982; Geraghty *et al.*, 1982; Hu *et al.*, 1982; Marks and Larkins, 1982; Pedersen *et al.*, 1982; Spena *et al.*, 1982; Marks *et al.*, 1985; Viotti *et al.*, 1985), β- zein (Pederson *et al.*, 1986), and γ-zein (Prat *et al.*, 1987). However, limited information is available as to which of these sequences correspond to which specific *in vivo* zein polypeptide (Galili *et al.*, 1987).

Alpha-zeins have similar amino acid compositions. There is little or no lysine or tryptophan, however, they contain about 20% glutamine, 20% leucine, 14% alanine, 9% proline and 7% serine (Wilson, 1983). The amino acid composition of the β-zeins and the 10 kD zein is similar; however, they have higher amounts of methionine, cysteine, tyrosine and glycine. The γ-zein differs from others by having higher amounts of proline, histidine, cysteine, glycine and valine, and is lower in glutamine and leucine. The absence of lysine and tryptophan as well as high leucine/isoleucine ratio in zein is responsible for the poor nutritional quality of these proteins for human consumption (Nelson, 1969).

Alpha-zeins contain a conserved peptide of 20 amino acids which is speculated to aggregate to form a polar surface distributed in three symmetrical sites (Argos *et al.*, 1982). Two of the polar surfaces could fold back on each other in an antiparallel fashion that could form hydrogen bonding with adjacent helices, leaving the third free for the aggregation of the polypeptides within the RER. Circular dichroism measurements and electron microscopic studies revealed that α-zein secondary structure is rich in α-helix and the folded protein has rod shape, respectively.

D. Glutelins

Glutelins are the second most abundant form of storage protein in maize endosperm, making up to 39% (Whitehouse, 1973). They were originally defined as those proteins which were soluble in dilute alkali or acid (Osborne and Mendel, 1914). However, they are soluble in such solvents as detergent (Nagy *et al.*, 1941; Foster *et al.*, 1950), sodium bisulfite in alkylbenzene sulfonate solution (Foster *et al.*, 1950), urea, dimethylaminoethanol and 2-chloroethanol (Nielsen *et al.*, 1970). In their native states they form extensive intermolecular disulfide bonds, thus the use of reducing agents such as 2-mercaptoethanol was required for extraction and solubility (Wu *et al.*, 1983).

The alcohol-soluble zein-like fractions may cross-link with one another by disulfide bonds only. Whereas, the main glutelin subunits may aggregate to form a disulfide-linked polymer. Wu *et al.* (1983) reported that alcohol-soluble reduced glutelins (ASG) had 51% α-helix and 14% β-pleated sheet structure. Alkali was a more efficient solvent than acid to extract glutelins (Nielsen, 1970). However, the alkylated reduced glutelin was soluble in acetic acid (Paulis and Wall, 1971). However, it is now believed that this fraction is not glutelin but represents cross-linked zeins (β and γ). To avoid partial degradation of protein by alkali as affected by temperature, pH and time, the Osborne-Mendel procedure was modified by Landry and Moureaux (1970). They instead employed the use of detergent and a reducing agent in their extraction scheme.

Starch gel electrophoresis of reduced and alkylated NaOH extracted glutelins showed that some components resembled those of globulins and zeins (Boundy *et al.*, 1967; Paulis and Wall, 1981). The bulk of these fractions appeared to be of very high molecular weight. Paulis and Wall (1971) found that the zein-like protein contained more cysteine than zein, and that it could be extracted with aqueous alcohol. The alcohol extractable fractions were given various names such as glutelin-1 (Moureaux and

Landry, 1968), ASG (Paulis and Wall, 1971), Zein-2 (Sodek and Wilson, 1971), and γ-zein (Esen, 1986 and 1987). They all showed a high degree of similarity by electrophoretic and amino acid analyses.

IV. Protein Degradation in Maize

Most seeds contain appreciable reserves of nutrients which may include, depending on taxonomic groups, carbohydrates, proteins, lipids, amino acids, organic phosphate esters and minerals. The function of these reserves is to sustain the seedlings until it is firmly established (Bryant, 1985). When cereal seeds germinate, the endosperm reserve proteins are degraded into their constituent peptides and amino acids by increasing levels of proteolytic enzymes. These hydrolysis products are then transferred to the scutellum where they are either directly or indirectly utilized by the growing embryo (Preston and Kruger, 1976).

The implications that the scutellum or the endosperm is the source of nutrient for the growing axis had been credited to C. Bonnet who was the first to study the germination of embryos detached from the cotyledon in his work with *Phaseolus multiflorus* in 1754 (Andronescu, 1919). Also mentioned was the work of Sachs in 1859 who observed that embryos when separated from their endosperms germinated poorly and concluded that the "embryo during germination has a parasitic relation towards its endosperm, digesting and sucking up the latter through its absorption organ, the scutellum". The first scientist to ascertain the dependence of the various parts of different organs upon the embryo and the dependence of the embryo upon the endosperm was P. Van Tieghem in 1873 (Andronescu, 1919).

The chemical composition of different anatomical parts of the maize kernel was determined by Hopkins (1903). He found that the embryo contained almost all the oil and the largest percentage of minerals, while the endosperm contained almost all the carbohydrates and protein of the kernel.

Andronescu (1919) demonstrated that in maize normal plants may be developed from the embryos without endosperm. The course of the germination followed exactly the same stages as those germinations of whole seeds, except that the process was slower than normal. The period for the plant to reach maturity was almost the same as the ones derived from whole seeds, but the number of internodes were reduced.

During maize seed germination, starch, hemicellulose, calories of heat and fat decreased, while the level of sugar increased (Malhotra, 1934). Proteins remained the same as determined by total nitrogen.

The gross nutritional contributions to the germination growth of the maize axis by the scutellum and the endosperm were investigated by germinating excised embryos and excised axes on different carbohydrate substrates (Dure, 1960). The early germination growth of the axis was dependent upon scutellar food reserves, and it occurred at the expense of the scutellar lipid reserves. The results also indicated that the mature embryo depended on the endosperm only for a source of carbohydrate and inorganic ions, and that it did not require hormones, vitamins, or other factors originating in the endosperm for successful germination. Dure (1960) also showed that in the germinating maize, there was a steady loss of endosperm nitrogen from three to ten days. By day 10, maize endosperm had lost 71% of its nitrogen content.

Although excised maize embryos showed normal increases in dry matter when cultured in the presence of glucose or sucrose, the accumulation of alcohol soluble and insoluble nitrogen was very low (Oaks and Beevers, 1964). With excision, the soluble pools of the neutral and basic amino acids were the first components to fall below the

control values. Levels of glutamic and aspartic acids and their amides were close to the control throughout. Leachate obtained from detached endosperm pieces were effective in restoring protein synthesis in the excised embryos. The amino acids most needed by the detached embryo were the same as those which were abundantly supplied by the endosperm. Synthetic mixture of *L*-amino acids with concentration corresponding to those of the hydrolyzed leachate completely restored the normal level of total and protein nitrogen. The results also suggested that small peptides rather than amino acids are the major hydrolysis products.

Ingle *et al.* (1964) determined the changes of various chemical components, nitrogen fractions, sugars, fat, and nucleic acid, in the embryo axis, scutellum, and endosperm of maize over a five-day germination period. Many changes in the chemical components were observed before any growth of the embryo axis occurred indicating that these changes were associated with processes responsible for the resumption of growth. The growth of the axis was largely at the expense of the reserves of the endosperm. Extensive loss of protein and insoluble carbohydrates (i.e. starch) from endosperm occurred over the five-day period. Although the growth of the axis may have been partially maintained by the fat supply of the scutellum, there was also a concurrent utilization of carbohydrates as indicated by the changes in sugars and dry weight. The fat content of the scutellum decreased after the second day with a concurrent increase in soluble carbohydrates and soluble nitrogenous components.

Oaks (1965) studied the regulation of nitrogen loss from maize endosperm by germinating the maize seeds on agar and then transferring them to a nutrient salt solution. The loss of nitrogen from the endosperm was apparent 12 hours after the transfer. By including a mixture of 15 synthetic *L*- amino acids known to be released by the endosperm, the loss of total and alcohol-soluble nitrogen was delayed for approximately 40 hours. Omission of leucine, valine, isoleucine or arginine did not alter the inhibition.

However, when the three branched- chained amino acids were omitted together, or when proline was left out, loss of nitrogen from the endosperm was similar to that observed in the basal medium. Additions and omissions, however, did not significantly affect the loss of dry matter from the endosperm, or the gain in dry matter and alcohol-soluble nitrogen of the embryo. She suggested that the degradation of storage protein in the endosperm is a process regulated by the demands of the embryo for amino acids.

The catabolism of carbohydrates and proteins in excised endosperms of maize was found to be stimulated by the introduction of exogenous gibberellic acid (Ingle and Hageman, 1965). The initiation of sugar production (starch degradation) was completely dependent on added gibberellic acid, whereas amino acid production was only partially dependent. The exogenous hormone replaced a component produced by the embryo axis, which was apparently received by the endosperm 36-48 hours after the start of germination. The increase in ribonuclease activity observed during germination could be accounted by activation of pre-existing enzymes. They concluded that the increase of 3′- nucleotidase activity could not be completely due to activation of enzymes, and nor was it dependent on exogenous gibberellic acid.

Sodek and Wilson (1973) studied the metabolism of [U- ^{14}C]leucine or [U-^{14}C]lysine during the germination of maize using seeds in which the storage protein was labelled. This was done by injecting labelled amino acids below the ear of maize plants during seed development. They showed that the seedling axis preferentially utilized leucine and lysine, and possibly proline, derived from storage protein for the synthesis of new protein.

The hydrolysis of endosperm protein in germinating half-seeds of maize was studied by Harvey and Oaks (1974b). They reported that the degradation of the major storage proteins, zein and glutelin, in maize endosperm began during the second day of germination. The protein most abundant in the mature endosperm, which is zein, was

degraded most rapidly as measured by the loss of nitrogen. About 65% of the endosperm nitrogen had been depleted after eight days. The patterns of protein loss were essentially similar in both the germinating seeds and excised endosperm. The degradation of the major storage protein fractions between three and eight days coincided with the appearance of a protease with an acid pH optimum. Total protein hydrolysis was measured by the release of total nitrogen from the endosperms into the incubation medium. Various fractions of the storage protein were extracted by differential solubility. All the measurements were made by Kjedahl digestion followed by Nesslerization. However, no electrophoretic studies were carried out to show what proteins in a given fraction were degraded and in what sequence.

Excised maize endosperm, when incubated in buffer, rapidly degrades its starch and protein reserves (Harvey and Oaks, 1974c). These processes were not markedly stimulated by the addition of exogenous gibberellic acid. However, proteases and α-amylase production were strongly inhibited by abscisic acid, which can be overcome by exogenous gibberellic acid. Endosperms of a dwarf mutant maize (d_5), which are deficient in endogenous gibberellic acid, produce only small amounts of protease and α-amylase. With these mutants exogenous gibberellic acid caused a three-to-five fold stimulation in hydrolase production. They interpreted these results as to suggest that maize endosperm contain sufficient gibberellic acid to stimulate maximal hydrolase production, and starch and protein breakdown.

The degradation of zein during germination was investigated in special reference to protease activity (Fujimaki *et al.*, 1977). Two main subunits of zein which coincide with the α-zein size components, were gradually degraded as germination proceeded, which was demonstrated by polyacrylamide gel electrophoresis. However, no new bands of lower molecular weight were observed suggesting that zein degradation produces very low molecular weight peptides and amino acids which could not be resolved

electrophoretically. The degradation of zein is followed by the production of free amino acids, especially phenylalanine and tyrosine. Protease activity increased during germination up to five days after germination.

Moureaux (1979) studied the changes in the endosperm protein fractions of germinating maize over a seven-day period. No major changes in total nitrogen, protein nitrogen, or nonprotein nitrogen were observed during the first two days. Following this lag period, there was a rapid loss of the major endosperm protein (prolamins and glutelins) over a five-day period. Other minor endosperm fractions consisting mainly of basic proteins (albumin, globulin, and basic glutelin) also showed decreases. In the first two days of germination, a slight disaggregation of G_3 glutelins into more simpler elements (albumins-globulins) were observed. Protein breakdown during germination of maize closely correlated with the appearance of protease activity.

Ethanol-soluble and insoluble nitrogen and protease activity in maize seeds during imbibition period were determined both in the light and in the dark by Bose and Srivastava (1980). In the embryo, they found that the level of soluble and insoluble nitrogen were the same both in the light and dark. However, in the endosperm, the increase in soluble nitrogen was higher in the light than in the dark, which correlated with the decrease in insoluble nitrogen. It was also found that light increased the proteolytic activity in the endosperm. Among various light qualities, red light was most effective in inducing proteolysis and loss of nitrogen from the endosperm. In addition, the growth of primary leaves from seedlings when grown from light pretreated seeds were better than the dark pretreated seeds.

Simpson *et al.* (1981) estimated the rate of protein degradation in *Zea mays* leaves by using tritiated water and [^{3}H]-acetic anhydride as the labelling agents. The half-life of ribulose-1,5-bisphosphate carboxylase protein in the second leaf of 13-day old seedlings under continuous light was found to be 7.8 days by the tritiated water tech-

nique, and 6.5 days by the [^{3}H]-acetic anhydride method. The half-life, determined under a 14-hour light and 10-hour dark photoperiod, was 6.2 days with tritiated water and 5.4 days with [^{3}H]-acetic anhydride.

The composition and mobilization of the endosperm protein reserve in early seedling growth in normal and opaque-2 *Zea mays* L. cv. Maya were investigated by Metevier and Monteiro (1981). During endosperm depletion there was a net increase in the salt-soluble fraction prior to day three after imbibition. However, glutelins and zein decreased throughout, the former being degraded at initially faster rates. Regardless of the protein composition, generally, the most abundant fraction was utilized most rapidly.

During early seedling growth in maize, there was an initial lag period before an increase in embryo nitrogen was seen, which was followed by a period of rapid nitrogen increase, and finally a period of relatively minor change (Srivastava *et al.*, 1976). This was observed when they investigated the effect of nitrate on early seedling growth in maize. They found that additions of NO^{3-} neither alter the nitrogen content of the seedlings during the phase of nitrogen increase nor affect the loss of nitrogen from the endosperm. In the leaf, addition of NO^{3-} lengthens the period of rapid protein increase resulting in higher level of leaf protein. Srivastava *et al.* (1976) concluded that NO^{3-} had little effect on the endogenous synthesis of nitrogenous compounds in the embryo at a time when amino acids are supplied by the endosperm.

Bose *et al.* (1982) studied the changes in the ethanol soluble and insoluble nitrogen in the embryo and endosperm by allowing maize seeds to germinate in the presence of different nitrogenous salts for 72 hours. They found that a supply of $Ca(NO_3)_2$ enhanced germination and protease activity in the endosperm. This resulted in greater solubilization of protein to soluble nitrogen in the seeds. NH_4NO_3 and $(NH_4)_2SO_4$, however, were less effective.

Oaks *et al.* (1983) examined the possibility of yet undetermined peptide hydrolases involvement in the initial hydrolysis of the storage proteins, since zeins are insoluble in water and the hydrolases they were working with were water soluble. By treating dried endosperm powder with commercial peptide hydrolases, they showed that both carboxypeptidase and pronase were required for a maximum and complete digestion of the endosperm protein. They also showed the restrictive behavior of proline in the primary structure of storage proteins on endogenous peptide hydrolases. This result agreed with that observed by Oaks and Beevers (1964). This conclusion was based on the observation that initially proline was not present in the commercial enzymes hydrolyzed digests even though it represents 11% of the amino acid content of the endosperm. However after 48 hrs of digestion, α- NH_2N in the medium was equal to total nitrogen which suggested a complete hydrolysis of endosperm protein. The inhibition of peptide hydrolases by the primary structured proline was confirmed when proline was detected after only 4 hrs of hydrolysis at 120 °C with 6 N HCl.

Cherry and Hageman (1960) isolated and identified acid-soluble nucleotides from etiolated maize seedlings using ion-exchange chromatography, paper chromatography, paper electrophoresis, and spectral and chemical analyses. Changes in nucleotide content as a function of growth was determined. Monophosphate nucleotides (AMP, UMP, and CMP) and diphosphopyridine nucleotide per unit of dry material increased rapidly during the first four days of germination, but remained nearly constant during the last two days. Diphosphate nucleotides (ADP, UDP, and GDP) increased with seedling growth through the third day of germination, remained constant on the fourth day, and decreased thereafter. Triphosphate nucleotides (ATP, CTP, GTP, and UTP) increased through the first four days of germination and then remained at about the same level for the next two days. These suggested a gradual shift from high energy di- and

triphosphate nucleotide to the monophosphate nucleotides with germination of the etiolated seedlings.

Ingle and Hageman (1965) determined the soluble nucleotides, RNA, DNA, total ribonuclease, and 3′-Nucleotidase from the axis, scutellum, and endosperm of maize at daily intervals during a five-day germination period. They showed that the kernel contained little reserve nucleic acid; the increase of nucleotides and nucleic acid material in the growing axis was due to *de novo* synthesis of these compounds. There was a large increase in total ribonuclease and 3′-Nucleotidase activities. The latter was largely confined to the axis, while the former was only contained within the endosperm.

Quiescent maize embryos were found to contain significant amounts of poly-A-rich preformed RNA (De Jiminez *et al.*, 1981). ^{14}C-amino acid incorporation into trichloroacetic acid precipitable material was at a slow rate at the beginning of imbibition and rapidly increased near 18 to 24 hours. Polysome formation was also determined during this period. Addition of α-amanitin to the incubation system at two six-hour-pulse periods showed significant inhibition of the ^{14}C-amino acid incorporation for the 18-24 hour period, but not for the zero-to-six hour period.

De Jimenez and Aguilar (1984) investigated the relevance of old and new messenger RNA in germinating maize embryos. They studied this by observing the protein synthesized during the first hours of seed imbibition in axes and scutellum of maize embryos saperately. They followed the increase in fresh weight in the embryonic axes through the germination period. Pulse labelling experiments with ^{14}C-amino acids were carried out at two stages of development: zero to six and 18 to 24 hours in the presence and absence of α-amanitin. The proteins were analyzed by two-dimensional gel electrophoresis and fluorography. Their results showed a major pattern of proteins common to both the axes and scutellum, which they termed "house keeping" proteins, apart from the specific proteins synthesized by each tissue. In the axes, the changes in proteins observed be-

tween the periods of zero to six and 18 to 24 hours of development seem to be due to both newly synthesized mRNA as well as to delayed translation of stored mRNA species.

V. Classes of Proteases

A. Proteases

The enzymes that play the central role in the degradation of proteins by hydrolyzing peptide bonds are known as *proteases*, which is equivalent to *peptide hydrolases* (Waldschmidt-Leitz, 1931). Later the proteases that act on *intact proteins* were called *proteinases* while those acting on *peptides* were called *peptidases*, as reviewed by Barrett (1986). These depended on the acceptibility of the amino or carboxyl termini of polypeptides in the specificity sites of the enzymes. For proteinases, the end groups are not required in the specificity sites and the enzyme acted well on long chains and away from the termini. Peptidases on the other hand, require at least one of the termini to occupy the specificity site (Linderstrom-Kang, 1949). Alternatively, *proteinases* are also named *endopeptidases*, and *peptidases* as *exopeptidases* (Barrett, 1985). The classification of proteolytic enzymes is summarized in Figure 3 on page 30 (Barrett, 1986).

B. The Active Sites

The active site is subdivided into catalytic and specificity sites. Since the substrate specificities of proteinases are difficult to define, their classifications are based on their

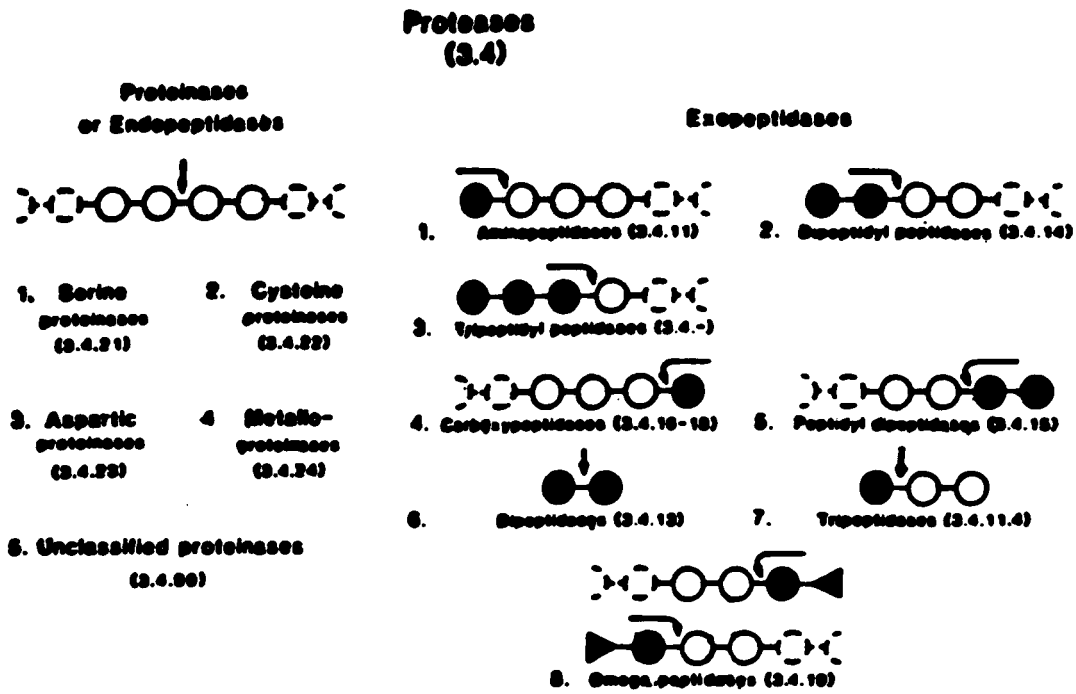

Figure 3. **The classification of proteolytic enzymes:** The enzymes that hydrolyze peptide bonds, all of which are termed proteases, are divided into endopeptidases (proteinases) and exopeptidases (peptidases). The endopeptidases are classified on the basis of their catalytic mechanism, which tends to reflect their evolutionary relationships, whereas the exopeptidases are classified on the basis of their substrate specificity. Numbers in parentheses indicate the divisions into which the enzymes have been placed in the enzyme nomenclature scheme of the International Union of Biochemistry. In the diagramatic representation of the types of activity of exopeptidases, the open circles represent amino acid residues of the substrate, and the filled circles are those of the fragment released (Barrett, 1986).

catalytic mechanisms or active sites. By this system they are classified as *serine, cysteine (thiol), aspartic (acid), metallo-*, and *unclassified* proteinases (Barrett, 1980).

As with other enzymes, proteinases catalytic mechanism appeared to be a two-step process ((Hartley and Kilby, 1954) which is suggested by the observation of the hydrolysis of 4- nitrophenyl acetate by chymotrypsin. The various types of proteases can be distinguished by (1) the requirements of the substrate binding sites that first allign the substrate into the right orientation for the activity of the enzyme's catalytic site, (2) the nature of the group in the enzyme that forms the temporary enzyme-product bond in the enzyme-substrate complex, and (3) the identity of the groups that catalyze the decay of this intermediate.

With active site-directed inhibitors, the various proteinases can be easily distinguished (Barrett, 1977a). *X*-ray crystallography showed that the catalytic site lies in a cleft on the surface of the enzyme molecule. The substrate polypeptide chain lies along the active site cleft, and on either side of the catalytic site are specificity subsites adapted to binding amino acid side chains or the polypeptide backbone. Berger and Schechter (1970) proposed a terminology for the specificity subsites and for the complementary parts of the substrate structure for proteinases (Figure 4 on page 32).

C. Serine Proteinases

Serine proteinases is the most successful of the endopeptidases that have evolved because it comprises the most numerous group and extremely widespread in nature. There are two superfamilies of serine proteinases: the *trypsin* and the *subtilisin* superfamilies (Young *et al.*, 1978). The trypsin types are found in both prokaryotic and eukaryotic microorganisms, plants, vertebrate and invertebrate animals while the subtilisin types are found only in bacteria. The catalytic site consist of a serine residue

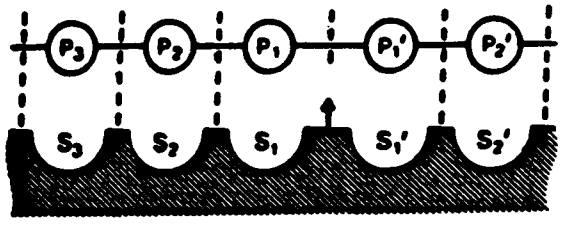

Figure 4. Scheme for proteases subsites and substrate complements terminology: Scheme for the terminology of specificity subsites of proteases, and the complementary features of the substrate. This scheme, for the active site of an endopeptidase, shows how substrate-binding subsites are considered to be located on either side of the catalytic group, in the active site cleft. The subsites are numbered S_1, S_2, etc. away from the catalytic site toward the amino terminus of the substrate, and S_1', S_2', etc. toward the carboxyl terminus. The subsites are usually thought of as binding amino acid side chains, but there are also important interactions with the polypeptide backbone and with terminal blocking groups of synthetic substrates. In exopeptidases, the cleft may be "blind" on one side, so as to prevent binding of extended polypeptides (Berger and Schechter, 1970).

whose hydroxyl group attacks the carbonyl carbon atom of the substrate with a general base catalysis by histidine. Activity of serine proteinases is commonly maximal at slightly alkaline pH (Polgar and Halasz, 1982). The *S* rather than the *S'* subsite fitting is important for the specificities of serine proteinases (refer to Figure 4). The subsite *S* must be occupied by a side-chain of arginine or lysine as a requirement for most serine proteinases. However, some require large hydrophobic or small aliphatic side-chain of the substrate (Young, *et al.*, 1978).

D. Cysteine (Thiol) Proteinases

Cysteine proteinases are found in bacteria, eukaryotic microorganisms, plant and animals, consisting of at least four superfamilies, the most common ones being papain, clostripain, streptococcal proteinase and calpain (Lowe, 1976). The active residue in the active site is cysteine (cys-25 in papain) forming a reactive nucleophile with histidine (Light *et al.*, 1964). All cysteine proteinases have acid pH optima, and require a low molecular weight thiol compound as an activator. The dominant specificity subsite is the requirement for phenylalanine side-chain of the substrate to occupy the hydrophobic pocket of the S_2 subsite (refer to Figure 4 on page 32). It also has a minor requirement for the arginine side-chain (Drenth *et al.*, 1976).

E. Aspartic (Acid) Proteinases

Aspartic proteinases are confined only to the eukaryotes (North, 1982). The most thoroughly studied is pepsin. They are secreted as zymogens, when at low pH, activation is initiated by the cleavage of the *N*-terminal extension of the polypeptide. Two

aspartic residues form part of the catalytic system of the active site (Knowles and Wybrandt, 1968). Low pH optimum facilitate the unfolding of the substrate. They act best on peptide bonds between large hydrophobic amino acid residues, such as, Leu-Tyr, Tyr-Leu, Phe-Phe, and Phe-Tyr bonds. Cleavage have been shown to occur within groups of hydrophobic residues (Barrett, 1977b; North, 1982).

F. Metallo-Proteinases

Metallo-proteinases have a wide and diverse distributions occurring in bacteria, streptomycetes, fungi, and higher organisms (Hartley, 1960). Most of the known metallo-proteases are of the exopeptidase types (Barrett, 1986). The main catalytically active metal is zinc but can be replaced by cobalt. The active site contains a zinc atom bounded by two histidine side-chains and one glutamic acid. Stabilization of the enzyme is achieved by binding to four Ca^{2+} (Kester and Mathew, 1977). They commonly cleave between amino acids with non-polar side- chains and often bulky hydrophobic residue (Leucine or phenylalanine) which is favored in the P_1 subsite of the substrate and S_1 subsite of the enzyme (refer to Figure 4 on page 32).

G. Unclassified Proteinases

Certain proteinases seem to cleave peptide bonds by mechanisms that are different from those described above. They are allocated to proteases of unidentified catalytic mechanisms in the section 3.4.99 of the enzyme nomenclature (1978), as mentioned by Barrett (1986).

H. Exopeptidases (Peptidases)

A glossary and bibliography of mammalian exopeptidases was prepared by McDonald and Barrett (1985). This is also applicable to exopeptidases from plants and other organisms. They may be categorized as follows (refer to Figure 3 on page 30):

1. *Aminopeptidases* hydrolyze the peptide bond between α-amino acyl residue (*N*-terminal) and the polypeptide to which it is attached, releasing the *N*- terminus peptide.

2. *Dipeptidylpeptidases* and *Tripeptidylpeptidases* liberated the *N*-terminal dipeptide and tripeptide sequentially from a polypeptide chain, respectively.

3. *Carboxypeptidases* release the *C*- terminal residue from a polypeptide.

4. *Peptidyldipeptidases* cleave off a *C*- terminal dipeptide sequentially from a polypeptide chain.

5. *Dipeptidases* and *Tripeptidases* require both a free α-amino group and an α-carboxyl group in their substrates. Dipeptidases cleave dipeptide with varying specificity for one or both of the amino acid residues. Tripeptidase cleave the *N*-terminal residue of a tripeptide.

6. *Omega Peptidases* cleave terminal peptide (*N*- or *C*-terminal) that lacks a free α-amino or α-carboxyl group, or is linked through a bond that is not an α-peptide bond (blocked terminal residues).

VI. Roles of Proteolytic Enzymes in Storage Protein Hydrolysis

Recent research of Winspear *et al.* (1984) indicates that the proteolytic enzymes present in such cereals as maize, sorghum, and rice, which tend to be grown in tropical and subtropical climates, had a much higher ratio of endopeptidase to carboxypeptidase activity than temperate zone cereals such as wheat and Barley. Comparison of trichloroacetic acid (TCA)- soluble products derived from the hydrolysis of hemoglobin showed that carboxy-terminal amino acids (histidine, arginine and tyrosine) were released when treated with extracts from wheat and barley endosperm. Extracts from maize endosperms released much more of the TCA-soluble ultraviolet-absorbing material, however, very little was released as free amino acids within the first two hours. Because of this, Winspear *et al.* (1984) suggested that it can be expected that mobilization of the endosperm reserves could be quite different.

In maize, there are at least three endopeptidases in the endosperm with pH optima of 3.8, 5.4, and 7.5, in addition to carboxypeptidase enzymes. Using hemoglobin as substrate, Harvey and Oaks (1974a) were able to show that the enzyme has a pH optimum of 3.8 and a temperature optimum of 46 °C. The enzyme extract also degraded gliadin, glutelin and partially hydrolyzed zein under standard assay conditions, and the enzyme exhibited endopeptidase activity with all substrates tested. The protease activity increased from day three to day eight after imbibition.

Using $(NH_4)_2SO_4$ fractionation, CM-cellulose chromatography, DEAE-cellulose chromatography, Sephadex G-100 gel filtration and preparative polyacrylamide gel electrophoresis, Abe *et al.* (1977) were able to purify a protease which denatured hemoglobin at a pH optimum of around 3.0. Abe *et al.* (1978) investigated the substrate specificity of a sulfhydryl protease (*P-Ia*) purified from germinating maize using syn-

thetic substrates and oxidized insulin β- chain. *P-Ia* showed a potent activity for *p*-nitrophenyl esters of various amino acid derivatives, except for those of carboxy-*L*-proline and carboxy-*L*-valine. Basing on these substrate specificity studies, they concluded that *P-Ia* is a sulfhydryl protease which seemed to have a characteristic similar to pepsin rather than papain.

Feller *et al.* (1978) determined the profile of pH dependence and activities of amino- and carboxypeptidase, and endopeptidases using casein as substrate in crude extract from various organs of corn seedlings during germination and early development. They found that activities of endosperm endopeptidases increased concurrently with loss of endosperm nitrogen during germination at all pH. In scutellum extract the major activity was at pH 5.4. High pH (5.4 and 7.5) activity was barely detectable in the shoot. The roots contained endopeptidase which increased optimally at pH 7.5. Carboxypeptidase activity was relatively low in young tissues (root tips and 3-day old shoots). Aminopeptidase activity was highest in organs with high metabolic activity (scutellum, shoot, and root tips). Because of the increases in activity of carboxypeptidases were concurrent with the decrease in nitrogen in endosperm and scutellum, Feller et al. (1978) suggested that this enzyme may be cooperatively involved with endopeptidases in the mobilization of reserve protein.

As in Barley, there were also endopeptidase inhibitors in maize that disappeared upon germination. A trypsin inhibitor is known to be present in maize kernels (Hochstrasser *et al.*, 1970; Melville and Scandalios, 1972; Ott and Scandalios, 1976). Abe *et al.* (1980) purified two cysteine proteinase inhibitors in maize endosperm, having molecular weights of about 13,000 (Inhibitor I) and 9,500 (Inhibitor II). Abe *et al.* (1980) followed the effect of these inhibitors on *P-Ia* and papain during germination. The inhibitory activities on both proteinases decreased during germination with the concurrent increase in proteinases activity. These observations led them to suggest that

the protease inhibitors suppress the proteolytic activity in dormant kernels and a proteolytic activity appears as a result of the decrease in the inhibitory activity upon germination.

Results obtained by Harvey and Oaks (1974b) indicated that at least part of the endopeptidase enzyme was synthesized *de novo* in the maize endosperm during germination. Addition of cycloheximide at the beginning of the incubation period prevented the development of α-amylase and protease activities and the disappearance of starch and protein reserves. Late additions (70 hours after incubation) of cycloheximide still inhibited the increase in starch hydrolase (α-amylase) activity but had no effect on the hydrolysis of storage reserves.

When the proteinases syntheses were initiated, there was no real control over the total production and they may be over- produced. This was observed when cycloheximide was added to the incubation medium (Oaks *et al.*, 1983). Proteolytic activity was totally inhibited when cycloheximide was added to the growth medium prior to three days after germination. After three days of germination, addition of cycloheximide did not effect the proteolytic activity. The increases in protease activities with time in de-embryonated, water incubated maize endosperm did not normally respond to gibberellic acid (Harvey and Oaks, 1974c; Winspear, 1981). Abscisic acid inhibited the development of the starch hydrolases, however, the inhibition was overcome by addition of gibberellic acid (Oaks *et al.*, 1983).

A number of the above studies reported that endopeptidase activity, which increased in the maize endosperm during germination, coincided with decreases in total nitrogen and protein breakdown, but the timing of these events was somewhat uncertain. For example, Harvey and Oaks (1974a) had found that zein and glutelin degradation in the endosperm began after 20 hr of germination, and the loss in total protein took place between three to eight days, coinciding with increased in endopeptidase activity. Simi-

larly, Feller *et al.* (1978) found that the endopeptidase and carboxypeptidase activity increased rapidly from day two onward, reaching a plateau between day four and day six. This was accompanied by a simultaneous decreased in endosperm nitrogen. Moureaux (1979) on the other hand, found that within the first two days of germination, disaggregation of a portion of the glutelins into albumins and globulins can be observed. This was followed, between two and 2.5 days, by extensive breakdown of storage protein fractions coincident with the rate of appearance of proteolytic activity that had a maximum at 3.5 days, and thereafter decreases. This suggested that the level of protease formed during this time period was sufficient to hydrolyze the storage proteins.

Evidence of a limited role for the carboxypeptidase enzymes relative to the endopeptidase enzymes in maize storage protein catabolism came from the studies using hemoglobin as substrate. Extracts from maize, sorghum, and rice liberated a great deal less carboxy-terminal amino acids than that from wheat or barley (Winspear *et al.*, 1984). This did not preclude some role for the carboxypeptidase system. Feller *et al.* (1978) suggested that this enzyme system could work synergistically with the endopeptidase to increase the rate of storage protein hydrolysis. It was established that hydrolysis of zein during germination was accompanied by the formation of free amino acids, in particular, phenylalanine and tyrosine (Fujimaki *et al.*, 1977). This suggested the definite participation of enzymes with exopeptidase activity. Decreased in the two main zein subunits which correspond to α-zein were also followed during germination by electrophoresis. No evidence of intermediate polypeptides with electrophoretically different mobilities were detected (Fujimaki *et al.*, 1977).

VII. Protease Characterization

A. Maize Leaf

In senescing maize leaves, it was observed that *in vitro* endopeptidase (casein assay) activity assayed at pH 5.4 and 7.5 increased as *in vitro* leaf protein decreased (Feller *et al.*, 1977). Activity at pH 5.4, but not 7.5, was stimulated by sulfhydryl groups or ethylenediamine tetraacetic acid (EDTA) in the extraction and reaction buffer.

Lin and Wittenbach (1981), in an experiment carefully conducted to obtain pure vacuoles, found that these organelles contained 4% of the maize leaf protoplast proteins and all of the proteolytic activities (endopeptidase activity on purified RuBPCase or casein). The maize leaf protoplasts retained 12% of the protoplast proteolytic activity, but studies with ^{125}I-labelled trypsin showed this was due to contamination during protoplast preparation and chloroplast isolation.

The turnover rate of RuBPCase in maize leaves was measured by Simpson *et al.* (1981). By using tritiated water and [^{3}H]acetic anhydride as labelling agents, they reported that the half-life of RuBPCase was about seven days. However, no further enzymology was done.

B. Maize Seeds

Endopeptidase appeared to be the major enzymes which degraded reserve protein in germinating maize endosperm (Feller *et al.*, 1978; Moureaux, 1979). Of all the enzymes tested, only carboxypeptidase activity was markedly (in excess of 50%) inhibited by phenylmethylsulfonylfluoride (Feller *et al.*, 1978). In the endosperm and

scutellum of dry kernels only aminopeptidase activity was found in appreciable amounts. However, aminopeptidase was highest in organs with high metabolic activity (scutellum, shoot and root tips) and decreased in plant parts undergoing rapid loss of nitrogen (endosperm and senescing leaves).

Three acid proteases (P_{11}, P_{21} and P_{22}) from germinating maize endosperms were isolated and partially characterized by Moureaux (1979). P_{11} (MW 40,000) was present in ungerminated seeds, could not hydrolyze prolamins, and was insensitive to reducing agents. P_{21} (MW 36,000) and P_{22} (MW 12,000) appeared on day three of germination and were able to degrade prolamins *in vitro*. Reducing agents enhanced their activity and prevented their aggregation and denaturation. Using comparative assay with other substrates (hemoglobin and BAPA), Moureaux (1979) suggested that the enzyme preparations were principally of the endotype proteases with little contaminating carboxypeptidase activity.

Ryan and Walker-Simmon (1981) reported two cysteine endopeptidases having pH optimum of 3.0 (hemoglobin substrate) and 7.5 in maize seeds. Their molecular weights were 21,000 and 52,000-58,000, respectively. The smaller enzyme was inhibited by PCMB and antipain, unaffected by DFP or diazoacetyl-*D*- *L*-norleucine or pepstatin, and activated by thiols. The larger enzyme was inhibited by PCMB, *N*-ethylmaleimide, and TLCK, but was unaffected by PMSF, TPCK, or thiols. The wide difference in pH optima between the two enzymes suggested different physiological roles in maize seedlings and their possible presence in different cells, or perhaps compartments of the same cell. Makoto *et al.* (1978), however, reported a protease sensitive to cysteine endopeptidase reagents from germinated maize was found to be similar to pepsin, but not papain by its substrate specificity.

C. Maize Roots

Shannon and Wallace (1979) reported that maize roots contained at least two endopeptidases called proteinase I and II. Proteinase I was designated as a serine type of enzyme which degraded azocasein optimally at pH 9-10. It also degraded maize root proteins in the neutral pH range. Perhaps proteinase I was several enzymes. Proteinase II degraded hemoglobin at pH 4, but not azocasein. It was inhibited by serine and thiol reagents. Thus it was suggested that proteinase I, but not II, was responsible for nitrate reductase inactivating activity in maize roots (Shannon and Wallace, 1979). The confirmation of this was made later after the enzyme was further characterized (Wallace and Shannon, 1981). Other investigators found that this enzyme (proteinase I) degraded hemoglobin optimally at pH 4.0. This condition was highly unusual for a serine type of proteinase (Knight, 1977; Ryans and Walker-Simmon, 1981).

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Chapter 2: Zein Degradation in the Endosperm of Germinating Maize Seeds

Abstract

The pattern and sequence of zein degradation in the endosperm of germinating maize seeds were investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blots developed with a monoclonal antibody to α-zein and polyclonal antibodies to β- (17 and 18 kD), γ- and 10-kD zeins. The results suggested: (1) The degradation of the predominant α-zein fractions (22 and 24 kD) started on the fifth day after germination (DAG) and continued gradually until 10 DAG with a small fraction remaining undegraded on the 26th DAG, (2) Beta-zeins (17 and 18 kD) began to be degraded on the second day after germination, and the degradation of the 17 and 18 kD polypeptides was completed by 7 and 4 DAG, respectively, (3) gamma-zein (27 kD) was the first zein component to be degraded and its degradation was complete 3 DAG, and (4) The degradation of the 10 kD zein began on the fourth DAG and was completed by 8 DAG. Based on these results, the following model was postulated for the arrangement of zein polypeptides within the protein bodies. It assumes that the proteolytic events start at the periphery and proceed towards the core of the protein body: (1) Gamma-zein would be situated around the periphery of the protein bodies and could possibly be a structural component of the protein body membrane or directly anchored in the membrane, (2) Beta-zeins would be internal to γ-zein with the 17 kD being more internal to the 18 kD polypeptides, and (3) α-zein and the 10 kD size class would be in the protein body core. This arrangement is consistent with published data on the immunocytochemical localization of zeins. It is interesting that the different size classes of zein are not randomly organized within the lumen of the protein body.

Introduction

The prolamins of maize, zeins, are a group of alcohol soluble proteins which constitute the major storage proteins in maize endosperm. They can be separated into three fractions by differential solubility (Esen, 1986). These three distinct fractions are designated as α-zein (22 and 24 kD zeins), β-zein (17 and 18 kD zeins), and γ-zein (27 kD zein) (Esen, 1987). Based on calculated molecular weights from nucleotide sequence data, the sizes of 22 and 24 kD α-zeins have been changed to 23.8 and 26.7 kD (Esen, 1987). Also present is a minor 10 kD zein polypeptide, which exhibits solubility characteristics similar to those of α-zeins. During the course of seed development zein is deposited in distinct membrane-bound subcellular compartments called protein bodies (Wolf, Khoo and Seckinger, 1967; Larkins and Hurkman, 1978).

Harvey and Oaks (1974) showed that zein and glutelin degradation in maize endosperm took place between 3 and 8 days after germination (DAG) and coincided with the appearance of an acid pH optimum protease. The involvement of protease activity in the degradation of α- and β-zeins during germination increased remarkably during germination up to 5 DAG, and resulted in small peptides and individual amino acids without any intermediate size fragments detectable by electrophoresis (Fujimaki *et al.*, 1977; and Moureaux, 1979). Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques, the results showed that the degradation of the various zein size classes during germination is differential and not random and proposes a model for the arrangement of zeins within maize protein bodies.

Materials and Methods

Seed Germination and Preparation of Endosperm

Seeds of the maize inbred K55 were surface sterilized using 1.5% hydrogen peroxide for 10 minutes followed by a commercial strength bleach for 15 minutes and rinsed five times with sterile distilled water. The seeds were germinated in vermiculite (10 seeds per pot) in the dark. Ten seeds or seedlings were sampled daily up to ten days after germination (DAG), and the embryos, growing axes and seed coats were removed and discarded. The endosperms were freeze-dried, ground to a very fine powder (corn meal) using a stone mortar and pestle, and kept at -20 °C until used.

Zein Extraction

The whole zein was extracted from the corn meal with 60% isopropanol (IPA) containing 1% 2-mercaptoethanol (2-ME) at a ratio of 50 mg meal to 1 ml solvent. The extraction was performed overnight at room temperature and with constant shaking. Zein was recovered in the supernatants after centrifugation at 5000 x g for three minutes. Zein content in the extract was monitored using the spot test of Esen (1978).

Antisera Production

Antisera to α-zein were prepared in our laboratory, and a monoclonal antibody (MAb) to α-zein was prepared in collaboration with Dr. Gerhardt G. Schurig of the College of Veterinary Medicine at Virginia Polytechnic Institute and State University.

The antibodies used were designated as R3030, R2026, R309, and MAb 3944 (a monoclonal antibody which is reactive with all 22 and 24 kD α-zeins). The antiserum R3030 was to a homogeneous 17 kD β-zein but it cross-reacted weakly with all other zein components but extensively with 18 kD β-zein. This antiserum was also employed to probe for the 18 kD β- zein. The antiserum R2026 was to a homogenous 27 kD γ-zein. It cross-reacts to some extent with all the zein components except the 10 kD component, and very extensively with the 18 kD β-zein. The antiserum R309 was to a homogeneous 10 kD zein. It cross-reacts with all other zein components except the 27 kD γ-zein, and extensively with the 17 and 18 kD zein components.

Electrophoresis

The zein, extracted as described above, was evaporated to dryness and reconstituted in an equivalent volume of SDS sample buffer (Laemmli, 1970). Prior to electrophoresis, the samples were heated in a water bath at 90 °C for 10 minutes. The samples were electrophoresed on a 12% SDS-polyacrylamide gel (17 cm long resolving gel) according the procedure of Laemmli (1970) using the Biorad Protean II apparatus. Zeins were detected by staining with Coommasie blue R250, and de-staining in a solution of 25% IPA and 10% acetic acid in water.

Western Blotting

After electrophoresis, the proteins were electro-transferred from the gel onto a nitrocellulose (NC) membrane according to Towbin *et al.* (1979) and using a Tris-glycine-SDS buffer (25 mM, 192 mM, and 0.25%, respectively), containing 10%

methanol. Electroblotting was performed at constant current of 60 V for 45 minutes followed by 100 V for 1.5 hours. The NC filters containing the proteins were rinsed in several changes of distilled water, air dried, and developed immunologically using the procedure of Esen *et al.* (1983) with the modifications indicated in the Figure legends. The filters were reacted for 1 hr (room temperature) or overnight (4 °C) with MAb, anti-α-zein, anti-β-zein, anti-γ-zein and anti- 10 kD-zein in the presence of PBST (1.59 mM KH_2PO_4, 8.4 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, and 0.05% Tween 20). All incubations with the secondary antibody (Goat-anti- mouse, GAM) or protein A peroxidase (PAP) and subsequent washes (4 times, 5 minutes each) were also conducted in PBST. The peroxidase substrate, 4-chloro-1-naphthol dissolved in methanol, was used at a final concentration of 0.6 mg/ml of PBS (1.59 mM KH_2PO_4, 8.4 mM Na_2HPO_4, 2.68 mM KCl, and 137 mM NaCl) containing 0.05% H_2O_2.

Results and Discussion

The Content of Whole Zein During Germination

The progressive decrease in zein concentration as germination proceeded was demonstrated by using the Commasie blue dye-binding spot test (Esen, 1978). Figure 5 on page 61 shows that the onset of zein degradation takes place on the first DAG and proceeds until the seventh DAG. This observation suggested: (1) zein degradation might be an important source of free amino acids and amide nitrogen during the early developmental stages of maize seed germination. This conclusion is supported by the observation that seeds of opaque-2 maize genotypes, having low zein amount and zein degradation rate (Metevier and Montero, 1981), also show a slower germination

rate as compared to normal inbreds (data not shown), and (2) the possible involvement of a proteolytic activity responsible for zein degradation in maize seeds post-germination.

The Differential Degradation of Zein Size Classes

The time-course of degradation of zein size-classes was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Figure 6 on page 63 shows a general decrease in all zein classes during the course of seed germination. However, the degradation of different zein size classes after germination was differential. That is, different zein classes exhibit different rates and time courses of degradation as shown from their electrophoretic profiles on SDS-PAGE. Moreover, the sequence of the degradation of different zeins was not random. The predominant zein fraction, the 22 and 24 kD α-zein, was degraded more slowly than other zeins (Figure 6, arrows b and c, respectively). The degradation of α-zein was evident on the fifth DAG and continued until after the tenth DAG. Although most of the the α-zein polypeptides were degraded by the seventh DAG, a small fraction seemed to resist proteolysis and persisted for a long period of time. The 17 and 18 kD β-zein components began to show detectable degradation on the second DAG (Figure 6, arrows d and e). The pattern of degradation appeared to be gradual with the 18 kD being degraded more rapidly (completed by the fourth DAG) than its 17 kD counterpart (completed by the seventh DAG). As for the minor 10 kD zein, it began to be degraded on the fourth DAG and appeared to follow the course of degradation of α-zein (Figure 6, arrow f). Its degradation was complete by the eighth DAG. The 27 kD γ-zein showed the most rapid degradation and was the first zein component to be degraded completely (Figure 6, arrow a). The degradation of γ-zein was evident at the onset of imbibition

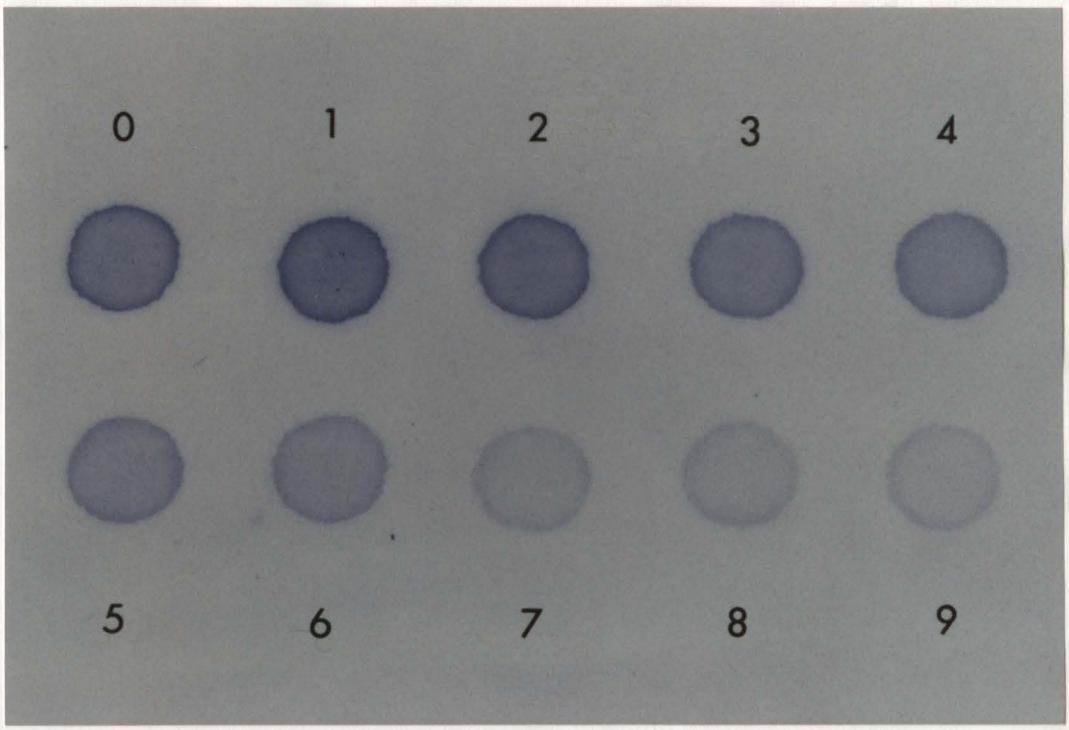

Figure 5. **Spot test analysis of extracted zeins:** Five μl of zein extract was spotted on filter paper and allowed to dry in the oven (60 °C). The filter paper was stained with Coommasie blue according to Esen (1978). Numbers 0 - 9 denote days after germination (0 was from dry seeds).

(1 day in germination) and was completed by the third day after germination (DAG). Between 1 and 2 DAG, the degradation seemed to progress rather rapidly, and γ-zein completely disappeared by the third DAG. Figure 6 also shows that products of zein degradation could be detected after SDS-PAGE as novel, low molecular weight alcohol-soluble components (Figure 6, unmarked arrows). In order to elucidate from which zein size class the lower molecular weight polypeptides originate, immunoblots of SDS polyacrylamide gels were probed with antibodies prepared against various zeins as described below.

Antibodies to α-zein: The monoclonal antibody MAb3944 was used to study the degradation pattern of the 22 and 24 kD α-zein components (Figure 7, arrows a and b). They were most stable and their degradation was very gradual when compared to other zeins. The onset of degradation was evident by the appearance 20 kD fragment beginning the fourth DAG. After the seventh DAG most of the α-zein components were degraded (Figure 7, arrow c). The remaining components persisted for a long period of time and were degraded more gradually than the rest of the α-zeins. Such intact α- zein was detected even on the 26th DAG in a longer time-course study (data not shown). There are two possible explanations for the presistence of small amounts of α-zein. The first of these is the possibility that an island of endosperm splits physically from the rest and it is no longer subject to signals from the embryo, and the second, the germinating maize seedlings are capable of synthesizing their own carbohydrate and become independent of the endosperm reserves after 10 days or so. Fragments of about 20 and 21 kD appeared around the fourth DAG. Fragments with sizes of 19, 17.5, 12, 10, and 9 kD could be detected beginning the fourth DAG (Figure 7, unmarked arrows). These lower molecular weight fragments could no longer be detected after seventh DAG.

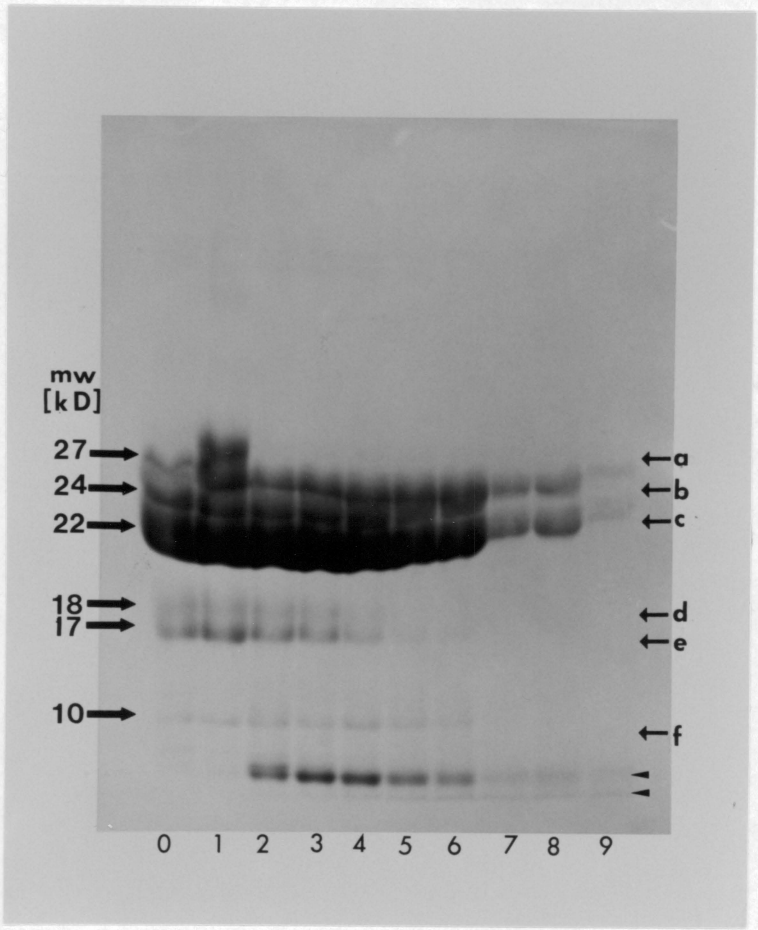

Figure 6. **Whole zein profiles on SDS-PAGE:** Analysis of total zein fractions from endosperms of germinating maize seeds by SDS-PAGE. Arrows a, 27 kD γ-zein; b, 24 kD α-zein; c, 22 kD α-zein; d, 18 kD β-zein; e, 17 kD β-zein; f, 10 kD zein; unmarked, novel degradation products of zeins degradation. Numbers 0 - 9 denote days after germination (0 was zeins extracted from dry seeds). The SDS-PAGE (12%, 17 cm) was performed according to Laemmli (1970) on a BioRad Protean II apparatus.

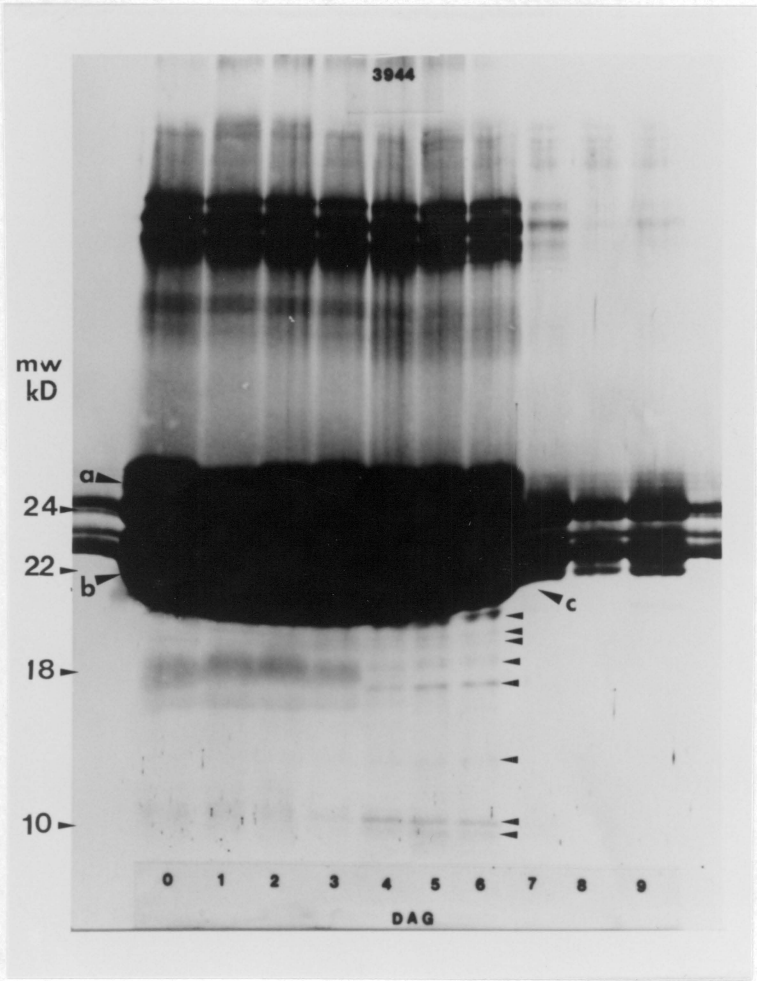

Figure 7. Immunological analysis of Western blot with MAb 3944: Monoclonal antibody 3944 (anti-22 and 24 kD α-zein) was used to probe the Western blot. Arrows a, 24 kD α-zein; b, 22 kD α-zein; c, time when major α-zein degradation; unmarked, products of α-zein degradation. Numbers 0 - 9 denote days after germination (0 was from dry seeds). Western blotting was performed as described by Towbin *et al.* (1979) and immunological assay was performed as described (Esen *et al.*, 1983).

Two main subunits of zein which correspond to the size components of α-zein were also shown to be degraded gradually by SDS-PAGE (Fujimaki *et al.*, 1977). However, since no new bands of other molecular weight sizes were observed, this led to the conclusion that the proteins were hydrolyzed directly to low molecular weight peptides that ultimately release free amino acids especially phenylalanine and tyrosine. Moreover, their procedure extracted only α-zein since a reducing agent was not included in the solvent. Fujimaki *et al.* (1977) followed zein degradation only by staining the gel for protein. My procedure, however, employed a more sensitive and specific antibody probes in addition to staining. This together with the more reliable method of extracting total zeins from endosperm meal (Esen, 1986) had enabled me to obtain a clearer and meaningful picture of the degradation of various zein fractions.

Antibodies to β-zein: The degradation of the 17 and 18 kD components were followed by probing the blot with R3030 antiserum (Figure 8 on page 67) since this antiserum (anti-17 kD) extensively reacts with 18 kD. Evidence of β-zein degradation became apparent as early as the second DAG with the appearance of novel low molecular weight components detectable by immunostaining. By the fourth DAG, the 18 kD component was completely degraded (Figure 8, arrow a), while fragments of 7, 14 and 15 kD persisted until the fifth DAG which are thought to be products of 18 kD proteolysis (arrows c and d). The 17 kD component (Figure 8, arrow b) gradually decreased and the degradation was complete by the seventh DAG. The increase of immunostaining intensity of fragments detectable around the 10 kD region began on the second DAG and they disappeared by the seventh DAG. Minor fragments around the 9 kD are thought to be the degradation products of the 17 kD β-zein (Figure 8, unmarked arrows) because it appeared on the fourth DAG when the degradation of 17 kD component was apparent. The disappearance of the 18 kD component after the fourth DAG, subsequent to that

of γ-zein, suggested that it is located internally next to the γ-zein component assuming that zein proteolysis begins on the periphery of the protein body and proceeds inward towards the core. The new low molecular weight fragments of 14 and 15 kD detectable by antibody were probably its degradation products since they were no longer detectable after the 18 kD component itself disappeared. The 17 kD component is degraded more gradually than its 18 kD counterpart. A 10 kD fragment that began to appear on the third DAG must be the product of 17 kD degradation since its staining intensity increased in parallel with the decrease of the 17 kD component and it disappeared together with the 17 kD component.

Antibodies to γ-zein: Immunostaining the blots with R2026 antisera showed evidence of 27 kD γ-zein degradation from the first DAG (Figure 9, γ-zein indicated by arrow a). The fragments resulting from γ-zein degradation migrated into the zone of α-zein components. In addition, a series of smaller low molecular weight fragments were observed around the regions of 16, 10 kD and lower regions (Figure 9, unmarked arrows). On the second DAG massive degradation of γ-zein occurred as evident from smearing starting at the 27 Kd region and extending to the dye front on the blot (Figure 9, arrow c). By the third DAG, γ-zein was completely degraded (Figure 9, arrow b); however, the remaining fragments can be detected around the 16, 10, 9, and 7 kD regions (Figure 10, see unmarked arrows). After the third DAG, no γ-zein fragments could be detected by immunostaining with anti-γ- zein serum.

In order to obtain a more precise pattern of γ-zein degradation additional experiments were performed with more frequent sampling, especially between the second and third DAG. The electrophoretic data from such experiments showed that γ-zein (Figure 10, arrow a) degradation was complete around the 63rd and 65th hour after imbibition (Figure 10, arrow b).

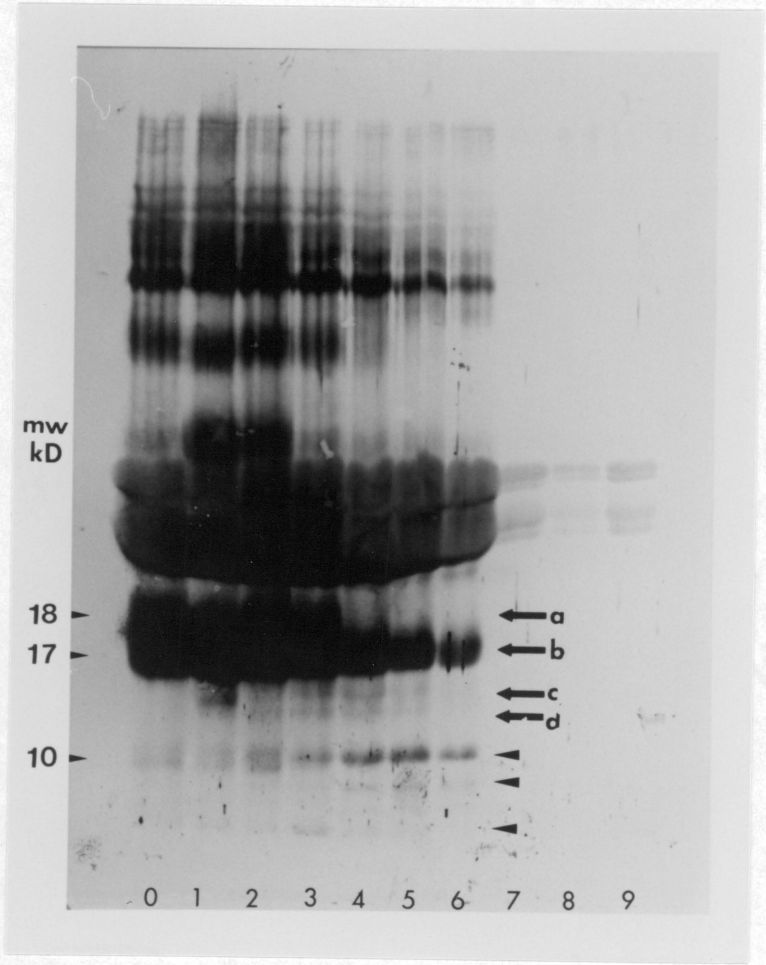

Figure 8. Immunostaining of Western blot with anti-β sera: Antiserum R3030 (anti-17 kD β-zein) was used to probe the Western blot. Arrows a, 18 kD β-zein; b, 17 kD β-zein; c and d, products of 18 kD β-zein degradation; unmarked, products of 17 kD β-zein degradation. Numbers 0 - 9 denote days after germination (0 was from dry seeds). Western blotting was performed as described by Towbin *et al.* (1979) and immunological assay was performed as described (Esen *et al.*, 1983).

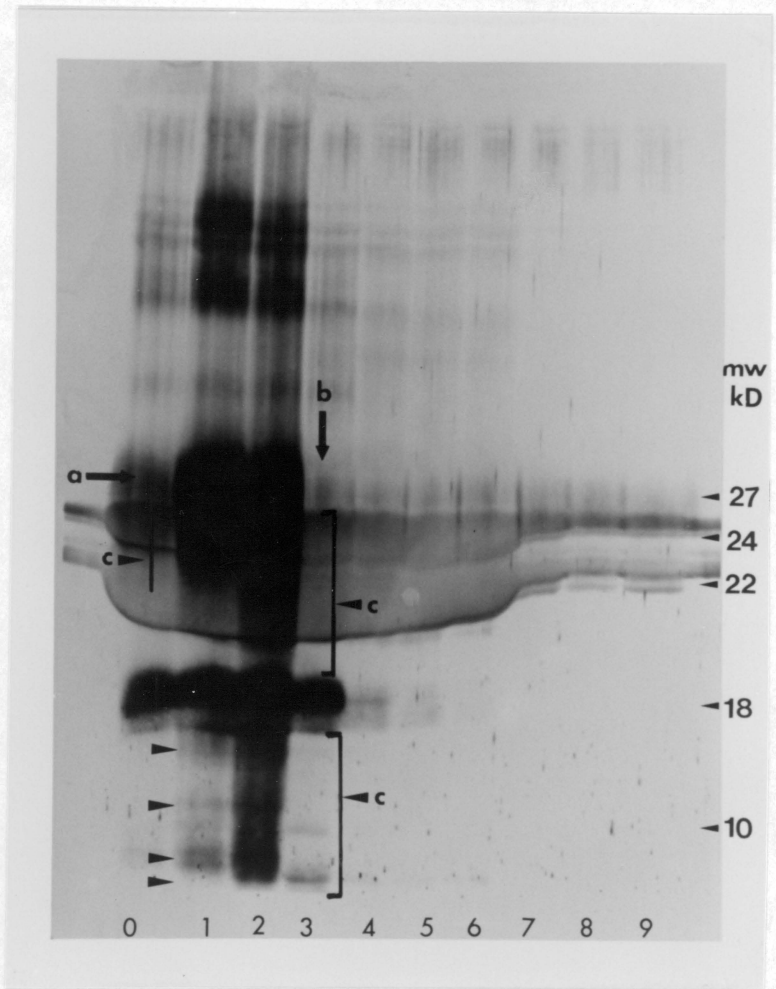

Figure 9. Immunological staining of Western blot with anti-γ sera: Antiserum R2026 (anti-γ-zein) was used to probe the Western blot after SDS-PAGE. Arrows a, 27 kD γ-zein; b, time of disappearance of γ-zein; c, smearing effect; unmarked, novel degradation products of γ-zein. Numbers 0 - 9 denote days after germination (0 was from dry seeds). Western blotting was performed as described by Towbin *et al.* (1979) and immunological assay was performed as described (Esen *et al.*, 1983).

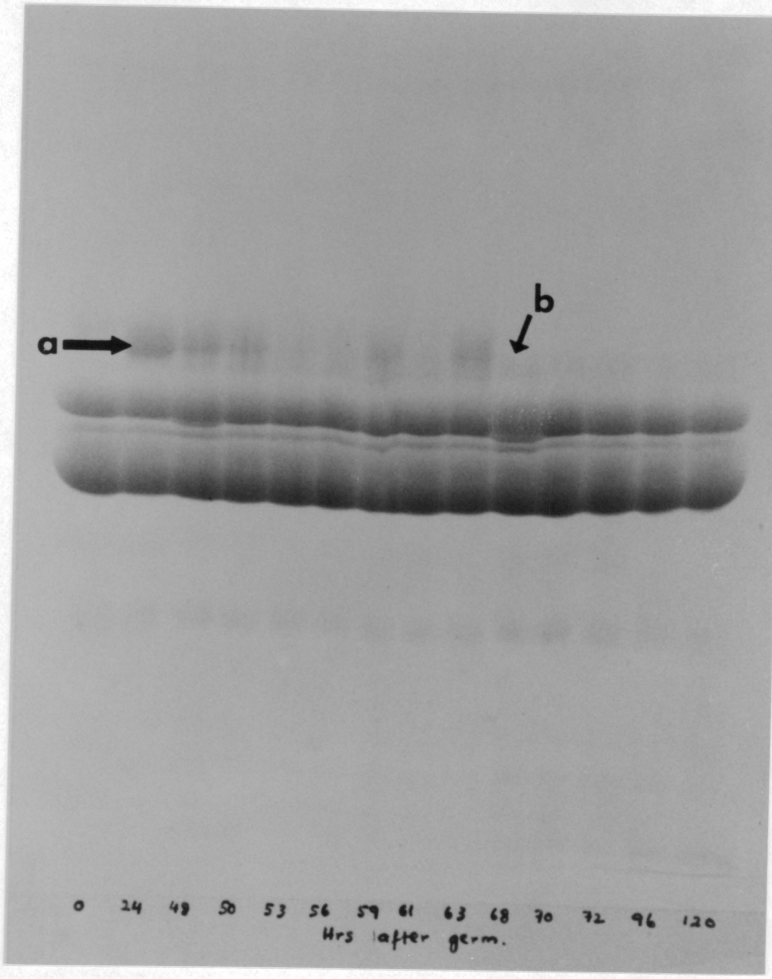

Figure 10. Time of completion of γ-zein degradation by SDS-PAGE: Arrows a, 27 kD γ-zein; b, time of completion of γ-zein degradation. Arrows a, 27 kD γ-zein; b, time of γ-zein disappearance. Numbers 0 - 120 denote hours after germination (0 was zeins extracted from dry seeds). The SDS-PAGE (12%, 17 cm) was performed according to Laemmli (1970) on a BioRad Protean II apparatus.

If one uses the Kjedhal procedure to measure total nitrogen during the early early phases of germination, a lag period of total nitrogen loss in the endosperm occurs especially during the first three days of germination (Dure, 1960; Harvey and Oak, 1974; Moureaux, 1979). Since the measurements made in these studies are by monitoring the total nitrogen, the lag period was observed because nitrogen values would also include the products of γ-zein degradation. Without accompanying electrophoretic studies, the products of zein degradation would be accounted for in total nitrogen determination and one would not be able to determine accurately the time of initiation of zein degradation.

Antibodies to 10 kD zein: Degradation of the minor 10 kD component was probed with R309 (Figure 11, arrow a). The degradation pattern followed the course of the α-zein degradation and was very gradual. Its degradation was complete by the eighth DAG and a fragment of 9 kD was detected beginning on the fourth DAG and disappeared at the same time as the 10 kD component (Figure 11, arrow b). The low molecular weight fragment of about 9 kD was the only degradation product detected.

A Postulated Model for Zein Arrangement in the Protein Bodies

A postulated model for the arrangement of zeins in the protein bodies should account for the non-random and differential degradation of the different zein size-classes during the germination of maize seeds. During seed development zein is synthesized on polysomes attached to protein bodies. The growing chain of the polypeptide is then transported into the protein bodies where it is stored (Burr and Burr, 1976; Burr *et al.*, 1978; Larkins and Hurkman, 1978; Viotti *et al.*, 1985). During germination, protein bodies undergo either internal or peripheral degradation, with the latter proceeding from the protein body periphery inwards, and may originate from several locations of the

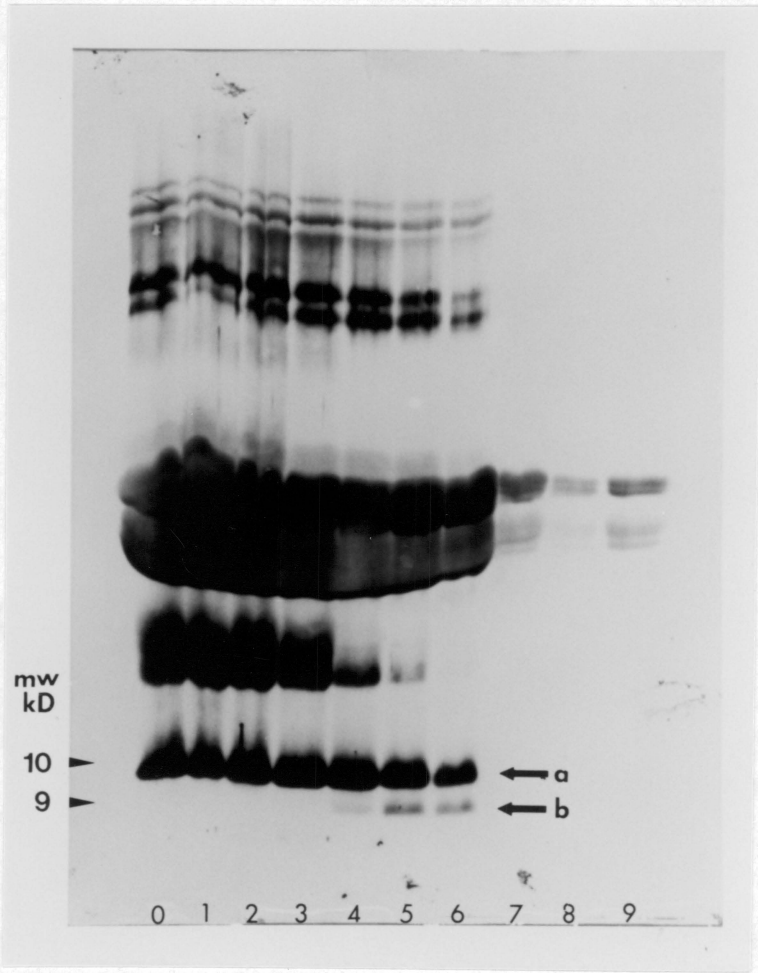

Figure 11. Immunostaining of Western blot with anti-10 kD sera: Antiserum R309 (anti-10 kD zein) was used to probe the Western blot. Arrows a, 10 kD zein; b, 10 kD zein degradation product. Numbers 0 - 9 denote days after germination (0 was from dry seeds). Western blotting was performed as described by Towbin *et al.* (1979) and immunological assay was performed as described (Esen *et al.*, 1983).

protein body surface (Horner and Arnott, 1965; Mollenhauer and Totten, 1971). The results of this study define the pattern and mode of degradation of various zein components and allows me to postulate the location of various zein components within the protein bodies and also the pattern of protein breakdown. Since α-zein was degraded very gradually and its remains were detectable even after the other zein components (γ-, β-, and 10 kD zeins) were mostly or completely degraded (Figure 6), it suggested that (1) α-zein is located centrally, forming the core of the protein bodies, and (2) the rate and sequence (slow and late) of α-zein degradation might be due to its inaccessibility to the proteolytic enzyme(s). On the other hand, the rate and sequence of γ-zein degradation (rapid and early, respectively) suggests that it is located around the periphery of the protein bodies and is fully accessible to proteolysis. The time-course of β-zein degradation suggested that β-zein was situated in the layer after γ-zein towards the center of the protein body. Since the degradation of the 10 kD progressed at a more gradual pace than that of the β-zeins, and seemed to follow that of α-zeins, one could postulate that the 10 kD zein is located more centrally within the protein bodies and might occur either interspersed with α-zein or on the surface of the α-zein core. According to this model, γ-zein degradation would expose the other zein components that are situated more centrally within the protein body for proteases. Gamma-zein may be a structural component of the protein body membrane by virtue of its likely peripheral location. Moreover, the smearing observed on the blot of γ- zein during its degradation suggest that some of the enzymes that are responsible for its degradation belong to the exopeptidase group. Otherwise, a series of distinct fragments would have been generated during the course of its degradation. Immunocytochemical data of Ludevid *et al.* (1984), Larkins (1986) and Lending *et al.* (1988) agrees with this model suggesting that γ-zein is located on the periphery of protein bodies. However, immunocytochemical staining of protein body sections showed that β-zein is located in the central portion of the pro-

tein body (Larkins, 1986; Lending *et al.*, 1988). It should be noted that there is varying degrees of immunological cross-reactivity between β-zein and other zeins (α and γ). Therefore, the location of β- zein by immunocytochemistry may not be done unequivocally unless one uses monospecific antibodies.

Future research should deal with the isolation and characterization of the protease(s) that are degrading various zein components. Whether the various zein components are being degraded by a single group of proteases, by different groups of proteases, or by several groups of proteases working synergistically, and how these proteases are induced and regulated will be of great interest.

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Chapter 3: Partial Characterization of Proteases in Germinating Maize Endosperm

Abstract

The protease activities involved in the degradation of α-, β- and γ-zeins in the endosperm of germinating maize seeds were partially characterized with respect to their modes of synthesis and action. The use of transcriptional, translational and protease activity inhibitors indicated that the mRNAs synthesized and stored during seed development (pre-formed mRNA) appear to be sufficient to direct the *de novo* synthesis of either of these enzymes or the protein factors required for the induction of their activities. The data also suggest that different proteases were active in the degradation of the different zein classes as follows: (1) serine protease play a major role in the degradation of α- and β-zeins with a little involvement in γ-zein degradation, (2) aspartic (acid) protease plays some role in β-zein degradation, (3) cysteine (thiol) protease may be involved in a limited way in γ-zein degradation, and (4) proteases assayed from different stages of germination showed different activities in the degradation of different zein components.

Introduction

Zeins, the prolamins of maize are alcohol soluble storage proteins constituting the major protein content in maize endosperm. Based on differential solubility, these proteins can be separated into three fractions; that is, α-zein (22 and 24 kD zeins), β-zein (17 and 18 kD zeins), and γ-zein (27 kD zein) (Esen, 1986 and 1987). Based on molecular weight deduced from nucleotide sequence data, 22 and 24 kD α-zeins are referred to as 23.8 and 26.7 kD (Esen, 1987). A minor component of zein (10 kD) exhibits solubility characteristics similar to those of α-zeins. During the course of seed development, zein is deposited in distinct membrane-bound subcellular compartments called protein bodies (Wolf, Khoo and Seckinger, 1967; Larkins and Hurkman, 1978). In the previous chapter, I reported the sequence and time course of degradation of various zein components (α, β, γ, and 10 kD zeins) and proposed a model for the arrangement of the various zein size classes in the protein bodies.

The involvement of proteolytic activities in the post- germinative degradation of zeins was reported by several investigators. Abe *et al.* (1977 and 1978) purified a sulfhydryl protease with a pH optimum of around 3.0 from maize endosperm. A protease sensitive to cysteine endopeptidase reagents and similar to pepsin in substrate specificity was reported for germinating maize seeds (Makoto *et al.*, 1978). Proteases occurring in germinating maize seedlings have a pH optima of 3.8 (endosperm), 5.4 (scutellum) and 7.5 (root and shoot) (Feller *et al.*, 1978; Moureaux, 1979). Ryan and Walker-Simmon (1981) reported two cysteine endopeptidases having pH optima of 3.0 and 7.5 in maize seeds. The wide difference in the pH optima of these enzymes suggests their different physiological roles and/or their possible presence in different cells, or perhaps compartments of the same cell. The present study describes the use of specific

inhibitors in the characterization of protease activities involved in zein degradation, as well as an *in vitro* proteolytic assay using different zeins as model substrates.

Materials and Methods

Seed Preparation

Seeds of the maize inbred K55 were used in all experiments. The seeds were surface sterilized using 1.5% hydrogen peroxide for 10 minutes and with commercial strength bleach for 15 minutes. The treated seeds were rinsed five times with sterile distilled water.

For protease inhibition studies the seeds were laid down on 3 mm filter paper circles in Petri dishes containing 10 ml of distilled water with the test chemicals. Ten seeds were placed in each Petri dish. The Petri dishes containing the seeds were placed in the dark. The solutions were changed every two days. The samples were harvested at one (1), three (3), six (6) and ten (10) days after germination. The embryo, the growing axis, and the seed coat were removed and the endosperm was saved and freeze dried. The freeze-dried endosperm was grounded to a very fine powder in a stone mortar and pestle. The powder was kept at -20 °C until used. Whole zein was extracted from these meals with 60% IPA containing 1% 2-ME.

For protease extraction the seeds were planted in vermiculite, 10 seeds per pot, and were allowed to germinate in the dark. The pots were watered daily. 100 seeds were harvested each day up to nine days after germination (DAG). The embryo, the growing axis and the seed coat were removed and the endosperms were saved and frozen at -20

°C. The frozen endosperms were homogenized in the appropriate buffer with a polytron at 4 °C. The supernatant was saved and stored at -20 °C until used.

Protease Inhibition

Maize seeds were incubated with various chemicals that inhibit protein synthesis, transcription of mRNA, and specific inhibitors of proteases. The solution was changed every two days due to the instability of some of the chemicals. The chemicals used were as follows:

1. The inhibition of protein synthesis was achieved by germinating the maize seeds in the presence of cycloheximide (CHI). CHI was dissolved in distilled water and added to 10 ml distilled water at a final concentration of 0.01 mM.

2. For inhibition of transcription, Actinomycin D (AmD) was added to the germination medium at a final concentration of 0.1 mM.

3. Phenylmethyl sulfonylfluoride (PMSF) was added at a final concentration of 0.5 mM for the inhibition of serine proteases (James, 1978).

4. Ethylenediaminetetraacetic acid (EDTA) was included in the incubation medium at a final concentration of 0.5 M to inhibit metallo-proteases (Maniatis *et al.*, 1982).

5. For the inhibition of cysteine (thiol) proteases, Leupeptin (LEUP) was added to incubation medium at a final concentration of 1 μM (Umezawa, 1976).

6. Pepstatin (PEP) was added to the incubation medium at a final concentration of 1 μM for the inhibition of aspartic (acid) proteases (Umezawa, 1976).

In addition to the above treatments, the specific protease inhibitors were mixed in several combinations to show if a particular zein component is being degraded by a single type of protease or by several proteases functioning synergistically. As a control, seeds were also germinated with only distilled water as the incubation medium.

Zein Extraction

Fifty mg of corn meal from each DAG and treatments were weighed and placed in a 1.5 ml Eppendorf tube. Whole zeins were extracted by adding 1 ml of 60% isopropanol (IPA) containing 1% 2- mercaptoethanol (2-ME) to each tube. The extraction was continued overnight with constant shaking at room temperature. Zeins were recovered in the supernatants after centrifugation at 5000 x g for 3 minutes.

Electrophoresis

As described in Chapter 2, extracted whole zeins were evaporated to dryness and reconstituted in an equivalent volume of SDS sample buffer (Laemmli, 1970). Prior to electrophoresis, the samples were heated in a water bath at 90 °C for 10 minutes. The samples were electrophoresed on a 12% SDS- polyacrylamide gel (17 cm long resolving gel) according to the procedure of Laemmli (1970) using the BioRad Protean II apparatus. Zeins were detected by staining with Coommasie blue R250, and destaining in a solution of 25% isopropanol and 10% acetic acid in water.

Protease Extraction

Protease from the endosperms of maize at various stages of germination were extracted using three different buffer systems according to published procedures. All extractions were done over ice with cold buffers, and samples were homogenized using a polytron (Brinkman). The homogenates were placed on a shaker for 15 minutes at 4 °C (about 100 rpm). The buffer systems used were as follow: (1) 0.2 M sodium acetate, pH 3.8 (acetic acid)/0.005 M 2-ME (Harvey and Oaks, 1974a); (2) 0.025 M phosphate buffer, pH 7.4/0.005 M 2-ME (Abe *et al.*, 1977); and (3) 0.05 M phosphate buffer, pH 5.4/0.01 M 2-ME/1% soluble PVP (Feller *et al.*, 1978). The homogenates were centrifuged at 8,000 x g in the case of the pH 3.8 and 7.4 buffers and at 3,000 x g in the case of pH 5.4 buffer for 10 minutes, and the supernatants were dialyzed against the buffer used for extraction at 4 °C overnight. The extracted proteases were kept at -20 °C until used. Aliquots were kept at 4 °C to avoid constant thawing and freezing of the samples.

Zein Degradation Assay

Nine sets of assays were performed, each zein component (α, β and γ) was dissolved in different buffers (as in protease extractions above). Substrate gels were prepared for each zein components in different buffers and pHs.

Agar gels containing the substrate protein (α-, β-, and γ-zeins, respectively) were prepared according to Harvey and Oaks (1974a) with minor modifications. Equal volumes of 3% agar in buffer were mixed with solutions containing 0.025% zein in the same buffer (as in protease extraction above) containing 8 M urea. Aliquots of 20 ml of the

mixture were poured into Petri dishes and allowed to set. The gels were immersed in several changes of the corresponding buffer for 24 hours to remove the urea from the gels. Holes 7 mm in diameter were punched in the gels with a cork borer. 150 μl of the enzyme extract were pipetted into each well in the gels. The gels were incubated for 24 hours at 38 °C, to allow the enzyme to diffuse out of the wells and degrade the substrate suspended in the gels. At the end of the incubation period, the gels were washed with 7% acetic acid, and then stained with nigrosine (0.02% in 0.2 M phosphate buffer, pH 8.0). Clear rings around the wells indicated proteolytic activity against the substrate.

Results and Discussion

Modes of Protease(s) Synthesis and Action

Inhibition of transcription: Actinomycin D is a potent inhibitor of RNA transcription. When AmD was added to the incubation medium, it was found that the degradation of γ-zein proceeded as normal (Figure 12C, arrow a). However, the degradation of the other zein components (α, β and 10 kD) were slightly retarded (Figure 12C, arrows f, g, and h, respectively). The results showed that the pre- existing mRNAs present in the dry seeds were sufficient for translation into proteases that completely degraded γ-zein when compared to the control (Figure 12A, arrow a). As for β-zein, the pre-existing mRNAs were not sufficient to maintain the protease for its complete degradation, especially the 17 kD β-zein because it was still detectable on the tenth DAG. Also the 10 kD degradation was slightly retarded (Figure 12C, arrow g). Substantial amounts of α-zeins were still detectable on tenth DAG (Figure 12C, arrow f). These results suggest that the enzyme and the mRNA required for the synthesis of these proteases were pre-

existing within the dry seeds. De Jiminez *et al.* (1981) and De Jiminez and Aguilar (1984) showed that this "house keeping" proteins were due to both the translation of new mRNA and delayed translation of stored mRNA species. This was evident when α-amanitin did not inhibit the incorporation of ^{14}C-amino acid during the early stages of germination. However, they never show the specific proteins which were translated. Some of these house keeping proteins may be some of the proteases which are required for degrading some of the zein components during the initial stages of germination.

Inhibition of protein synthesis: In order to find out whether the proteases needed for the initial stages of degradation of some or all of the zein components are already present in the dry mature seeds (prepackaged) or being synthesized *de novo* upon initiation of germination, CHI was added to the incubation medium of the maize seeds. The results suggested that the degradation of all the zein components (α, β γ and 10 kD) was inhibited (Figure 12B, see arrows a, b, c and d) in the presence of CHI. The inhibition of degradation can be clearly seen by the absence of novel low molecular weight fragments present in the control (Figure 12A, see arrow e). In fact, the incubated seeds fail to germinate altogether (data not shown). Cycloheximide is a chemical that inhibits protein synthesis. It also inhibits zein degradation in germinating maize seeds (Harvey and Oaks, 1974b; Oaks *et al.*, 1983). Inhibition was dependent on the time of CHI addition. Addition of CHI at the beginning of the incubation period prevents degradation of endosperm reserves. Late additions have no effect on the hydrolysis of storage reserves. This showed that the protease required for zein degradation was synthesized *de novo* in the endosperm of germinating maize seeds. Our results therefore confirmed the earlier findings of Harvey and Oaks (1974b) and Oaks *et al.* (1983). Some literature mentioned that some proteases were being packaged within the protein bodies during the synthesis of the storage proteins in maize and therefore are present in ungerminated

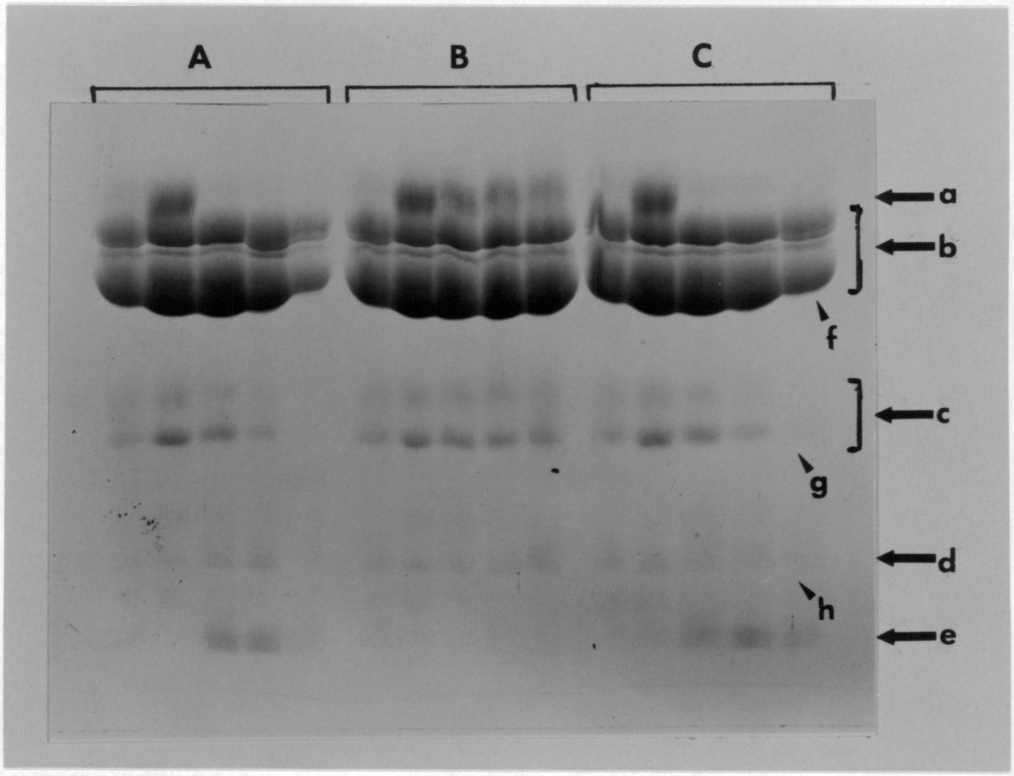

Figure 12. Effect of CHI and AmD on zein degradation: SDS-PAGE profiles of zein degradation in maize endosperms incubated with CHI and AmD. A, control; B, incubation medium containing CHI; C, incubation medium containing AmD. Arrows a, γ-zein; b, α-zeins; c, β-zeins; d, 10 kD- zein; e, some degradation products; f, g and h, retarded α-, β- and 10 kD zeins, respectively. In the control only water was included in the incubation medium. In each panel the lanes show zein profiles at day 0, 1, 3, 6, 10, respectively, in germination. Total zeins were extracted with 60% IPA containing 1% 2-ME. SDS-PAGE (12%, 17 cm) was done according to Laemmli (1970).

seeds (Feller *et al.*, 1978; Moureaux, 1979). However, they were not able to degrade prolamins. The results of Feller *et al.* (1978) and Moureaux (1979) were consistent with mine because the inhibition of new protein synthesis also inhibited protein degradation. The other possibility is that, even though proteases were present, the factor required for their activation are not produced.

Inhibition of specific protease activities: To find out if each zein component is being degraded by a specific protease, various protease inhibitors were included in the incubation medium. When PMSF was added to the incubation medium, degradation of γ-zein was retarded (Figure 13B, arrow f). The other zein components (α, β and 10 kD) were not degraded at all (Figure 13B, arrows b, c and d). The germination of the seedlings was abruptly inhibited shortly after the protrussion of the radicle. PMSF is a potent inhibitor of the serine type of proteases (James, 1978). The results suggest that serine protease is the major type of enzyme that are degrading the α-, β and 10 kD-zeins in the maize endosperm. The degradation of γ-zein is somewhat delayed which indicates that this enzyme also play some role in the degradation of γ-zein. In Chapter 2, immunostaining of Western blots showed a smearing and also defined smaller molecular weight bands as the result of γ-zein degradation. This suggests that a major portion of γ-zein is being degraded by exopeptidases and endopeptidases. Feller *et al.* (1978) showed that most of carboxypeptidases (in excess of 50%) were inhibited by PMSF and that the increase in their activity coincided with nitrogen lost from the endosperm. One experiment was done in which maize seeds were germinated with exogenous amino acids complementary to those of γ-zein which were shown to be predominant in either the *N*- or *C*-terminus were excluded in the incubation medium. The result showed that when the amino acid complement from the *C*-terminus was included in the medium, the rate of γ-zein degradation was delayed and proceeded as normal when excluded (data not

shown). This result is consistent with that of Feller *et al.* (1977 and 78) which showed that carboxypeptidase work cooperatively with endopeptidase in degrading the endosperm reserves.

Using EDTA in the incubation medium (Figure 13C), the degradation of all the zein components proceeded as normal when compared to the control in which no inhibitor was added. Inhibition by EDTA is probably negligible, if any. Since EDTA is an inhibitor of metallo-proteases, it can be concluded that metallo-proteases are not responsible for the degradation of the zein components in the endosperm of germinating maize seeds.

Figure 14B showed the zein degradation profile in the endosperm of maize seeds germinated in the presence of pepstatin (inhibitor of aspartic (acid) proteases). Gamma-zein was degraded normally (as compared to control in Figure 14A). Hydrolysis of the α-, 17 kD β- and 10 kD-zeins were slightly delayed (Figure 14B, arrows f, g and h). The retardation of α-zeins degradation could be largely due to their inaccessibility to the proteolytic enzymes due to the delayed degradation of β- zein since it was shown earlier that β-zein was located on the outer layer of the α-zein core of the protein bodies (chapter 2). This would suggest that acid protease play a minor role in the β-zein degradation. Acid proteases are present in germinating maize seeds, and are referred to as P_{21} and P_{22}. They are endopeptidases appearing on the third DAG which are enhanced by reducing agent (Moureaux, 1979). Beta-zein degradation studies of Chapter 2 did not reveal any smear on immunostained SDS blot suggesting that this acid protease belongs to the endopeptidase group.

In another set of inhibition studies, leupeptin was included in the incubation medium to inhibit the action of cysteine (thiol) proteases (Figure 14C). The degradation of γ-zein was slightly retarded. However, the degradation of other zeins were not affected (Figure 14C, arrow i). This showed that the cysteine proteases played a minor

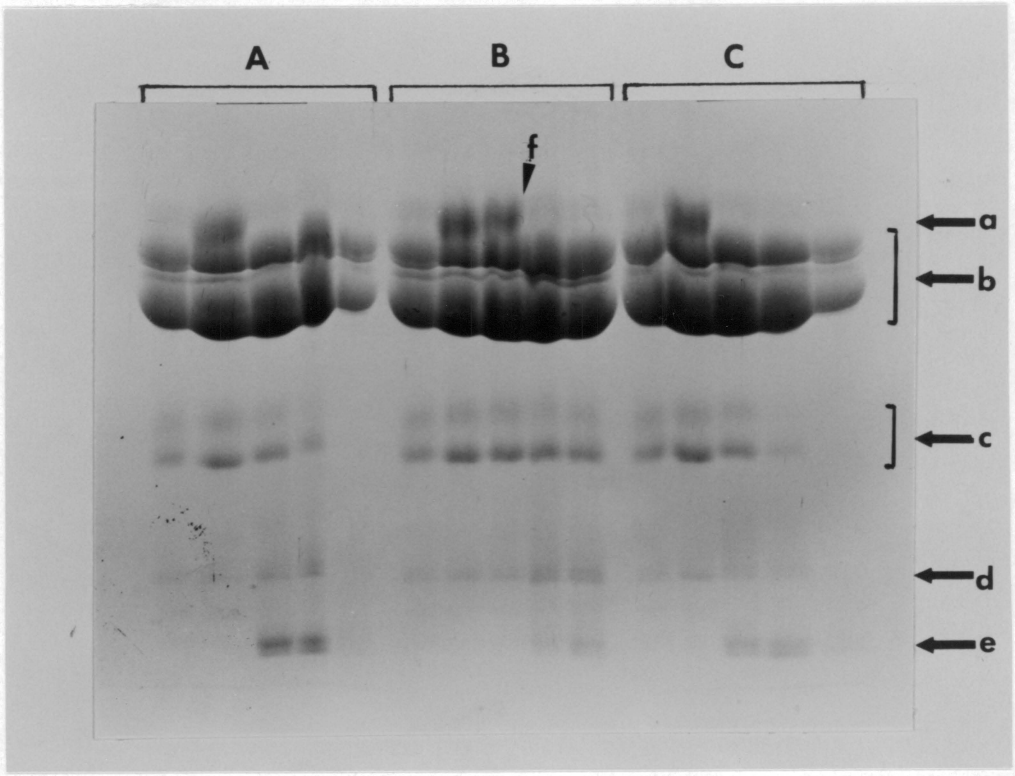

Figure 13. Effect of PMSF and EDTA on zein degradation: SDS-PAGE profiles showing the effect of various protease inhibitor on zein degradation in maize endosperms during germination incubated with PMSF and EDTA. A, control; B, incubation medium containing PMSF; C, incubation medium containing EDTA. Arrows a, γ-zein; b, α-zeins; c, β-zeins; d, 10 kD-zein; e, some degradation products; F, retarded γ-zein degradation. In the control only water was included in the incubation medium. In each panel the lanes show zein profiles at day 0, 1, 3, 6, 10, respectively, in germination. Total zeins were extracted with 60% IPA containing 1% 2-ME. SDS-PAGE (12%, 17 cm) was done according to Laemmli (1970).

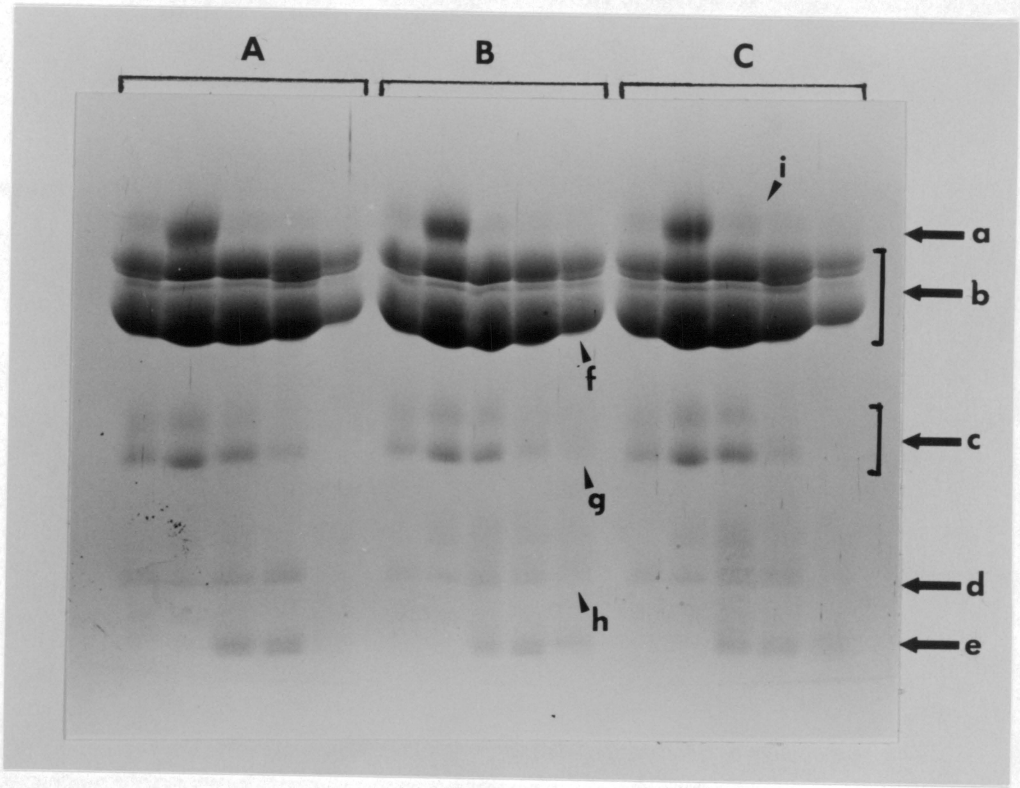

Figure 14. Effect of PEP and LEUP on zein degradation: SDS-PAGE profiles showing the effect of various protease inhibitor on zein degradation in maize endosperms during germination incubated with PEP and LEUP. A, control; B, incubation medium containing PEP; C, incubation medium containing LEUP. Arrows a, γ-zein; b, α-zeins; c, β-zeins; d, 10 kD-zein; e, some degradation products; f, g, h and i, retarded α-, 17 kD β-, 10 kD and γ-zeins, respectively. In the control only water was included in the incubation medium. In each panel the lanes show zein profiles at day 0, 1, 3, 6, 10, respectively, in germination. Total zeins were extracted with 60% IPA containing 1% 2-ME. SDS-PAGE (12%, 17 cm) was done according to Laemmli (1970).

role in the degradation of γ-zein. Germinating maize seeds contained cysteine endopeptidases (Makoto *et al.*, 1978; Ryan and Walker-Simmon, 1981) which were characterized by using artificial substrate (hemoglobin) in an *in vitro* assay. My results, however, showed the *in vivo* inhibition of the cysteine protease.

When PMSF and EDTA were present in combination in the incubation medium, γ-zein was retarded slightly, and the degradation of all other zein components were inhibited (Figure 15B). The pattern was similar when PMSF was present by itself (refer to Figure 13B). This confirms my earlier result (refer Figure 13C) that metallo-protease does not have any role in zein degradation.

The combined treatment with PMSF and leupeptin cause (Figure 15C) γ-zein degradation to decrease more than when PMSF was used alone. Degradation of all other zeins were inhibited. This clearly showed that at least serine and cysteine proteases are working together or in sequence to degrade γ-zein. Most of the proteases inhibited by PMSF belong to the carboxypeptidase type (Feller *et al.*, 1978). Feller *et al.* also suggested that the carboxypeptidases act synergistically with endopeptidase in the mobilization of storage reserves of maize endosperm. This conclusion was reached because the loss of endosperm nitrogen was concurrent with the increase in activities of these two types of proteases.

Protease Assay in Agar Gels

To determine proteases activity on their natural substrates, various zein components were suspended in agar gels. When α- zein was used as the substrate, the proteases that degrade it were evidently present in the extract beginning from day one after germination (Figure 16, arrow) as demonstrated by the formation of a clear ring around the well. The proteolytic activity was present through the ninth DAG. However, the proteolytic

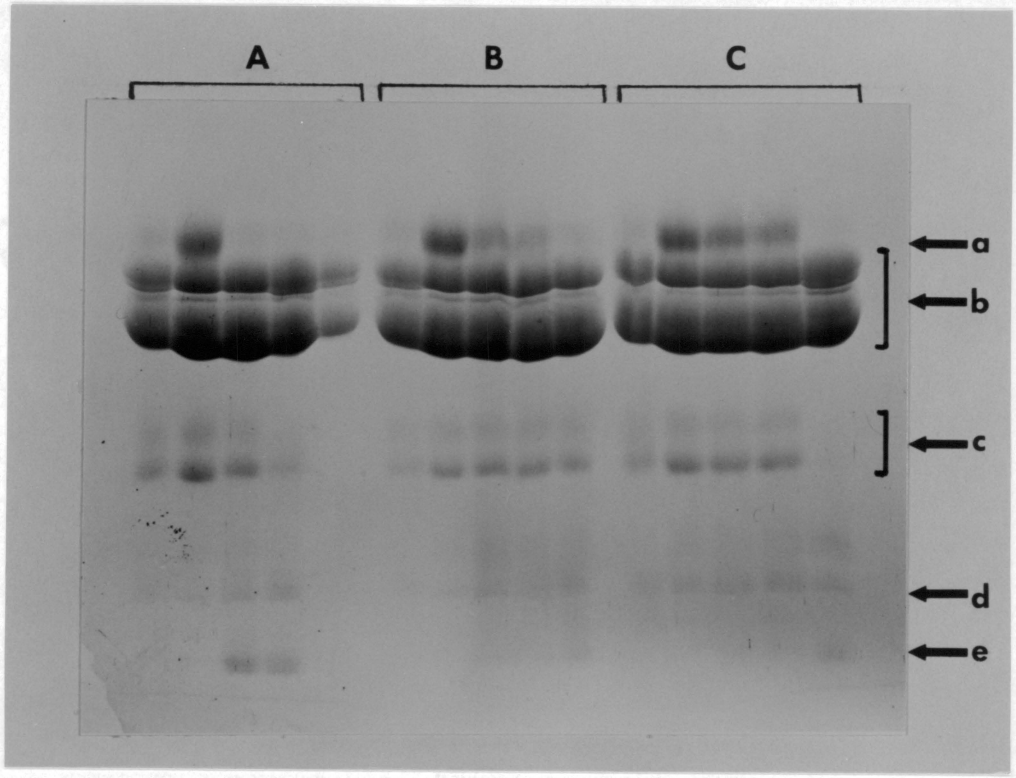

Figure 15. Incubation with protease inhibitor mixture: SDS-PAGE profiles showing the effect of various protease inhibitor mixture on zein degradation in maize endosperms during germination incubated with PMSF, EDTA and LEUP. A, control; B, incubation medium containing PMSF and EDTA; C, incubation medium containing PMSF and LEUP. Arrows a, γ-zein; b, α-zeins; c, β-zeins; d, 10 kD-zein; e, some degradation products. In the control only water was included in the incubation medium. In each panel the lanes show zein profiles at day 0, 1, 3, 6, 10, respectively, in germination. Total zeins were extracted with 60% IPA containing 1% 2-ME. SDS-PAGE (12%, 17 cm) was done according to Laemmli (1970).

activity remained constant throughout, but the degradation was gradual. Gradual degradation of α-zein subunits have been monitored SDS-PAGE analysis (Fujimaki *et al.*, 1977). Activity was not present in the extract from dry seeds. Although proteases are present in dry seeds, they cannot hydrolyze prolamins (Feller *et al.*, 1978; Moureaux, 1979). To show that the degradation was not due to solubility in the buffer system used, one well was incubated in buffer alone, and no evidence of clear ring formation. This suggested that the clear ring formation was due to the proteolytic action of the enzyme extract. The activity can only be detected in the extractions which were done in 0.2 M sodium acetate buffer (pH 3.8). The other two buffer extracts used did not show any evidence of proteolytic activity. A typical example of a negative reaction was also included (Figure 17 on page 94).

When β-zein was used as substrate, proteolytic activity was also detected in the 0.2 M sodium acetate (pH 3.8) buffer system (Figure 18 on page 95). Enzyme extracted with other buffer systems did not yield any proteolytic activity. Proteolytic activity was first detected in the extract from three DAG endosperms, and increase progressively until the seventh DAG. After this, the activity decreased. A control in which the well was incubated with buffer alone did not reveal any evidence of the formation of the clear ring.

When γ-zein was used as the substrate, the extract showed evidence of proteolytic activity beginning on the second DAG (Figure 19 on page 97). From the third DAG until the seventh DAG, there was a progressive increase in proteolytic activity. The activity abruptly decreased on the eighth DAG and little activity remained on the ninth DAG. The proteolytic activity was also observed only in the 0.2 M sodium acetate (pH 3.8) extraction buffer system.

A proteolytic enzyme with endopeptidase activity (pH optimum of 3.8 and temperature optimum of 46 °C) that can degrade partially hydrolyzed zein is present in

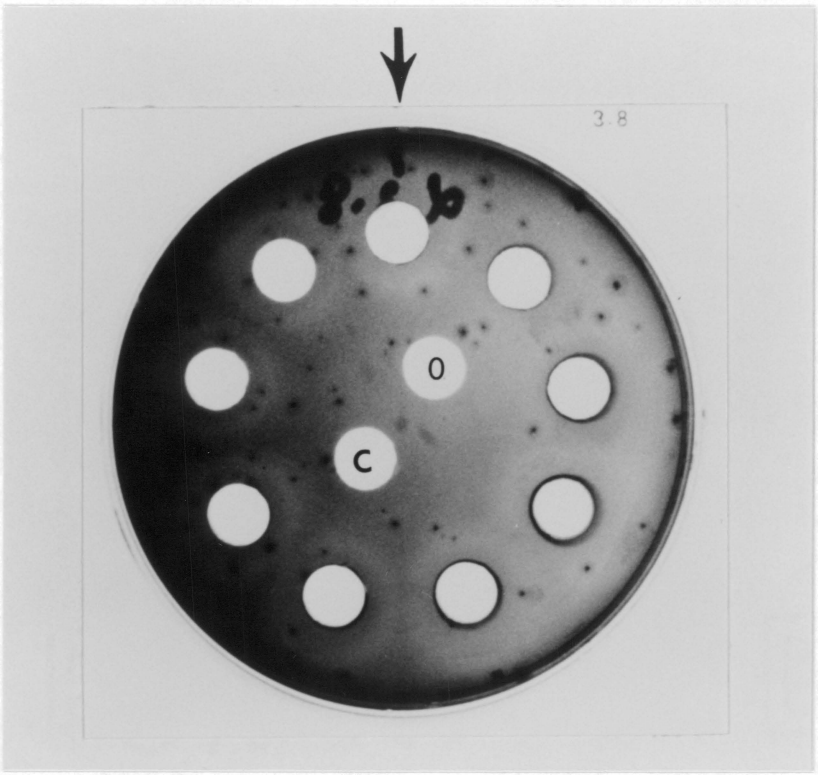

Figure 16. Agar gel assay for protease activity with α-zein: Arrow indicate well #1. Moving clockwise from arrow, the wells contained enzyme extracted with pH 3.8 buffer from 1 - 9 DAG. 0, extract from dry seeds; c, control in which the well was incubated with buffer only. The gel was incubated at 40 °C for 24 hours, washed with 7% acetic acid and stained with 0.02% nigrosine. Clear rings around the wells indicate protease activity.

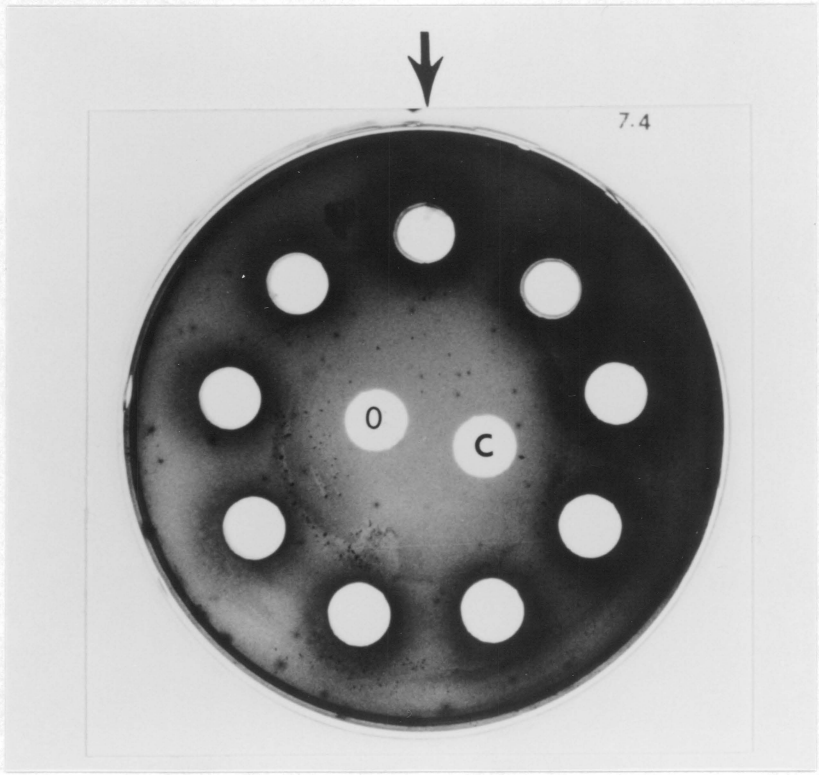

Figure 17. Agar gel assay showing a negative reaction: This is pH 7.4 extract with α-zein as the substrate. Arrow indicate well #1. Moving clockwise from arrow, the wells contained enzyme extracted with pH 3.8 buffer from 1 - 9 DAG. 0, extract from dry seeds; c, control in which the well was incubated with buffer only. The gel was incubated at 40 °C for 24 hours, washed with 7% acetic acid and stained with 0.02% nigrosine. Clear rings around the wells indicate protease activity.

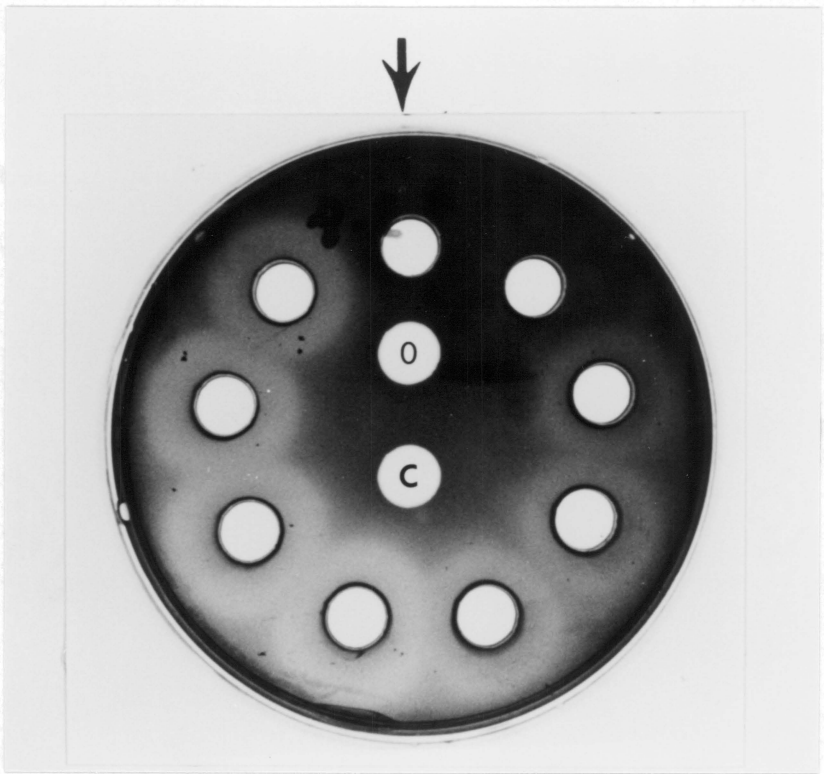

Figure 18. Agar gel assay for protease activity with β-zein: Arrow indicate well #1. Moving clockwise from arrow, the wells contained enzyme extracted with pH 3.8 buffer from 1 - 9 DAG. 0, extract from dry seeds; c, control in which the well was incubated with buffer only. The gel was incubated at 40 °C for 24 hours, washed with 7% acetic acid and stained with 0.02% nigrosine. Clear rings around the wells indicate protease activity.

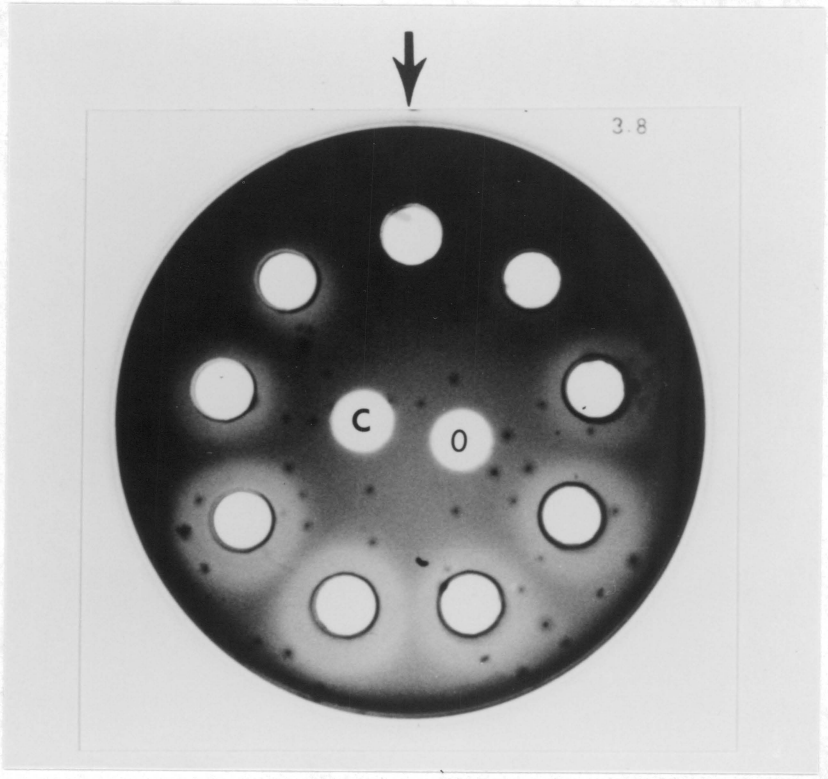

Figure 19. Agar gel assay for protease activity with γ-zein: Arrow indicate well #1. Moving clockwise from arrow, the wells contained enzyme extracted with pH 3.8 buffer from 1 - 9 DAG. 0, extract from dry seeds; c, control in which the well was incubated with buffer only. The gel was incubated at 40 °C for 24 hours, washed with 7% acetic acid and stained with 0.02% nigrosine. Clear rings around the wells indicate protease activity.

germinating maize seeds (Harvey and Oaks, 1974a). The activity increased from three to eight DAG. Similar increases in proteolytic activities through day eight after germination were reported by several investigators (Harvey and Oaks, 1974c; Fujimaki *et al.*, 1977; Feller *et al.*, 1978; Moureaux, 1979) which are consistent with my gel assay results.

From these studies, it can be concluded that the proteases required for degradation of various zein components or the protein factors required for the activation of these proteases are synthesized *de novo* during seed germination. The pre-formed mRNAs that are synthesized and stored during seed development are sufficient to direct the translation of proteolytic enzymes and/or activators during the initial stages of seed germination. Moreover, serine protease are responsible for a major role in the degradation of α- and β-zeins while it plays a minor role in γ-zein degradation. Aspartic (acid) protease plays a minor role in β-zein degradation. Cysteine (thiol) protease also play a minor role in γ-zein degradation. All the proteases that were extractable in 0.2 M sodium acetate buffer system (pH 3.8) were able to degrade α-, β- and γ-zeins.

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Chapter 4: General Discussion

The major focus of this study was concerned with a group of alcohol-soluble storage proteins (zeins) which occur in the protein bodies of maize endosperm. Most of the studies on zeins to date have been on the developmental aspects (Randolph, 1936; Bressani and Conde, 1961; Duvick, 1961; Khoo and Wolf, 1970; Sodek and Wilson, 1971; Christiansen *et al.*, 1974; Burr and Burr, 1976; Burr *et al.*, 1977; Larkins and Hurkman, 1978; Tsai *et al.*, 1978; Tsai, 1979; Lee and Tsai, 1984). However, literature concerning zein degradation is scanty and unrefined (Harvey and Oaks, 1974b; Fujimaki *et al.*, 1977; Moureaux, 1979; Metevier and Montero, 1981; Oaks *et al.*, 1983). This study was therefore undertaken to obtain a more refined and detailed data on the pattern of zein degradation during germination, and also to partially characterize the associated proteases by *in vivo* and *in vitro* assays.

The results of the germination study suggest that germination was delayed in *Opaque-2* mutant maize seeds when compared to normal seeds. *Opaque-2* mutant maize seeds have low zein, but high lysine content (Mertz *et al.*, 1964). Zein is a dynamic nitrogen sink in developing seeds and can be readily manipulated by nitrogen fertilization and genetic means (Tsai *et al.*, 1978 and 1980). Metevier and Montero (1981) showed that *opaque-2* mutant maize seeds had a lower rate of zein degradation during germination. A detached embryo has delayed development but can be remedied when leachate from endosperm or amino acid complements (as from zein) is added to the growth medium (Oaks and Beevers, 1964; Oaks, 1965). All these data suggest that zeins are important for the growth and productivity of the maize plant as a whole. Zeins may be the major source of amino acids and amide nitrogen during early stages of germination because a lower zein content leads to delayed germination. This would suggest that future manipulation of zein in maize endosperm should not be attempted before the importance of zein to germination and early stages of development is clarified.

In the initial test for protein (spot test) of zein degradation studies, the progressive decrease in zein content was apparent after second DAG. This is consistent with the data of several investigators which showed a lag period of one to two days before total nitrogen, protein nitrogen and non-protein nitrogen (NPN) were rapidly lost thereafter (Oaks, 1965; Harvey and Oaks, 1974; Srivastava, 1976; Moureaux, 1979). It should be noted that all the measurements, made although quantitative, were not very informative. My measurements were by the staining of total protein with Coommasie blue stain while their measurements were for total nitrogen by Kjeldhal procedure (which will also include NPN such as free amino acid).

The degradation pattern was more profound in the SDS-PAGE profiles of zeins extracted from various stages of germination. There was a progressive decrease in all zein components except α-zein which was degraded gradually. Most zein components had been completely degraded by the seventh DAG. Alpha-zein was the only exception because after this time a minor portion remained and could still be detectable on 26th DAG. Fujimaki *et al.* (1977) had reported the gradual degradation of two main subunits of zein which coincided with α-zein size components (based on their extraction procedure without the addition of any reducing agent). There are two possible explanations for the persistence of α-zein: (1) it is possible that an island of endosperm splits physically from the rest and it is no longer subjected to signals from the embryo, and (2) the germinating maize seedlings are capable of synthesizing their own food and become independent of the endosperm reserves after 10 days or so. Oaks (1965) suggested taht the degradation of storage proteins in the endosperm is a process regulated by the demand of the embryo for amino acids. This is consistent with the above-mentioned possibilities. It is interesting to note that during the early stages of germination, α-zein was degraded very gradually. The bulk of its degradation started only after all the other zein components (β, γ and 10 kD) had been nearly or completely degraded. This is highly suggestive

that the order in which different zein classes are degraded reflect the order in which they are localized within the protein body and thus accessibility to proteolytic enzymes. For example, the degradation of γ- zein was rapid and complete by the third DAG. In fact, the elextrophoretic and immunological data showed that γ-zein degradation was complete around the 63rd to 65th hour after imbibition suggesting that it is the first zein accessible to proteases and thus resides in the outermost layer of the protein body mass. The next zein to be degraded were the 17 and 18 kD β-zeins with the 18 kD being completely degraded by the fourth DAG and the 17 kD by the seventh DAG. The 10 kD was degraded more gradually and was completed by the eighth DAG.

The sequential pattern of degradation of zeins from maize endosperm during germination suggested that various zein components were arranged or located in a specific order within the protein bodies. The result of this study therefore allows me to hypothesize and deduce the arrangement of various zein fractions within the protein bodies as shown in Figure 20A. The proposed arrangement is consistent with the data published by other researchers. Gamma-zein being the first to be degraded would be situated around the periphery of the protein bodies and could possibly be a structural component of the protein body membrane or directly anchored in the membrane. Gamma-zein may be a transmembrane protein (David Johnson, personal communication). The immunocytochemical data of Ludevid *et al.* (1984), Larkins (1986) and Lending *et al.* (1988) have suggest that γ-zein is located on the periphery of protein bodies. Their results were consistent with my data since γ-zein was degraded rapidly suggesting that it is located around the periphery of the protein bodies and is the first one to be accessible to proteolytic attack. Gamma-zein degradation, in turn, would expose the other zein components that are situated more centrally within the protein body for protease attack. Beta-zeins would be internal to γ-zein with the 18 kD in the interface between the 17 kD and γ-zein. Larkins (1986) and Lending *et al.* (1988) showed by

immunocytochemical staining of protein body sections that β-zein is located in the central portion of the protein body. It should be noted that there is varying degrees of immunological cross-reactivity between β-zein and other zeins (α and γ). Therefore the location of β-zein by immunocytochemistry cannot be determined unequivocally unless one uses monospecific antibodies. Since the 10 kD zein follows a course similar to that of α- zein, it is probable to be located between the 17 kD β- zein and α-zein or interlacing with α-zein in the protein body core. Alpha-zein, being the last to be degraded especially after other zeins were essentially completely degraded, would be in the protein body core.

Protein bodies were thought to undergo either internal or peripheral degradation. Peripheral degradation proceeds from the protein body periphery inwards, and may originate from several locations of the protein body surface. This proceeds until a large central vacuole remains (Horner and Arnott, 1965; Mollenhauer and Totten, 1971; Rost, 1972). The internal mode of protein body breakdown is proceeded by organelle swelling followed by flocculation of its content (Bagley *et al.*, 1963; Horner and Arnott, 1965; Opik, 1966; Smith and Flinn, 1967). At later stages of degradation, the fusion occurred between vacuoles to form unorganized protein masses (Briarty *et al.*, 1970). However, none of the work done by these researchers was on maize protein bodies. Although the most commonly reported fate of protein body is an internal mode of breakdown, my hypothesized structural arrangement of zeins within the protein body would fit the peripheral mode of protein body breakdown. If the content of maize protein bodies in maize were to follow internal degradation, then α-zein would be the first to be degraded whereas γ-zein would be degraded last. If the breakdown proceeded simultaneously from both interior and periphery, then degradation of α-zein would occur more rapidly at an earlier stage rather than after all the other zeins are essentially hydrolyzed. These two possibilities are not valid in this case. Therefore, based on these observations and those of others (Ludevid *et al.*, 1984; Larkins, 1986; Lending *et al.*, 1988), I propose that the

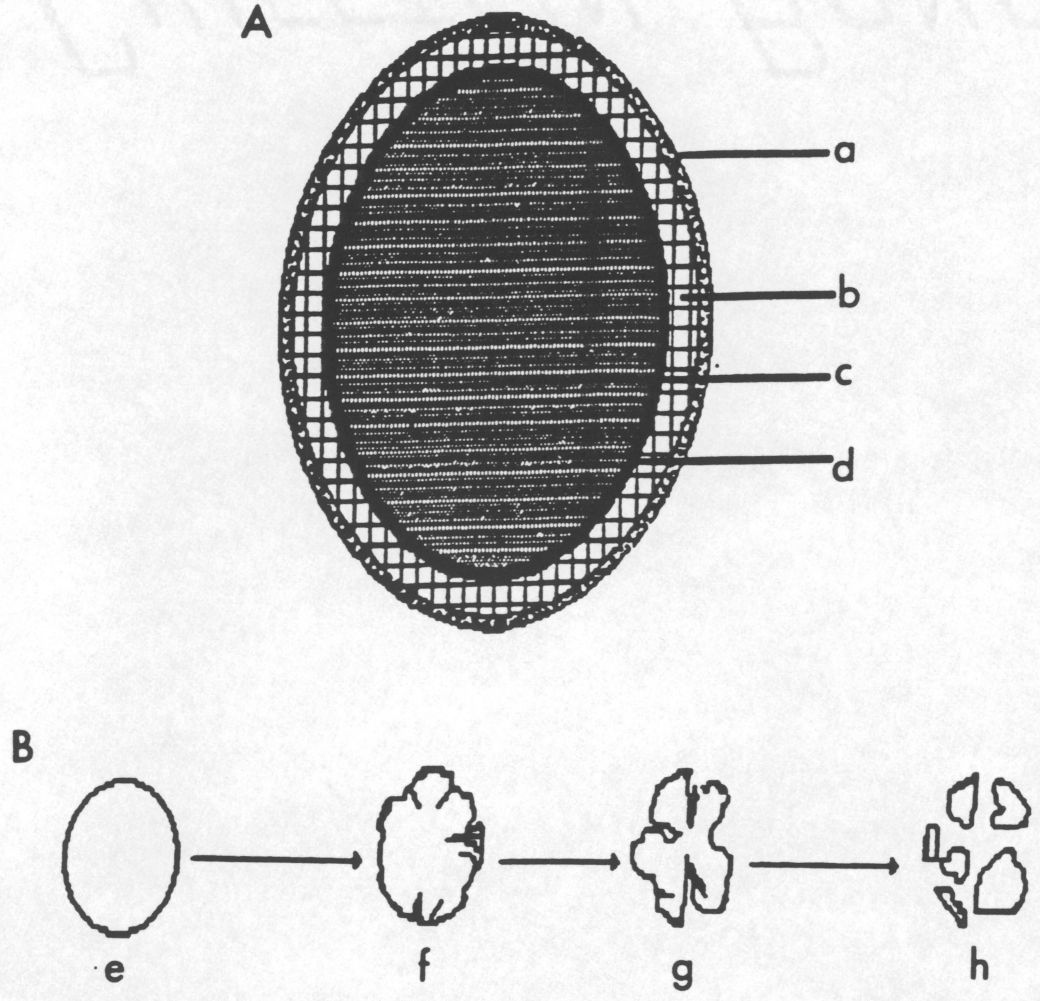

Figure 20. **A proposed model of protein body and its breakdown:** A, proposed model of protein body with the location of various zein components; B, proposed mode of protein body breakdown. a, γ-zein; b, β-zein (17 and 18 kD, with 18 kD on the outer layer); c, α-zein core; d, 10 kD zein; e, intact protein body; f, degradation starts from the periphery; g, complete degradation of zeins with the exception of α-zein; h, α-zein remaining after 10 DAG. The protein body model was based on the observed pattern of zein degradation. Based on this model, the mode of protein body breakdown was hypothesized.

proteolysis of the protein in protein bodies would start from the periphery and proceed towards their core. The proposed models for this mode of degradation is presented in Figure 20B.

The use of the Kjedhal procedure alone to measure total nitrogen during the early phases of germination revealed a lag period of total nitrogen loss in the endosperm, especially during the first three days of germination (Dure, 1960; Harvey and Oak, 1974a; Moureaux, 1979). Since the measurements in these studies were made by monitoring the total nitrogen, the observed lag period was due to nitrogen values of γ-zein degradation products. Without accompanying electrophoretic studies, the products of zein degradation would be accounted for in total nitrogen determination and one would not be able to determine accurately the time of initiation of zein degradation.

Eventhough the use of electrophoresis helps one to determine the initiation of zein degradation, the answer is still not complete. This is because as degradation proceeded, the products of degradation appeared as novel low molecular weight bands on the gel. Without knowing from which zein the new bands were derived from, the findings will not be very meaningful. Fujimaki *et al.* (1977) show two main subunits of zein which correspond to the size components of α-zein to be degraded gradually by SDS-PAGE analysis. However, no new bands of other molecular weight sizes were observed. This led them to conclude that the proteins hydrolyzed produced small peptides which would ultimately release free amino acids. Moreover, their procedure extracted only α-zein since a reducing agent was not included in the solvent. Fujimaki *et al.* (1977) followed zein degradation only by staining the gel for protein. The main disadvantage here is the limited resolution of the staining procedure.

My procedure, however, employed a more sensitive, specific antibody probes in addition to staining. This together with the more reliable method of extracting total zeins from endosperm meal (Esen, 1986) enabled me to obtain a clearer and more meaningful

picture of the degradation of various zein fractions. To elucidate the origin/source of low molecular weight components, immunoblots of SDS-PAGE runs were probed with antibodies prepared against various zeins. Since all the antibodies tested reacted with varying degrees to all or some of the heterologous zein components, they were used in the detection of its cross-reacting zeins (e.g. antibodies against 17 kD β-zein can also be used for detection of 18 kD β-zein). The use of antibody probes resulted in some significant findings, e.g., the degradation of γ-zein. On the second DAG massive degradation of γ- zein occurred as evidence from smearing starting at the 27 Kd region and extending to the dye front. The first DAG revealed a slight smear and a series of fragments varying in size. The series of degradation fragments generated and smearing observed on the blot of γ-zein suggested that the enzymes that are responsible for γ-zein degradation belong to both the endo- and exopeptidase groups, respectively. The degradation of other zein components, however, seemed to be due to endopeptidases.

The inhibition of zein degradation by cycloheximide (CHI) clearly suggested that the proteases involved in zein degradation are synthesized *de novo* in the endosperm of germinating maize seeds. The incubated seeds failed to germinate. The inhibition correlated with the time at which the seeds were exposed to cycloheximide (Harvey and Oaks, 1974b; Oaks *et al.*, 1983). The inhibition was dependent on the time of CHI application. Cycloheximide only inhibited zein degradation when it was present in the incubation medium from the time of imbibition. Late additions (70 hours after incubation) of cycloheximide have no effect on the hydrolysis of storage reserves. Some literature mentioned that some proteases are being packaged within the protein bodies during the synthesis of the storage proteins in maize and therefore are present in ungerminated seeds (Feller *et al.*, 1978; Moureaux, 1979). If this is the case, such preformed proteases are not able to degrade prolamins. There are two possible reasons for the CHI inhibition. The first, cycloheximide inhibit the *de novo* synthesis of the pro-

teases. Secondly, even though proteases are present in ungerminated seeds, the production of protein factors required for their activation are inhibited.

Actinomycin D, a potent inhibitor of RNA transcription, did not affect degradation of γ-zein. However, the degradation of the α- and β-zein components were slightly retarded. This suggested that the pre-existing mRNAs present in the dry seeds were sufficient to direct the synthesis of proteases that completely degraded γ-zein. The enzymes and the mRNA required for the synthesis of these proteases must also be pre-existing within the dry seeds. De Jiminez *et al.* (1981) and De Jiminez and Aguilar (1984) showed that the "house keeping" proteins were due to both the translation of new mRNA and delayed translation of stored mRNA species. However, they never showed the specific proteins which were translated. Some of house keeping proteins could very well be some of the proteases required for degrading the zein components during the initial stages of germination.

The use of specific protease inhibitors enabled me to identify various classes of proteases that degrade specific zein components. PMSF identified that serine protease are responsible for the degradation of α- and β-zeins. Most of the proteases inhibited by PMSF belong to the carboxypeptidase type (Feller *et al.*, 1978). They also suggested that the carboxypeptidase act synergistically with endopeptidase in the mobilization of storage reserves of maize endosperm which is consistent with my Western blot results of γ-zein degradation. This conclusion was suggested because the loss of endosperm nitrogen was concurrent with the increase in activities of these two types of proteases. The proteases that degrade β- and α-zeins cannot be of the carboxypeptidase (an exopeptidase) because their degradation products did not generate a smear on the Western blot. Pepstatin showed that aspartic (acid) protease plays a minor role in β-zein degradation. Acid proteases are present in germinating maize seeds, and have been referred to as P_{21} and P_{22} (Moureaux, 1979). These appeared on the third DAG and

reducing agents seemed to enhance their activity. Using hemoglobin substrate, the enzyme exhibited an endopeptidase type of reaction. Leupeptin inhibition suggested that cysteine (thiol) protease work synergistically with serine protease in γ-zein degradation. Makoto *et al.* (1978) showed that germinating maize seeds contained cysteine protease that is similar to pepsin rather than to papain. Similarly, Ryan and Walker-Simmon (1981) reported two cysteine endopeptidases with pH optima of 3.0 and 7.5 occurring in maize seeds. However, both groups of reasearchers characterize these enzymes by using an artificial substrate (hemoglobin) in an *in vitro* assay.

Almost all of the protease characterization work in maize is based on the proteolysis of artificial substrates such as hemoglobin. If zein was used, the specific component was not identified. Since most of the zein extraction procedures did not include any reducing agent, the zein involved is most likely α-zein. Furthermore, all the tests were performed in an *in vitro* system. This is the first report in which the proteases responsible for zein degradation are characterized in an *in vivo* systems.

The use of an agar gel in which various zein components were suspended demonstrated the activity of the various proteases on their natural substrate. Of all the extracts tested, only the extracts using 200 mM sodium acetate buffer (pH 3.8) possessed proteolytic activity that was able to degrade all the three major components of zeins (α, β and γ). Results from the agar gel assay showed a gradual degradation of α-zein which is consistent with SDS-PAGE analysis of Fujimaki *et al.* (1977). Activity was not present in the extract from dry seeds. Although protease is present in dry seeds, it cannot hydrolyze prolamins (Feller *et al.*, 1978; Moureaux, 1979). Proteases degrading β-and γ-zeins showed increased activity beginning from three DAG. This is consistent with the results of others which demonstrated a proteolytic enzyme with endopeptidase activity. This enzyme had a pH optimum of 3.8, temperature optimum of 46 °C, and could partially hydrolyze zein. It is present in germinating maize seeds (Harvey and Oaks, 1974a).

The activity increased from three to eight DAG. Similar increased proteolytic activities up to day eight after germination were reported by several investigators (Harvey and Oaks, 1974c; Fujimaki *et al.*, 1977; Feller *et al.*, 1978; Moureaux, 1979; Winspear, 1981). However, the proteases that degrade β-zein remained very active until the ninth DAG.

Concluding Remarks

In summary, I have shown that the degradation of the various zein components in the endosperm of germinating maize seeds is differential and not random. Based on these observations a model for the arrangement of various zein components within the protein body and the mode of protein body degradation is proposed. Several proteases are responsible for the degradation of the various zein components. The proteases are also studied for their extractability and ability to degrade the various zein components using several buffer systems.

Further Research Suggestions

1. Verification of the protein body model and mode of protein body breakdown by immunocytochemistry studies using monospecific antibodies from both developing and germinating endosperms.

2. Further characterization of the proteases using other different inhibitors and expand the combinations of mixtures.

3. Since the classes of proteases that are degrading specific zein component are known, isolation and purification of these enzymes will be the next logical step.

4. Production of antibody against native and denatured enzyme for further enzyme isolation, purification and molecular studies.

5. Once a monospecific antibody is obtained, isolate mRNA, perform *in vitro* translation, and immunoprecipitate. This will lead to the gene isolation, and further characterization can be done at the molecular level.

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